Induction of Abscopal Anti-Tumor Immunity and Immunogenic Tumor Cell Death by Ionizing Irradiation – Implications for Cancer Therapies

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Abstract: Although cancer progression is primarily driven by the expansion of tumor cells, the tumor microenvironment and anti-tumor immunity also play important roles. Herein, we consider how tumors can become established by escaping immune surveillance and also how cancer cells can be rendered visible to the immune system by standard therapies such as radiotherapy or chemotherapy, either alone or in combination with additional immune stimulators. Although local radiotherapy results in DNA damage (targeted effects), it is also capable of inducing immunogenic forms of tumor cell death which are associated with a release of immune activating danger signals (non-targeted effects), such as necrosis. Necrotic tumor cells may result from continued exposure to death stimuli and/or an impaired phosphatidylserine (PS) dependent clearance of the dying tumor cells. In such circumstances, mature dendritic cells take up tumor antigen and mediate the induction of adaptive and innate anti-tumor immunity. Locally-triggered, systemic immune activation can also lead to a spontaneous regression of tumors or metastases that are outside the radiation field - an effect which is termed abscopal. Pre-clinical studies have demonstrated that combining radiotherapy with immune stimulation can induce anti-tumor immunity. Given that it takes time for immunity to develop following exposure to immunogenic tumor cells, we propose practical combination therapies that should be considered as a basis for future research and clinical practice. It is essential that radiation oncologists become more aware of the importance of the immune system to the success of cancer therapy.

Keywords: Abscopal effects, anti-tumor immunity, cancer, danger signals, dendritic cells, immune editing, immunogenic tumor cell death, radiotherapy.

INTRODUCTION

Tumor cells that have escaped immune surveillance are capable of forming vascularized tumors which have their own complex microenvironment [1]. Although radiotherapy, chemotherapy and surgery are standard treatments for established tumors, treatments that also include single or adjuvant immune therapy are being increasingly considered. It is now well accepted that specific immune responses can contribute to the elimination of small tumor masses, recurrent tumors and metastases. However, it remains unclear why tumors that one presumes have previously escaped immune surveillance can be treated by activating the immune system. This review will focus on how a tumor can be modified by local treatment with radiotherapy (ionizing irradiation) and be consequently rendered susceptible to anti-tumor immune attack.

1. EFFECTS OF RADIOTHERAPY ON CELLS

The ionizing irradiation (X-ray) which is delivered by standard radiotherapy (RT) induces various forms of DNA damage [2], either directly *via* the generation of electrons or indirectly *via* the production of radicals. DNA double-strand breaks in tumor cells caused by RT often cannot be repaired adequately [3] and this damage frequently leads to chromosomal aberrations that finally resut in cell death [4]. Most of the DNA damage which is induced by RT occurs in clustered lesions [5] and the multiple DNA and base damage effects impede adequate repair by mechanisms such as homologous recombination and non-homologous end-joining. Furthermore, RT-induced DNA damage leads to temporary cell cycle arrest in the G2 phase which renders cells highly susceptible to further irradiation. The application of fractionated RT therefore increases the likelihood of irradiating cells in this more sensitive phase, as well as reducing the side-effects of high single doses of X-ray on normal tissue. Current fractionation schemes have been developed empirically and typically contain five single doses of around 2 Gy per week [6]. Nothwithstanding the above, it should also be noted that recent data implicate an *increased* DNA repair capacity in tumors, at least in tumor initiating (stem) cells [7] and the implications of this on the curative capacity of RT is currently under investigation.

X-irradiation primarily leads to a halt in the proliferation of tumor cells. One commonly used *in vitro* assay for detecting cell cycle block is the clonogenic "survival" assay. This assay defines the proliferative and colony formation capacity of irradiated cells, but not their death [8]. Based on this clonogenic "potential" (a less confusing term than "survival"), the term *radiosensitivity* usually defines the relative susceptibility of cells, tissues, organs or tumors to irradiation [3]. The primary goal of standard RT is the local control of tumors and one approach for achieving *radiocurability* is *via* the elimination of proliferating (clonogenic) tumor cells. However, it has to be kept in mind that the induction of cell death is safer than merely stopping the proliferation of tumor cells [9]. The forms of tumor cell death that are induced by X-ray will be discussed later.

In many cases, the extent of local tumor control is not sufficient to predict cure due to the potential for a tumor to reoccur or establish metastatic disease. Although historically, efforts have focused on improving the accuracy of local radiation delivery, in the last decade researchers and clinicians have started to focus more strongly on the genetics and microenvironment of the tumor [10]. It is important to appreciate that *non-targeted effects* which influence "bystander" cells and tissues that are outside of the irradiation field exist in addition to the *targeted effects* of X-rays that have been detailed above [11]. Distant bystander effects, the so-called abscopal effects [12] that will be explained in more detail below, can manifest in the form of protective anti-tumor immunity which has been induced by tumor cells that have been modified by standard therapies like RT or chemotherapy (CT) (Fig. **1**).

2. ABSCOPAL EFFECTS

Although RT is widely used for local tumor control in various malignancies, a spontaneous regression of tumors, metastases or

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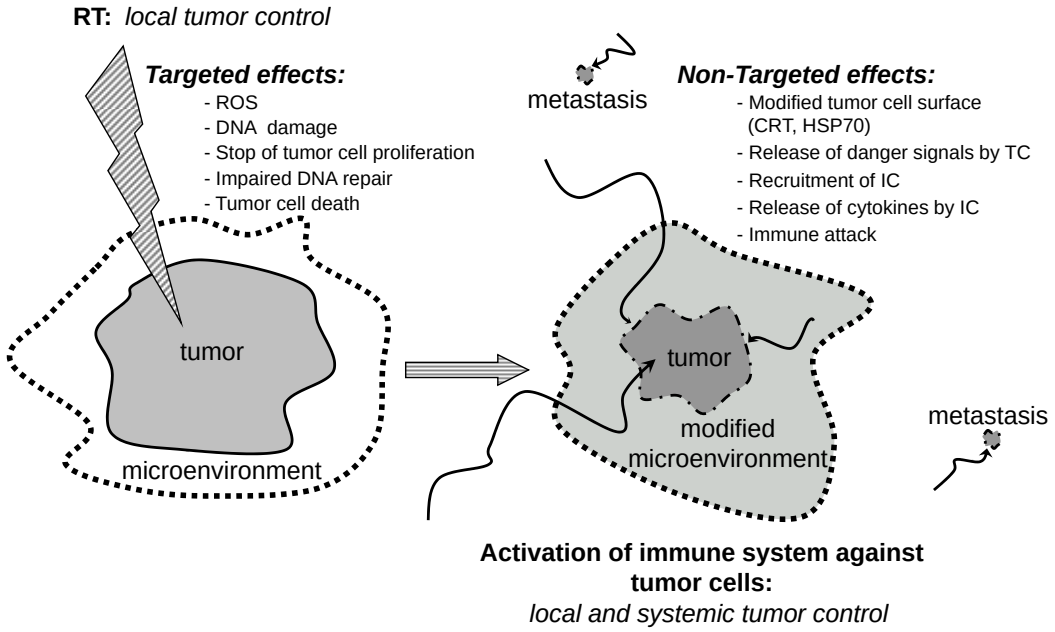

Fig. (1). Targeted and non-targeted (systemic) effects exerted by ionizing irradiation. Local irradiation of a tumor induces DNA damage(s) *via* direct and indirect (production of ROS) mechanisms. The tumor cells stop proliferating and unrepaired DNA damage finally leads to tumor cell death and to shrinkage of the whole tumor. Furthermore, local treatment of the tumor with ionizing radiation is capable of modifying the tumor cell surface (e.g. increased exposure of CRT and HSP). Massive tumor cell death results in necrotic tumor cells that release danger signals that are normally hidden inside the tumor cells. In addition, cytokines are secreted by infiltrating immune cells following interactions with dying tumor cells. Local RT treatment, therefore, modifies the tumor microenvironment. Activated immune cells mediate the induction of specific adaptive anti-tumor immunity against the primary tumor, as well as metastases (for more mechanistic details see also Fig. **2**).

