INTERPLAY BETWEEN IMMUNE AND TUMOR CELLS IN THE OUTCOME OF RADIOTHERAPY

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Introduction:

Radiotherapy is a commonly used treatment modality for a vast majority of cancer patients. This can be achieved through (a) external beam therapy, in which a focused beam of radiation from a radioactive source is directed onto the tumors, (b) brachytherapy, in which a radioactive source is positioned internally close to the tumor, or (c) nuclear medicine, in which radioisotope-tagged compounds are injected into the patient's body. Due to the high cost involved in setting up of radiotherapy facilities as compared to surgery or chemotherapy, there is a substantial disparity in the distribution of these devices around the world, with the majority found in high-income nations and less than required equipment found in middle- and low-income countries. Bhabha Atomic Research Centre and Panacea Medical in India have crossed that gap by delivering indigenously manufactured Bhabhatron and Bhabhatron II units to many nations[5-7]. Currently India has approximately 545 teletherapy machines, 22 advanced therapy machines and 250 brachytherapy units[11]. Board of Radiation and Isotope Technology (BRIT), an independent unit under Department of Atomic Energy supplies encapsulated Cobalt-60 sources utilised in these teletherapy equipment and sources for brachytherapy such as Iridium-192 and Cesium-137. Nuclear medicine utilises radiopharmaceuticals that are injected into the patient’s body whose signals are detected by gamma camera, an instrument that can detect gamma radiation. The first gamma camera for nuclear medicine was installed in 1969 at Radiation Medicine Centre (RMC), Bhabha Atomic Research Centre. Currently there are 233 functioning gamma cameras (Single-photon emission computed tomography [SPECT]/SPECT–computed tomography [CT]) units, 222 positron emission tomography (PET)-CT, 3 PET-magnetic resonance imaging scanners, and 19 cyclotrons in India. SPECT uses a rotating gamma camera to obtain multiple image of the organ under investigation[12].

Traditionally, radiotherapy was considered immunosuppressive, with only a direct cytotoxic effect on the tumor cells. But in recent times, it has been appreciated that radiotherapy also

initiates innate and adaptive immune responses resulting in systemic anti-tumorigenic effects. This is believed to be due to the radiation-induced remodelling of the tumor microenvironment as dying cancer cells engage with innate immune cells triggering off a series of events resulting in immune system activation. A deeper understanding of the immunological consequences of radiation therapy can assist us in developing appropriate therapeutic combinations with other treatment modalities and identifying new ones that can be used in conjunction with radiotherapy. For this, basic research is being conducted at Immunology Section, Radiation Biology & Health Sciences Division to understand how the immune cells and the tumor interact in the microenvironment following exposure to radiation and some of the factors that affect radiotherapy outcomes.

1. Factors that affect radiation therapy: Radiotherapy outcomes are influenced by three main factors (Figure 1)[13]. These are (a) treatment characteristics or features of the radiation therapy, that is, the radiation dose, fractionation regimen, and the sequence of combination with other treatments. (b) The tumor characteristics have a significant impact on the outcome of radiotherapy. These parameters include the cell type, its p53 status, the cell cycle phase during radiotherapy, the extent of immune infiltration during radiotherapy, as well as microenvironment factors like oxygen tension, pH, angiogenesis, etc. (c) Another key factor that influences the outcome of radiotherapy is the host characteristics, which include the immunological status of the individual prior to radiotherapy as well as single nucleotide polymorphisms (SNPs), which determine the DNA repair response.

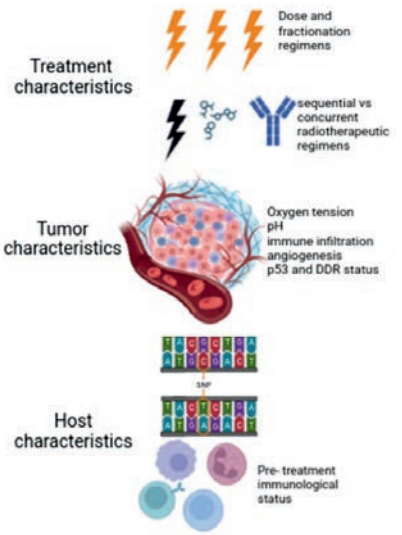

Figure 1: Factors that affect the radiation therapy outcome (adapted from [8]). DDR- DNA damage response.