CRT: Calreticulin; HSP: heat shock protein; IC: immune cells; ROS: reactive oxygen species; RT: radiotherapy with ionizing radiation; TC: tumor cells.

enlarged lymph nodes (LN) outside the radiation field has also been reported [13-15]. This phenomenon was termed the abscopal effect by R.H. Mole in 1953 [12] on the basis of the Latin prefix ab- (position away from) and –scopus (mark or target for shooting at). He defined it as being "at a distance from the irradiated volume, but within the same organism", which in other words means the appearance of systemic effects in non-irradiated areas after treatment with localized RT. It is important to note that the abscopal effect is a synonym for distant bystander effect and differs from the radiation-induced bystander effects that occur in non-irradiated cells that neighbor irradiated cells and is mediated *via* cell-to-cell gap junctions or by the secretion of soluble factors [16, 17]. Bystander effects are also called non-targeted effects in order to distinguish them from the direct, targeted effects of radiation on cellular DNA.

Taken together, X-rays exert both local (targeted and bystander effects) and systemic effects (abscopal effects) in tumor-bearing hosts. Elucidating the cellular and molecular mechanisms that lead to abscopal effects might assist the development of better therapeutic strategies for the treatment of systemic metastases.

Several pre-clinical studies have shed light on how local RT might induce systemic anti-tumor responses. Nobler and co-workers considered the abscopal effect in the context of lymphocyte circulation [18]. Demaria and co-workers demonstrated that the systemic outcome is mediated by the immune system and that T cells are required for distant tumor regression after combined local radiation and Flt-3 Ligand treatment in a mouse model of mammary carcinoma. Flt-3 Ligand is a potent stimulator of dendritic cells (DCs) and natural killer (NK) cell generation. They suggested that radiation-induced death of some tumor cells triggers the production and release of cytokines and other inflammatory stimuli which promotes the local maturation and cross-priming of DCs [19]. Local radiation damages tumor cells and results in the release of tumor antigens (Ag) in form of cellular debris as well as necrotic and apoptotic tumor cells which have the potential to stimulate anti-tumor immune responses [20]. The direct administration of autologous DCs after the local irradiation of tumors had effects that were similar to Flt-3 Ligand administration and significantly increased tumor regression in mice [21]. Shiraishi and colleagues investigated the effect of ECI301, a human macrophage inflammatory protein-1 alpha variant, on the growth of different tumors at irradiated and non-irradiated sites in tumor-bearing mice. Their data clearly indicate that a combined therapy not only reduced the tumor size of the irradiated site, but also that a combination of RT with ECI301 retards the growth of distal non-irradiated tumors. The observed abscopal effect was independent of the genetic background of the mice and was independent of the tumor-type, thereby indicating that this treatment regimen has the potential to control metastases. Leukocyte depletion experiments suggest that $CD8^+$ T cells, $CD4^+$ T cells and NK1.1 cells are all involved in the local and systemic anti-tumor effects [22]. Using a similar experimental design as outlined before, Takeshima and colleagues demonstrated that local RT treatment of EG7 tumors in one leg of the mouse combined with Th1 cell therapy inhibits tumor growth in both legs. This experiment further proves the abscopal effects of RT when combined with supplemental immune stimulation/immune therapy [23]. Of particular clinical relevance are the studies of Dewan and colleagues which have shown that a fractionated RT in combination with anti-CTLA-4 antibody induces an abscopal anti-tumor effect in two carcinoma models. Blocking of CTLA-4 compensates for the inhibitory signals that are transmitted to T cells. This study also showed that the frequency of $CD8^+$ T cells that exhibit tumor-specific IFN-gamma production correlated with the growth inhibition of the non-irradiated tumor [24]. In contrast to the studies outlined above, Camphausen and co-workers

have demonstrated an abscopal effect with RT alone [25]. The effect was not tumor-specific, but was dose- and p53-dependent.

Based on the studies above, the exploration of the optimal radiation doses and fractionation schemes in combination with immunotherapy offers a promising approach for improving the efficacy of primary and metastatic cancer treatments.

3. BASIC IMMUNE MEDIATED TUMOR KILLING MECHANISMS

Nowadays, it is well accepted that the immune system plays a crucial role in the control of tumors and that it acts in concert with other killing mechanisms that emanate from standard therapies. NK cells and the specific triggering of cytotoxic T-lymphocytes against tumor cells are of immense importance in immunosurveillance and protective anti-tumor immunity [26].

NK cells belong to the innate immune system and are able to recognize transformed cells such as tumor cells without prior sensitization [27]. The primary trigger for NK cells to attack a target cell is the absence of major histocompatibility complex (MHC) class I complex, over-expression of self-proteins (NKG2D-ligands) or the appearance of "foreign" proteins on the cell surface. These proteins are recognized by inhibitory and activating receptors and the balance of the intracellular signals that are generated by these receptors trigger their activation or inactivation [28]. After activation, NK cell killing is mediated *via* two main ways. On the one hand NK cells use granule exocytosis, in which perforin and granzymes are delivered into the target cell to result in cell killing [29]. On the other hand, NK cells induce target cell apoptosis using a receptor-mediated pathway which involves Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) [30]. NK cell populations can also secrete a variety of cytokines and chemokines such IFN-gamma, TNF-alpha, CC chemokine ligand (CCL) 3 or CCL 4. These factors are key regulators of DC maturation and the recruitment of more immune cells into the tissue.

DCs are key immunoregulatory antigen presenting cells (APCs) that connect innate and adaptive immunity. APCs can recognize pathogen-associated molecular patterns (PAMPs), as well as damage-associated molecular patterns (DAMPs) *via* pattern recognition receptors [31, 32]. In addition, tumor cells treated with anthracyclines or gamma-irradiation display "eat-me" signals such as calreticulin (CRT) on their surface, and these further stimulate their engulfment by DCs or the secretion of DAMPs such as HMGB1 or HSP70 (see also Section 5) [33-35]. DCs take up tumor cell material and process tumor-associated antigens (TAAs), mature and migrate into the draining lymph node (LN), in which they present peptides in the context of their MHC complexes and deliver essential co-stimulatory signals to naïve $CD4^+$ and $CD8^+$ T lymphocytes [36]. $CD8^+$ T lymphocytes differentiate into cytotoxic T lymphocytes (CTLs), whereas naïve $CD4^+$ T cells differentiate into T helper cells (T_H). The naïve T cell subset which is primed by the DC is dictated by its MHC expression and the origin of the antigen. Antigens of intracellular pathogens such as viruses are presented on MHC class I to $CD8^+$ T cells, whereas extracellular-material such as TAAs are typically presented on MHC class II to $CD4^+$ T cells [37]. However, it is also possible for peptides that are derived from extracellular antigens to be presented on MHC class I molecules and prime $CD8^+$ T cells *via* a process called 'cross-presentation' or 'cross-priming'. This process can be supported by high-mobility group protein B1 (HMGB1) which is secreted by dying tumor cells.

Dying tumor cells and/or tumor-related material can be engulfed by DCs, and the TAAs processed and presented to naïve $CD4^+$ T cells which in turn support B cells to produce TAA-specific antibodies. Although NK cells can kill tumor cells directly or *via* antibody-dependent cellular cytotoxicity (ADCC), a crucial additional component in the immune control of a tumor involves the induction of adaptive immunity. Although the cross-presentation of tumor Ag and the priming of $CD8^+$ CTLs to TAAs are crucial to this protective mechanism, the priming of CTLs alone is not sufficient for effective killing of cancer cells. Activated CTLs require a second signal from $CD4^+$ T_H cells which is delivered *via* CD40-CD40L interactions in order to maintain a long-lived phenotype [38]. In the absence of the second signal, CTLs would swiftly die *via* apoptosis *via* the induction of TRAIL and the generated adaptive immune response would be very short-lived [39]. This highlights that a balance of priming of CTLs and T_H cells is required for effective killing of tumor cells by CTLs. If this balance is accomplished, CTLs recognize tumor cells *via* T cell receptors and the associated TAA for a lengthened period of time and induce cell death *via* the granule exocytosis pathway and the receptor-mediated pathway of the TNF family, as has already described for NK cells. Lysed or dying tumor cells can be phagocytosed and cleared from the organism by macrophages or lead to further priming of CTLs.

In addition to being essential to the priming of naïve T cell populations, activated DCs can regulate NK cell activity by secreting cytokines such as interleukin (IL)-12, IL-18 or Type I interferons. These events enhance the cytotoxicity, proliferation and IFN-gamma production by NK cells. Furthermore, NK cells can control DC "behavior" by providing signals for their maturation in a cell-to-cell contact dependent manner. These signals include pro-inflammatory cytokines such as TNF-alpha, IFN-gamma and natural killer cell p30-related protein (NKp30) [40].

Tumors often create an immune suppressive environment, under which DCs do not mature and may lack specific receptors for DAMPs. However, those receptors can then be provided by NK cells [41]. The latter recognize "danger cells" by their receptors and consequently stimulate DCs and contribute to the generation of an adaptive immune response [42].

In addition to priming naïve T-cells, *in vitro* experiments provide evidence that subpopulations of common human DCs can exhibit direct anti-tumor effects [43]. The growth inhibition of tumor cell lines by those natural killer dendritic cells (NKDCs) is accomplished *via* direct killing of cancer cells by TNF-alpha secretion, FasL or TRAIL expression. Interferons stimulate the NKDC dependent tumor cell killing [44-46]. In summary, the innate and adaptive immune systems are crucial for controlling tumor development and tumor defense after conventional cancer treatments. The interplay of NK cells, DCs and CTLs is essential for creating long-lasting and specific protective anti-tumor immunity.

4. IMMUNE EDITING AND THE TUMOR MICROENVIRONMENT

Historically, cancer was regarded as being a purely genetic disease and to be solely driven by tumor cells. The contribution of the immune system to the establishment, progression and elimination of cancer was considered by oncologists. Nowadays, the acquired capacity of developing tumors to escape immune control is an emerging hallmark of cancer, as it is the reprogramming of cellular metabolism [47].

Immune Editing

In the past, many pre-clinical studies were carried out using xenogeneic models in which human cancer cells are grown in nude mice that do not have the capacity to mount an immune response against the tumor. These studies do not consider the contribution of T cell-mediated adaptive immune responses against tumors. Tumor outgrowth experiments conducted with immune deficient mice coined the *immune editing* hypothesis of cancer. Immune deficient

RAG2$^{-/-}$ mice developed tumors with greater frequency and more quickly than wild-type mice which have an intact immune system [48]. This revealed that mature T and B lymphocytes contribute to tumor surveillance. Besides immune cells, cytokines such as IFN-gamma and transcription factors that mediate interferon signaling (STAT1) are also involved in this process. IFN-gamma-insensitive p53$^{-/-}$ mice develop a much broader spectrum of tumors than mice which only lack the tumor suppressor p53 [49]. Of note, the immunological phenotype of tumors that are derived from immune deficient mice is markedly different to that of tumors from competent animals. Whereas tumors derived from immune competent mice developed in almost 100% of wild type or immune deficient mice, only around 50% of tumors that were derived from immune deficient mice grew out in immune competent mice. This led to the hypothesis that immunogenic tumors (being attacked by the immune system) are not recognized in an immune deficient environment and are therefore unedited (summarized in [1]). The cancer immune editing concept –*elimination*, *equilibrium*, and *escape*- according to Schreiber and colleagues has arisen as follows [50]: Healthy tissue becomes transformed by carcinogens, radiation, viral infections, ongoing inflammation or genetic alterations and is then recognized as being ”foreign” or “dangerous” [31] for the organism and eliminated *via* innate and adaptive immune mechanisms. If this cancer immune surveillance fails to *eliminate* the cancer cells before they are clinically recognized, then those cancer cells that have not been recognized by the immune system can enter an *equilibrium* phase. Cytokines such as IFN-gamma and T cells regulate this cancer dormancy. Preventing IFN-gamma production by suppressor of cytokine signaling 1 assures the correct functioning of regulatory T cell populations [51]. When an outgrowth of the tumor is no longer blocked by the immune system, certain tumor cell variants escape to form an established tumor. The tumor has progressed and *escaped* the immune surveillance. An immune suppressive tumor microenvironment is beneficial in the escape phase, whereas chronic inflammation, which is in part coordinated at the mitochondrial level, might foster further tumor outgrowth [52]. Acute and chronic inflammatory events are nearly always interconnected [53]. While chronic inflammation favors the establishment of a tumor [54] by fostering neo-vascularization, cell proliferation and metastases [55], appropriately timed and restricted acute inflammation can be beneficial since released danger signals may mature and activate DCs, and result in the induction of anti-tumor immunity [56, 57].

One early, macrophage-derived inflammatory cytokine which is involved in malignant transformation is IL-1. IL-1 consists of two distinct proteins and has dual functions. IL-1 beta increases vascularization in tumors and metastases formation and IL-1 alpha activates anti-tumor effector mechanisms that are exerted by NK cells and CTL [58]. IL-1 may therefore contribute to tumor progression [59] and also control anti-tumor immunity [60]. Therapeutically-relevant doses of X-rays have been shown to significantly increase IL-1 alpha mRNA levels in mice [61]. It is therefore apparent that inflammatory factors can promote as well as inhibit tumor growth and radiotherapy might therefore be capable of inducing ‘inflammatory windows’ in the tumor microenvironment which allow professional APCs to become activated.

DCs in the Tumor Microenvironment

DCs are the most potent population of APCs and are central players in the induction of adaptive anti-tumor immunity [62]. Early “onco-immunological” studies showed that the infiltration of DCs in gastric tumors significantly lowers the incidence of lymph node metastasis [63]. More recent work suggests that distinct subsets of DCs favor anti-tumor responses [64]. In general, it appears that various states of DCs in addition to immature and mature exist, and that these depend on the distinct microenvironment [65], and relate to various forms of cell death that are defined by functional and molecular properties [66]. A greater prevalence of CD1a^{+}/DC-LAMP^{+} DCs correlates with a reduced thickness of melanomas and a high density of mature DCs around the tumor has been associated with significantly longer survival of patients with melanoma [67]. Overall, tumor infiltration by CD8^{+} T cells is associated with a positive outcome, while the frequency of infiltrating CD4^{+} T cells is often a negative predictor (summarised in [68]).

It must be stressed once again that a delicate inflammatory balance exists in the tumor microenvironment. Inflammatory cytokines may stimulate DCs to mature. Mature DCs can induce specific anti-tumor immune responses by cross-priming CD8^{+} T cells, but may also attract and foster the expansion of CD4^{+}CD25^{high} regulatory T cells which are known to attenuate anti-tumor immune responses [64]. However, regulatory T cells might also have a dual role, as the accumulation of regulatory T cells in various human carcinomas is associated with a poor prognosis, but not in colorectal cancer. The latest work of Ladoire and colleagues suggests that inflammation induced by microbial flora in the region of the colorectal tumor is attenuated by regulatory T cells *via* an inhibition of the Th17-cell-dependent tumor-enhancing response, the result of which is a reduction in tumor growth [69].

To conclude, in addition to the tumor itself, the "immune signature" of a tumor, as defined by genetic, molecular and functional profiles of immune cells in the tumor microenvironment should be taken into account when attempting to optimize therapeutic approaches [70].