1.a. Treatment characteristics: Radiotherapy for cancer started shortly following the seminal discoveries of X-rays in 1895 by Roentgen, radioactivity in 1896 by Henri Becquerel, and radium in 1898 by Marie Curie. When radiation was first used for therapy, it was applied widely without a complete understanding of the harm it could do to healthy

tissues. It was unknown how radiation interacted with biological matter or about the radiosensitivity of different tissues. The negative effects of radiation were lessened in the 1920s as a result of several developments. Among them were the recognition of the value of fractionation in cancer control, the discovery of dosimetry to measure the delivered dose, and the founding of the International Commission on Radiation Protection (ICRP) in 1928 to address the question of radioprotection.

The main objective of radiation therapy has always been to improve the precision and accuracy of dose distribution to the tumor that has largely been achieved due to key developments in radiation dose delivery and imaging modalities. The physical dose or biological dose to the tumor can be increased by the use of tissue compensators, wedges, 3-dimensional conformation radiation therapy (3D CRT), intensity modulated radiation therapy (IMRT), stereotactic radiosurgery (SRS), and stereotactic body radiotherapy (SBRT)[14]. The treatment regimen in conventional radiotherapy is designed in such a way that the target area is exposed to overlapping homogeneous beams that deliver a uniform dose to the tumor. However, factors like irregular patient surfaces can result in an uneven dose distribution. All the techniques mentioned above are used to adjust the beam in order to achieve a homogeneous dose distribution. A wedge or patient-specific compensators are made of high-density metals and are used to attenuate the beam. The radiation beams were previously matched to the tumor's height and width, which also exposed the surrounding normal tissues, and led to many side effects. But with advances in imaging techniques like 3D CRT, doctors are able to guide multiple beams to administer highly concentrated radiation to precisely target tumor shapes, particularly those that are irregularly shaped while sparing the normal tissue. IMRT is an advanced type of high-precision radiotherapy that uses computer-controlled linear accelerators to target the tumor with precise radiation dosages. The delivery of a precise customized radiation dose that will best conform to the tumor shape will allow for the delivery of the maximum dose to the tumor and minimum dose to the surrounding normal tissues. This is made possible by the use of 3-D computed tomography (CT) or magnetic resonance imaging (MRI) images of the patient along with computerized dose calculations. Stereotactic radiosurgery (SRS) is a non-invasive radiation therapy that treats cancers in the brain, neck, spine, lungs, and other regions of the body by using highly focused radiation beams. Other than the brain and skull, malignancies in various areas of the body can be treated with stereotactic body radiation (SBRT).

The biological dose can be altered by using (a) different fractionation schedules; (b) sequential or concurrent chemotherapy; targeted therapy; immunotherapies; and (c) various biological response modifiers to increase radiosensitivity. There are three main variations of fractionation that are used. These include (i) hyperfractionation, which delivers the total dose across a greater number of smaller individual treatment doses; (ii) accelerated fractionation, which shortens the time interval between fractions by increasing the number of individual treatments each day; and (iii) hypofractionation, a technique in which the total dose is provided in fewer fractions by increasing daily individual treatment doses. When two cancer treatment modalities are combined, they can be delivered either concurrently, that is, both the treatment modalities are given at the same time, or sequentially, where treatment modalities like chemotherapy are administered before or after the completion of radiotherapy. In general, concurrent chemoradiotherapy has been shown to be superior as compared to

sequential chemoradiotherapy; however, this would depend on the type, stage, and grade of cancer[15]. Targeted therapies are usually small-molecule inhibitors of a target enzyme (such as Gefitinib, a tyrosine kinase inhibitor). There are a number of treatment options for these patients that can be taken into account, including concomitant treatment, temporary drug withdrawal, radiotherapy plan adjustments, or radiotherapy dose decrease based on the toxicity or side effects, as the data regarding the regimen with which they can be combined with radiotherapy is sketchy.