Radiotherapy and Immune Activation

Ablative RT with a single dose of 20 Gy has been shown to induce a T cell-dependent growth retardation of the poorly immunogenic B16 melanoma tumor in Black/6 mice. This has led to the hypothesis that larger single doses of RT acting locally on the tumor switch the tumor microenvironment from an immune suppressive profile to an immune activating profile (Figs. **1** and **3**). RT with higher single dose further promoted the priming of antigen-specific DCs [71]. However, fractionated RT with clinically relevant single doses also increased the number of APCs within tumor-draining LNs [20], in which antigen presentation and the activation of CD8^{+} CTLs takes place. CTLs migrate back to the tumor and the tumor cells are killed by CTLs or NK cells by a process which is partially mediated by perforin [72]. Initial results providing evidence that the anti-tumor immunity which is induced by X-rays is inhibited in perforin-deficient mice again highlight that the immune system can be stimulated by RT and that it is involved in anti-tumor responses [23]. However, perforin is not required for CTL activity if tumor cells have been treated using chemotherapy [73]. Notwithstanding these findings, it is possible that chemotherapy enhances the killing efficacy of tumor-specific CTLs [74] and induces immunogenic cancer cell death [75].

Pattern or damage recognition receptors such as Toll like receptor 4 (TLR4) on DCs have clearly been shown to be crucial for the induction of anti-tumor T cell responses. Defects or polymorphisms in TLR4 in humans can be compensated for by the administration of chloroquine [76] which improves antigen cross-presentation by DCs [77]. Local RT of EL4 lymphoma, Lewis land carcinoma, B16 melanoma various tumors in mice leads to an induction of CD8^{+} CTL responses against the tumor. Application of anti-CD8 antibodies after local RT abolishes the tumor growth inhibitory effects of RT. Since draining LNs are critical for generation of CTLs [78], Takeshima and colleagues repeated their experiments using mice from which LNs had been removed, or alymphoplasia mutant mice. Of note, the anti-tumor effects of RT were strongly reduced in those animals [23]. From the practical point of view in clinical work, one might consider initially treating

patients with RT and later removing the LN by surgery in order to optimize the immune systems opportunity to react against the tumor (Fig. **3**). However, since LNs are one main source of tumor spread and metastases (summarized in [79]), a case-by-case decision regarding the length of time that a diseased LN should remain in the patient's body would have to be made by the clinical team. Very recently, Burnette and colleagues have demonstrated that RT drastically enhances the number and cross-priming capacity of tumor-infiltrating $CD11b^+$/$Ly\text{-}6C^+$/MHC class II^+ DCs in a type I interferon–dependent manner [80]. The type II interferon IFN-gamma also plays a role in radiation induced anti-tumor immunity as fractionated and ablative RT has been shown to increase the frequency of tumor infiltrating cells that secrete IFN-gamma in response to tumor-derived peptides [20]. IFN-gamma in the tumor microenvironment which is induced after RT leads to T cell infiltration and recognition of the tumor by immune cells [81].

Role of Interferons in Tumor Immunology

IFN-alpha and IFN-beta (Type I IFN) and IFN-gamma (type II interferon) might have non-redundant functions in the cancer immune editing process by affecting distinct target-cell populations [82]. Although the primary role of type I IFN was thought to be the defense against viral infections, recent studies have shown their importance as immune modulators, as is the case for IFN-gamma. Since gamma-delta T cell-deficient mice exhibited a significantly higher incidence of tumor development, Gao and colleagues suggested that this T cell subset is the main source of this type II interferon during the development of anti-tumor immunity [83]. The induction of IFN-gamma by IL-12 is one mechanism which drives the adjuvant activity of IL-12 [84]. One difference between type II and I IFN is that the latter do not act on tumor cells directly during an anti-tumor immune responses. Host cells such as NK cells and not the tumor cells themselves are the main target of type I interferons [85]. Host DCs in tumor draining LNs have recently been shown to produce type I IFN which is mandatory for priming the cross-presentation of tumor antigens by $CD8^-alpha^+$ DCs [86]. A detailed overview of the contribution of IFN to anti-tumor responses is given in the review article of Dunn and colleagues [82]. Tumors often secrete immune suppressive cytokines such as IL-10 or TGF-beta in order to induce or maintain immunological tolerance. Myeloid derived suppressor cells (MDSCs) are recruited to and generated at the tumor site and also contribute to an immune suppressive tumor microenvironment. It was recently shown by Apetoh and colleagues that treatment with the chemotherapeutic agent 5-Fluorouracil (5-FU) leads eliminates MDSCs and increases IFN-gamma secretion by tumor-specific $CD8^+$ T cells [87]. In addition to their immune modulatory functions, IFN might also have direct radiosensitizing effects on tumor cells, as has recently been demonstrated *in vitro* for pancreatic cancer cells [88].

5. IMMUNOGENIC TUMOR CELL DEATH INDUCED BY RADIOTHERAPY

Ionizing radiation damages tissue and thereby alerts the immune system to the appearance of "danger" [89]. Other work demonstrates that RT modified the phenotype of the target tissue in such a way that tumor cells become more susceptible to vaccine-mediated T cell immune attack [90]. *In vivo*, RT upregulates expression of the death receptor Fas and MHC class I molecules on the tumor cells and might further modulate MHC class I-mediated anti-tumor immunity by functionally affecting antigen presentation by DCs [91]. In addition, radiation enhances peptide production and the surface expression of MHC class I. Importantly, the repertoire of MHC class I peptides and radiation-specific peptides has been shown to be enlarged after X-ray treatment [92]. The local modulation of the tumor cell phenotype by RT might render the tumor visible to the immune system and immunogenic (Fig. **1**). RT is therefore capable of conditioning the tumor to subsequent immune therapy [93].

For many decades, radiation oncologists have considered apoptotic tumor cell death as being the sole form of cell death which is induced by X-rays. The paradigm which existed was that tumor cells are killed *via* apoptosis and/or lose their clonogenic "survival" by arresting in the cell cycle. However, it has become obvious over the last few years that tumor cell death after irradiation can occur *via* various alternative cell death modalities [34, 94].

Apoptotic Tumor Cell Death

The two best known forms of cell death are apoptosis and necrosis. The billions of cells that die in the human organism every day must be recognised and cleared swiftly and silently without inducing an immune response. Secreted "find-me" signals ensure the rapid recognition of apoptotic cells by macrophages. Failures in the clearance of the body's own dying cells have been demonstrated to activate the immune system and to contribute to the development of chronic autoimmunity [95]. The interaction of apoptotic cells with professional phagocytes, as macrophages are, is directly and indirectly mediated *via* the anionic phospholipid phosphatidylserine (PS) which becomes stably exposed on the outer cell membrane early in the apoptotic process (summarised in [96]). The scavenging of the dying cells by activated macrophages may modulate the immune system and induce an anti-inflammatory microenvironment [97]. Since the removal of apoptotic tumor cells is also conducted by macrophages, an anti-inflammatory microenvironment which promotes tumor growth may result [98]. PS is also capable of directly inhibiting the maturation of DCs and activated CTLs proliferate poorly to antigens which are presented by DCs that have been previously exposed to PS [99]. Furthermore, PS affects inflammatory signalling mechanisms in DCs by inhibiting NF-κB and p38 MAPK activation [100]. It is therefore possible that tumor cells that have been rendered apoptotic by radiation and which expose PS might contribute to oncogenesis. On the other hand, removal of huge tumor masses by surgery, RT or CT might reduce the immune suppressive properties of the tumor which regulate its escape during immune editing [101].

Necrotic Tumor Cell Death

Necrotic cell death is often, but not always, the outcome of non-physiological cell damage. Damaged cells expose DAMPs that activate inflammatory and immune effector mechanisms [35]. The massive release of DAMPs such as HMGB1 from the large numbers of tumor cells that die during CT and RT is rapid and can mediate DC recruitment and CTL activation [102]. A low expression of HMGB1 in non-small cell lung cancer might therefore explain the poor therapeutic outcome which is observed during standard treatments [103]. Of note is the observation that not a single DAMP links tumor cell death to the outcome of inflammation and anti-tumor immunity [104]. Necrosis results in multiple immunological effects and the presence of necrotic cell death forms can be beneficial to the induction of protective anti-tumor immunity.

Necrotic cells might be a clinically relevant counter to tumor cells that are resistant to apoptosis. A type of necrotic cell death which can be present in apoptotic-deficient conditions and which is dependent on receptor interacting protein-1 (RIP1) kinase, the so called necroptosis, was discovered by Hitomi and colleagues [105]. Necroptosis can be induced by ligation of the TNF receptor or by caspase inhibitors and the necroptosis-inducing protein complex consists of RIP1 and RIP3. Becker and co-workers discovered that necroptosis is induced under caspase-8 deficient conditions in epithelial cells of the intestine [106]. This programmed form of necrotic cell death is characterized by the same morphological

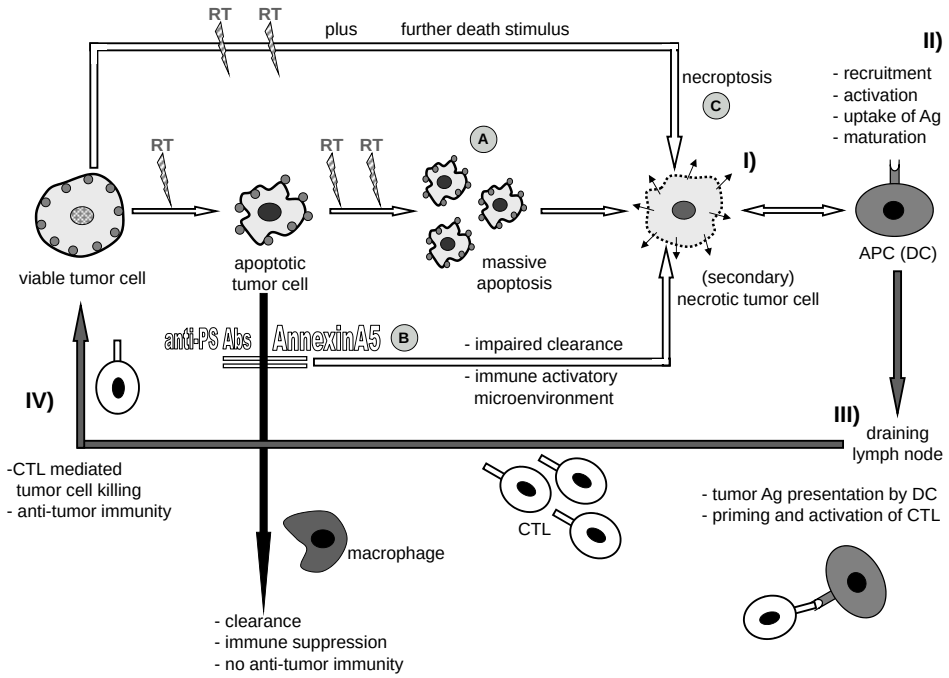

Fig. (2). Therapy-induced necrosis of tumor cells as an activator of specific adaptive anti-tumor immune responses. A tumor cell undergoes apoptotic cell death in response to RT. Apoptosis is characterized by the exposure of PS by the dying cells. PS holds anti-inflammatory properties and is the main recognition signal for macrophages. The latter clear apoptotic cells and induce immune suppression or no immune response. Continued RT leads to massive apoptosis which overwhelms the body's clearance system (**A**). Uncleared apoptotic tumor cells lose their membrane integrity during the apoptotic program and become secondary necrotic. Immune adjuvants like AnxA5 and antibodies targeting PS can block the PS-dependent clearance of apoptotic tumor cells and promote secondary necrosis (**B**). Massive death stimuli or the presence of defects in the apoptotic pathway lead to the direct induction of programmed necrosis in tumor cells, the so called necroptosis (**C**). Irrespective of the pathway to necrosis, cells release immune activating danger signals such as HMGB1, HSP70, ATP, uric acid, and S100 proteins (I). Danger signals foster the recruitment and maturation of DCs and their uptake and presentation of antigens (II). DCs migrate to the draining lymph nodes and cross- present tumor antigen with co-stimulation to $CD8^+$ T cells (III). Primed and activated $CD8^+$ CTLs migrate to the tumor and start their attack (IV).

Abs: antibodies; Ag: antigen; AnxA5: AnnexinA5; CTL: cytotoxic T lymphocyte; DC: dendritic cell; RT: radiotherapy with ionizing radiation.

features as unregulated primary necrosis, but can be specifically blocked by necrostatins. Although necrostatins are inhibitors of the RIP1 kinase [107], they might also inhibit T cell proliferation by a mechanism which is independent of RIP1 proximal T cell receptor signalling [108].

In addition to defining cell death on the basis of signalling pathways it is also of great importance for "onco-immunology" to define it on the basis of its immunological outcome, namely immunogenic or tolerogenic cancer cell death [9, 109]. Detailed information about the immunological potential of cell death and other cell death modalities such as autophagy and mitotic catastrophe have been considered elsewhere [8, 66]. Mitotic catastrophe can be induced by RT [94] and also result in necrotic-like cell death [110].

Returning to the immunological effects of necrotic tumor cell death; how can necrotic tumor cells be generated by RT? We suggest at least two main approaches for inducing a greater degree of necrotic cell death by RT: (i) combine RT with additional stress stimuli such as the application of temperature-controlled hyperthermia (41-43°C for 1 hour) [111] or (ii) block the uptake of RT-induced apoptotic cells by interfering with the PS-dependent tumor cell clearance pathway [112], as the blockade of apoptotic cell clearance results in secondary necrotic cells. Secondary necrosis is best defined by "a terminal process which is experienced by apoptotic cells when their clearance *via* phagocytes is absent or fails in certain *in vivo* conditions" [113]. The clearance deficiency could be due to impaired function of macrophages or to the amount of apoptosis, e.g. induced by long-lasting and strong death inductors (Fig. **2**). Secondary necrotic cells have lost their membrane integrity like primary necrotic cells and display similar phenotypes such as complement binding features [114] and the release of intracellular components including certain DAMPs [115]. As a consequence, the maturation and activation of DCs as APCs is (also) induced by secondary necrotic cells (Fig. **2**).

Interference with Tumor Cell Clearance – Blocking of PS with AnnexinA5 or Anti-PS Antibodies

The acquisition of a deeper understanding of the molecular and cellular mechanisms that influence the clearance of the apoptotic tumor cell populations which are induced by standard and immune therapies might enable outcomes to be improved [116]. We have previously demonstrated that the growth of syngeneic tumors is retarded by a single injection of AnnexinA5 (AnxA5) around the tumor [117]. AnxA5 binds to the PS which is exposed by dying tumor cells with high affinity and thereby inhibits the uptake of apoptotic tumor cells by macrophages (Fig. **2**). Furthermore, we found that the blockade of macrophage clearance results in the generation of secondary necrotic cells and an accumulation of apoptotic cells that are then accessible for phagocytosis by DCs [117]. The observation that AnxA5 did not block the uptake of the tumor cells by DCs indicates that macrophages and DCs, in part at least, utilize different clearance mechanisms. Hoves and colleagues used AnxA5 blocking experiments to demonstrate that PS is not critical for the uptake and subsequent cross-presentation of dying tumor cells by $CD8a^+$ DCs, and also that granzymes are involved in regulating the phagocytosis of killed tumor cells [118]. Vaccination

with irradiated tumor cells that have been preincubated with AnxA5 has been shown to result in the cure of established tumors in a pre-clinical mouse model [119]. *In vitro* experiments revealed that AnxA5 shifts the cytokine release by activated macrophages that are phagocytosing irradiated tumor cells from an anti-inflammatory phenotype to an inflammatory phenotype (increased TNF-alpha and IL-1 beta and decreased TGF-beta levels). Combining RT with AnxA5 results in the most effective inhibition of tumor growth , as compared to treatment with RT or AnxA5 alone [117]. Recent work has identified additional Annexins (AnxA3, AnxA4, AnxA13 but not AnxA8) that can bind to PS which is exposed on dying (tumor) cells [120]. Studies are also now examining the expression of AnxA5 during RT and its potential role in tumor clearance. For locally advanced rectal cancer, an increased mRNA expression of AnxA5, but not AnxA4 was found in tumor tissue after radiochemotherapy [121]. Elevated serum AnxA5 level have been found under inflammatory conditions that are associated with higher IL-1 beta concentrations [122] and it will be of interest to elucidate whether tumor cells can secrete AnxA5 during therapy and thereby mediate their recognition by DCs and subsequent killing *via* CTLs. The modulation of inflammation and immunity by dying cells and the influence of the PS ligand AnxA5 has been considered in more detail elsewhere [112, 123].

In addition to dying (tumor) cells, viable malignantly transformed cells and the tumor vasculature endothelium also expose PS [124]. Thorpe and colleagues have generated a murine monoclonal antibody (2aG4) which binds to PS which is exposed on the vasculature and tumor cells. 2aG4 can inhibit the immune suppressive activity of PS which is exposed on tumor cells and promotes the localization of macrophages to the tumor vessels and the consequential induction of ADCC [125]. The administration of 2aG4 alone has been shown to be as effective at increasing the survival of animals as RT in an orthotropic rat model of glioblastoma. A combined treatment of local RT and 2aG4 results in significantly prolonged survival of the rats and, of note, 13% of the rats were disease-free after the combined treatment and were also protected against tumor re-challenge [126]. Phase I and II clinical trials with the human-mouse chimeric antibody bavituximab (human analogue to 2aG4 antibody) in combination with standard therapies for the treatment of solid tumors like breast and lung cancer are currently ongoing [127]. RT and CT lead to exposure of PS and are therefore ideal therapy combination partners with such PS-targeting antibodies [128]. Very recently, a whole cell-based cancer vaccine, the likes of which often lack immune co-stimulatory signals, was combined with a fusion protein of 2aG4 and IL-2. Il-2 has potent immune activatory properties and efficiently expands NK and $CD8^+$ T cell populations [129]. The vaccination of mice with irradiated tumor cells that were coated with the fusion protein resulted in immune protection, the inhibition of lung metastases, an enhancement of tumor-specific CTL responses, and fostered tumor-specific IFN-gamma responses [125]. Another PS binding antibody which binds to anionic phospholipids in a beta2-glycoprotein I-dependent manner (3G4) has been shown to inhibit tumor growth in rodents by inducing ADCC which is targeted towards the tumor blood vessels. The 3G4 antibody thereby significantly enhanced the efficacy of chemotherapeutics in treating solid tumors [130].

We are currently investigating the effects of the PS-binding protein AnxA5 on anti-tumor responses when combined with clinically relevant doses of fractionated irradiation. The initial results suggest that this combination is capable of retarding tumor growth. Another clinically-relevant property of AnxA5 with respect to its use in anti-cancer therapy results from its ability to form a two-dimensional network of trimers when binding to PS on the cell surface. This results in membrane bending, vesicle formation and transport into the cytosol [131]. AnxA5 might therefore be used to deliver drugs into tumor cells that have been induced to expose PS [132] by interventions such as irradiation [133]. A very recent finding has also revealed that PS is preferentially exposed by non-dying tumor cells in metastases. PS could therefore serve as marker of metastases as well as being a target for novel therapeutic approaches [134].

In summary, therapies which selectively induce apoptotic tumor cells might not be beneficial to the induction of protective anti-tumor responses due to the potential for promoting an immune suppressive environment. Agents that target PS and thereby stimulate an immune response should be considered as adjuvants in standard therapies. Although the induction of an appropriately timed period of inflammation by necrosis might favour the induction of specific anti-tumor immune responses by releasing danger signals (Fig. **2**), it may also foster angiogenesis and cancer cell proliferation (reviewed in [47]). Insight into cell death and clearance mechanisms should provide a basis on which to better understand how inflammation in general and cancer in particular can be modulated [104].

Immunogenicity Determining Code of Tumor Cells

It should be remembered that the concept of apoptotic cells inducing immune suppression and necrotic cells chronic inflammation is simplified. The finding that very early or pre-apoptotic cells expose calreticulin (CRT) and become immunogenic rather than immune suppressive by Obeid and colleagues [34] has opened a large area of research which is focussed on immunogenic cancer cell death. Although key experiments have revealed that tumor cell death induced by anthracyclines has immunogenic features [135], a number of factors and features define the immunogenicity of a dying cell.

Calreticulin (CRT)

CRT is a Ca^{2+}-binding protein which is present in the lumen of the endoplasmic reticulum and is therefore, under physiological conditions, always located intracellularly. Extracellular CRT was initially found to form a receptor complex with CD91 on phagocytes that drives the phagocytosis of opsonized apoptotic cells [136, 137]. Some years ago, CRT was also shown to become exposed, together with its binding partner ERp57, on cells very early and even before PS exposure following the encounter with a death stimulus from certain chemotherapeutic agents [34]. Exposed CRT/ERp57 consecutively facilitates the engulfment of the treated cells by DCs. Future research will have to define the mechanism(s) *via* which similar chemotherapeutic agents differentially induce CRT/ERp57 exposure on tumor cells [138].

In addition to rendering tumor cell visible to immune cells by inducing the exposure of normally hidden proteins, the release of immune modulating danger signals contributes to the induction of anti-tumor immunity. Often a combination of signals leads to immune activation. Mobilization of CRT and HMGB1 has been shown to efficiently foster the differentiation of monocytes into clinically effective DCs [139]. Different danger signals modulate the immune system *via* distinct mechanisms.

ATP

Adenosine triphosphate (ATP), the main energy-transfer molecule in the cell and important coenzyme, has been found to act as an immune modulating danger signal by fostering the maturation and activation of DCs [140]. Released ATP engages and acts on P2X purinoceptor 7 on APCs. Chemotherapeutic agents such as Oxaliplatin lead to ATP release and the activation of the inflammasome in DCs [141]. Activation of the inflammasome eventually results in the release of IL-1 beta [142, 143]. In addition to fostering vascularization and cell proliferation, IL-1 beta is mandatory for the priming of $CD8^+$ T cells to produce IFN-gamma [141]. A loss of function polymorphism which affects the P2RX(7) receptor in humans has been shown to have a significant negative

prognostic impact on the metastatic disease-free survival of women with breast cancer [141]. Besides activation of the inflammasome, ATP also attracts phagocytes to the site of dying cells [144] and caspase-dependent release of ATP by apoptotic tumor cells induced by RT might therefore lead to phagocyte attraction, and RT-induced necrosis to DC activation. The use of RT or CT to induce a balanced profile of apoptotic and necrotic tumor cells might therefore be most beneficial for initiating anti-tumor immune responses. The depletion of cytochrome c in colorectal tumor cells results in a mixture of cell death forms and has been shown to induce specific anti-tumor immune responses [145].

HMGB1

Like ATP, released HMGB1 acts on DCs in the tumor microenvironment. HMGB1 has been found to be present in the supernatants of necrotic tumor cells [146] and Sauter and colleagues have shown that HMGB1-containing supernatants from necrotic tumor cells foster antigen cross-presentation by DCs [147]. Furthermore, HMGB1 has been shown to be required for DC migration [148]. Glioblastoma-infiltrating $CD11c^+$ DCs have been shown to be derived from bone marrow and that their expression of TLR2 is mandatory for the induction of tumor regression. Curtin and colleagues have identified HMGB1 as a ligand for TLR2 and responsible for anti-tumor immune responses in glioblastoma. *In vitro* analyses have revealed that HMGB1 is released from various tumor cells after RT, CT or gene therapy [149, 150]. However, it should be noted that in addition to activating DCs and stimulating specific anti-tumor responses, HMGB1 could also foster tumor progression, angiogenesis, evasion of apoptosis, chronic inflammation and metastasis (summarised in [151]).

Heat Shock (Stress) Proteins (HSP)

Heat shock proteins (HSP) are also released by tumor cells under therapy conditions [152]. Extracellular HSP70 fosters DC maturation and an upregulation of homing receptors [153-155]. Srivastava and colleagues were the first to identify that antigenic peptides bound to HSP70 or HSP90 become strong ligands of MHC class I molecules and therefore discovered that intracellular chaperones can contribute to antigen processing [156]. Various intracellular chaperones have been shown to be capable of binding intracellular peptides and thereby form potent immunogenic chaperone-peptide complexes, examples of which are HSP70 and HSP90 [157, 158], CRT [159], HSP110 and glucose-regulated protein 170 [160]. However, antigen delivery to DCs assumes that HSP/peptide complexes are released and are taken up by DCs. The existence of receptors for HSPs on DCs has been identified by electron microscope studies that proved that HSP bind specifically to the surface of APC and are spontaneously internalized by receptor-mediated endocytosis [161]. The ability of HSPs to activate APCs and facilitate the priming of CTLs against the peptides that are associated with the HSP, is another important requirement for HSP-mediated specific immunity [162].

But how are HSPs released from tumor cells? It is certainly known that necrotic, but not apoptotic cells passively release HSPs [163]. During apoptotic cell death, HSP might be released in exosomes or blebs within membranous structures [164]. The release of microparticles and danger signals such as HMGB1 shows strong similarities since it occurs with cell death and both may lead to stimulation of distinct TLRs [165]. HSPs also interact with phospholipids in cell membranes and therefore might be shuffled across the membrane *via* regular flip-flop mechanisms [96]. Furthermore, the anchorage of a membrane form of HSP70 which is preferentially expressed by tumor cells has been shown to be enabled by the glycosphingolipid Gb3 [166]. Taken together, HSPs can be regarded as being involved in the stimulation of DCs and the presentation of tumor antigens to effector cell populations. A detailed overview into the role of HSPs in anti-tumor immune responses is given by Schmid and Multhoff elsewhere in this issue [167].

S100 Proteins

In addition to tumor cells, danger signals can also be actively secreted during the stress response by immune cells like macrophages [168, 169]. As oxidative stress is another main stimulus for the induction of active HMGB1 release [170], reactive oxygen species (ROS) that are generated by RT might also foster an active secretion of HMGB1 by macrophages and passive release by necrotic tumor cells. Calprotectin, a complex of S100A8 and S100A9 protein is also actively secreted by macrophages during stress responses [171]. Like some other DAMPs, these proteins lack a leader signal and are therefore secreted *via* non-classical pathways [35].

Uric Acid

Another alarmin or danger signal is uric acid. It stimulates DC activation and CTL responses [172]. Inflammatory diseases such as gout are characterized by an accumulation of uric acid crystals which, like ATP, activate the inflammasome and result in IL-1 beta and IL-18 secretion [173]. However, circulating IL-18 might also suppress NK cell-mediated tumor defence mechanisms [174]. Sodium overload inside the cell after phagocytosis of uric acid crystals and consecutive water influx is currently considered as being a mechanism for inflammasome activation [175].

Granulysin

Granulysin is present in the granules of CTL and NK cells and has recently been identified as exhibiting alarmin properties. It is the first alarmin which is released by lymphocytes, it attracts DCs and it induces their maturation and activation. The latter is dependent on MyD88 and requires TLR4 [176].

Extracellular Matrix Proteins

Apart from intracellular components, extracellular matrix molecules (ECM) that interact with TLR on phagocytes also function as (endogenous) danger signals. Of note is the observation that ROS formation is involved in biglycan-mediated activation of the inflammasome [177]. Various other ECM molecules can alert the immune system danger after RT and/or CT induced tissue damage. Matrix constituents that signal *via* TLR might therefore modulate immunity (summarized in [178]).

Radiotherapy Combined with Immune Activators

Ongoing research is attempting to identify additional immune modulating danger signals that are actively or passively released by tumor cells or surrounding immune cells after RT and CT. Engineered immune stimulators such as granulocyte-macrophage colony-stimulating factor (GM-CSF) [179] might further enhance the immunogenic potential of tumor cells that have been killed by standard therapies or used as whole-cell vaccines. Immune infiltrates in pre-existing metastases that have been promoted by the administration of GM-CSF are associated with tumor destruction [179]. Combined treatment with lower doses of CT plus GM-CSF secreting whole cancer cell vaccines might therefore overcome immune tolerance and induce anti-tumor immune responses [180]. It is important to note that immune responses against multiple antigens are associated with efficient tumor destruction [181].

Besides combining whole tumor cell vaccines with immune stimulators, the joint application of local RT and T_H1 therapy in order to stimulate cellular anti-tumor immunity has been shown to be beneficial. The frequency of tumor-specific CTLs is significantly enhanced when local X-ray treatment is combined with T_H1 cell transfer. This combination therapy has been shown to result in the eradication of EG7 tumors in mice and immunological memory against the tumor [23]. Radio-resistant lung tumors can also be

rendered radio-curable when cytosine-phosphatidyl-guanosine (CpG) is given in addition to RT [182]. The CpG dinucleotid is a TLR9 antagonist and might induce anti-tumor immunity *via* mediating a cross-talk between classical and plasmacytoid DCs [183]. Some work even suggests that surviving immune cells that have been in the radiation field undergo functional changes. In pre-clinical models, antigen presentation by surviving DCs has been found to be enhanced after RT [184].

Returning to the clinical situation, Schaue and colleagues have examined whether tumor-specific T cell immunity is modulated by RT or RCT. For these studies, they isolated lymphocytes from patients with colorectal or prostate cancer before, during and after therapy. A significant increase of survivin-specific T cells was detected, primarily in patients with colorectal cancer that had a tumor which was down-staged by RCT [185]. High rates of clinical responses of many tumor entities were found in patients when chemotherapy is combined with cancer vaccines. A high rate of objective clinical responses to CT in patients with small cell lung cancer (over 60%) was observed when CT immediately followed vaccination with p53 transduced DCs [186]. A pre-vaccination may result in longer response rates to the chemotherapeutic agent docetaxel in patients with metastatic, androgen-independent prostate cancer [187]. The best response rates against Glioblastoma multiforme have been observed in responders DC vaccinations who received post-vaccine CT [188, 189]. Pre-clinical models have revealed that CT is capable of rendering tumor cells more susceptible to the cytotoxic effect of $CD8^+$ T cells *via* a perforin-independent increase in the permeability to CTL-derived granzymes [73]. It appears that CT modifies tumor cell membranes and renders them more prone to other damaging agents. In a similar way to CT, irradiation of tumors may stimulate anti-tumor immunity and thereby also the targeting of metastases [190]. Certain drugs such as histone deacetylases inhibitors are able to enhance the expression of major histocompatibility complex class I-related molecules (MIC), MICA and MICB, on tumor cells [191]. Such stress ligands are also present on those tumor cells that became recognized by the immune system and eliminated during the immune editing process (as previously outlined in detail in section 4) [1]. We are currently investigating whether fractionated radiation also modifies MICA/B expression and thereby renders tumors immunogenic again. Just recently, Finkelstein and colleagues reported that the administration of DCs into the tumors, combined with conventionally fractionated external beam RT may lead to anti-tumor immune responses. DCs were injected once at the end of the week at the second, third and fourth treatment cycle with RT. More than 50% of the high grade soft tissue sarcoma patients developed anti-tumor immunity and more than 70% of the patients were free of progression after 12 months. Non-responders had high levels of MDSC in the tumor microenvironment, whereas responders displayed high levels of infiltrating $CD8^+$ and $CD4^+$ T cells. *In vivo* labelling assays revealed that DCs require 48 hours to migrate to the LN [188]. One has to conclude that a distinct period of time (at least 2 days) after the application of immune therapy should be allowed, during which no further irradiation takes place in order to ensure that activated DCs are not killed. We are currently combining fractionated RT with AnxA5 application in pre-clinical mouse models to evaluate the optimal time frame and sequence for combing RT with immune activators. A concept relating to how anti-cancer therapies might be further improved in the near and median future, based on the mentioned pre-clinical and clinical data and observations, is displayed in Fig (**3**). Innovative fractionation schemes of RT should allow the immune system space and time to act. Hypofractionation with higher single doses might return to routine clinical practice in the future. The current status of hypofractionation protocols in RT have been summarised elsewhere [192]. The strong connection of molecular radiobiology, immunology and clinical radiation oncology will exploit further synergies between RT and the immune system and should lead to further improvements in cancer therapies in the future [193].

6. SUMMARY AND OUTLOOK

In summary, the relevance of the immune system to the success of cancer therapy has mostly been evaluated in pre-clinical mouse

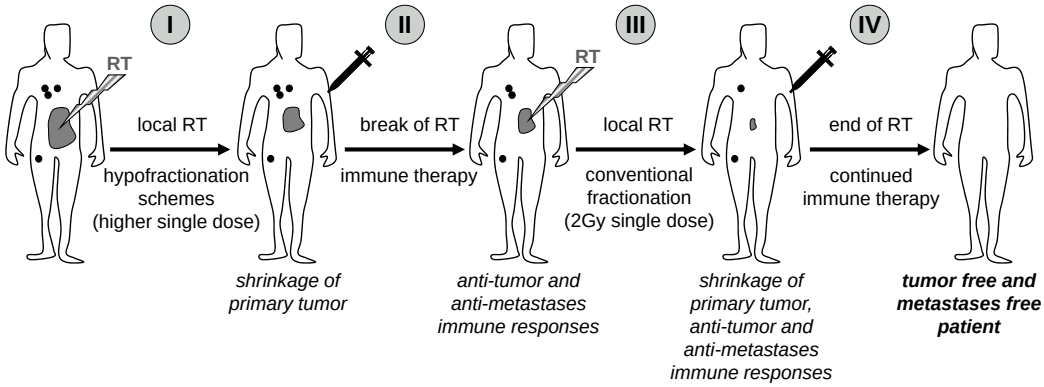

Fig. (3). Proposed therapy scheme for combining RT with immune therapy. The primary aim of RT has to date been local tumor control. However, it is now apparent that under distinct conditions RT alone or combined with immune modulators can modulate the tumor in a way which renders it visible to the immune system. The following scheme for inducing specific and long lasting anti-tumor immunity is therefore proposed: (I) Treatment should be started with a hypofractionated radiation of the tumor (e.g. 3 x 3.3 Gy, also resulting in conventionally applied weekly dose of 10 Gy) which has the capacity to induce tumor cell necr(opt)osis and the release of immune activating danger signals. After 3 radiation days, a break of 2 (radiation days saved compared to 5 x 2Gy) plus 2 (weekend days) days = 4 days gives the immune cells time to react. (II) Further immune stimulation (e.g. the application of AnxA5, see also Figure 2, or vaccination with inactivated tumor cells) promotes leukocyte infiltration, the uptake of tumor antigen by DCs, the migration of DCs to sentinel lymph nodes, the priming of CTLs by DCs, the migration of CTLs to the tumor and its metastases, and finally tumor attack. (III) Continuance of RT with conventional fractionation scheme (5 x 2Gy) further reduces the size of the tumor and has less effect on the immune cells that are already activated and distributed in the whole body. (IV) To boost the immune system, after a break of at least 2 days after the last irradiation of the primary tumor, a further immune therapy should follow the RT. Memory immune cells and newly generated ones now have the environment in which to specifically target the remainder of the primary tumor, metastases and clinically not detectable residual tumor cells.

Gy: Gray; RT: radiotherapy with ionizing radiation.

models. However, as discussed by Zitvogel and colleagues [194], this "onco-immunological" concept also applies to the clinical situation. It is clear that complex immunostimulatory and immunosuppressive forces are present in the tumor microenvironment [93]. The significance of DCs and pattern recognition receptors (TLRs) to protection against infections, cancer, and inflammatory diseases was recognized with the Nobel Prize in Physiology or Medicine 2011 for Drs. Beutler, Hoffmann, and Steinman [195-197]. The induction of tumor cell death by CT, RT, or RCT and its consequential enhancement of tumor immunogenicity and DC-dependent priming of CTLs should be considered as key steps for a successful cancer (immune) therapy (Fig. **3**). It will be important to shed more light into what effect conventional cancer interventions such as RT can have on the equilibrium phase of a tumor [1]. One of the biggest challenges will be to specifically trigger immunogenic tumor cell death and concomitantly avoid the induction of tolerance [198]. The interactions of dying tumor cells with immune cells of the tumor microenvironment provide a basis for the development of novel therapeutic approaches for inflammatory and malignant diseases [98]. Combined treatment strategies which have the capacity to efficiently induce tumor cell death, activate the immune system and concomitantly promote the expression of certain damage-associated molecular patterns should be elucidated [113]. Improved immune monitoring methods will provide a better understanding of the complex interplay between cancer parenchyma, stroma, and immune effectors [199]. The development of fast and easy to apply routine detection methods of immune-related parameters is mandatory for the clinical management of cancer patients and the adaptation and optimization of therapy, Of note, the mode of cancer cell death induced by standard tumor therapies and the further handling of the dying tumor cells by immune cells strongly contributes to the efficacy of anti-tumor immune responses [9].

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ABBREVIATIONS

Ab	=	Antibody
ADCC	=	Antibody-dependent cell-mediated cytotoxicity
Ag	=	Antigen
AnxA5	=	AnnexinA5
APC	=	Antigen presenting cell
ATP	=	Adenosine triphosphate
CCL	=	CC chemokine ligand
CpG	=	Cytosine-phosphatidyl-guanosine
CT	=	Chemotherapy
CTL	=	Cytotoxic T lymphocyte
CRT	=	Calreticulin
DAMP	=	Damage-associated molecular pattern
DC	=	Dendritic cell
DNA	=	Deoxyribonucleic acid
ECM	=	Extracellular matrix molecules
FasL/R	=	Fas ligand/receptor
5-FU	=	5- Fluorouracil
G1/2 phase	=	Gap1/2 phase of the cell cycle
GM-CSF	=	Granulocyte-monocyte colony-stimulating factor
Gy	=	Gray
IL	=	Interleukin
IC	=	Immune cell
HMGB1	=	High-mobility group protein B1
HSP	=	Heat-shock protein
INF	=	Interferon
LN	=	Lymph node
MDSC	=	Myeloid derived suppressor cell
MHC	=	Major histocompatibility complex
MIC	=	Major histocompatibility complex class I-related molecules
NKDC	=	Natural killer dendritic cells
NK cell	=	Natural killer cell
PAMP	=	Pathogen associated molecular pattern
PS	=	Phosphatidylserine
RCT	=	Radiochemotherapy
RIP-1	=	Receptor interacting protein-1
ROS	=	Reactive oxygen species
RT	=	Radiotherapy
TAA	=	Tumor-associated antigen
TC	=	Tumor cell
T_H	=	T helper
TLR	=	Toll like receptor
TNF	=	Tumor necrosis factor
TRAIL	=	TNF-related apoptosis-inducing ligand
X-ray	=	ionizing irradiation

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