Modern immunotherapy approaches target a variety of processes, but the most commonly used are checkpoint inhibitors, which neutralize inhibitory signals of T cell activation, promoting tumor cell destruction by host T cells. The anti-CTLA4 (cytotoxic T-lymphocyte-associated protein 4) antibody ipilimumab was first licensed for therapy in 2011, followed by monoclonal PD-1(programmed cell death protein 1) blocking antibodies, and anti-PD-L1 (programmed cell death ligand 1) antibodies. Many preclinical and clinical studies are being conducted to evaluate the efficacy of combining immunotherapy and radiotherapy, with promising results. However, the sequence in which they have to be delivered is not very clear. We carried out experiments to determine the efficacy of concurrent radio and immunotherapy in 4T1 mammary cancer and WEHI fibrosarcoma. There was no significant difference in tumor burden when anti-PD-1 antibodies were given concurrently with localized hypofractionated exposures (Figure 2). We have earlier demonstrated that cyclooxygenase (COX-2) inhibitor NS-398 and the transforming growth factor (TGF-β)R inhibitor SB431542 avert tumor induced immune dysfunction, are immunomodulatory as single agents, and cause a decrease in tumor burden[16, 17]. So, we combined COX-2 inhibitor NS-398 (Figure 2a) or TGF-βR inhibitor SB431542 (Figure 2b) with radiotherapy and immunotherapy regimen in a sequential manner, and observed a significant decrease in the combination treatment as compared to the single agents. These results demonstrate that using an immunomodulator adjuvant along with radio- immunotherapy may be more advantageous as compared to the combination of only radiotherapy and immunotherapy.

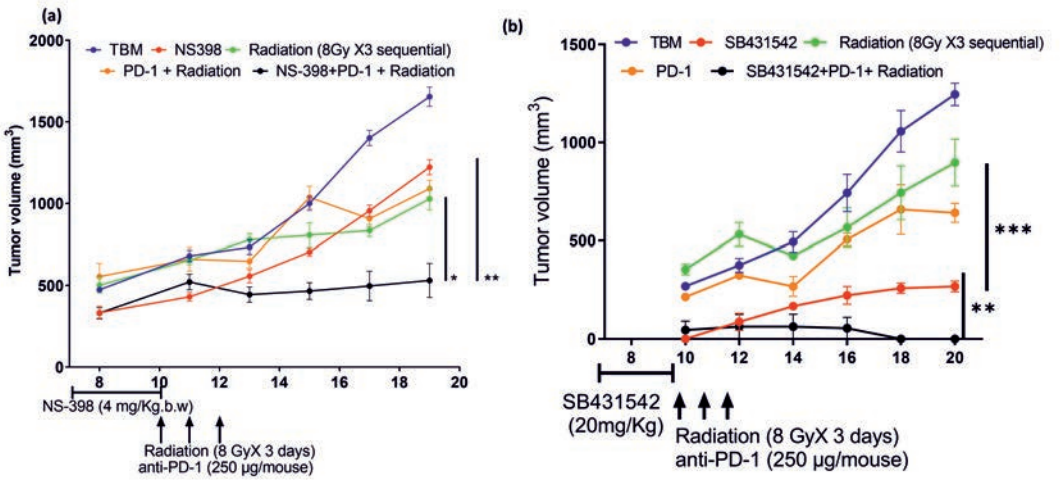

Figure 2: Effect of radiation and immunotherapy (anti-PD-1 antibody) on tumor burden in combination with (a) COX-2 inhibitor in 4T1 murine mammary cancer (b) TGF-βR inhibitor in WEHI-164 fibrosarcoma.

1.b. Tumor characteristics: Complete tumor control or total cure is possible only if the surviving tumor cells after radiation exposure becomes zero. Therefore, radiation-induced cell death is a crucial indicator of the therapeutic effect, and the survival fraction is widely considered to be a gold standard measurement. Cell survival curves depict the relationship between the surviving fraction of cells, that is, the proportion of irradiated cells that maintain their reproductive integrity (clonogenic cells), and the absorbed dose (Figure 3). Many radiobiological models have been devised to understand and explain the survival curves obtained with ionizing radiation exposure[18]. At the molecular level, it is generally considered that radiation-induced cell killing is caused by energy deposition in the nucleus, causing extensive DNA damage. The classic target theory, which postulates that DNA is the most important target for fatal effects of radiation exposure, was the first quantitative interpretative model for radiation-induced cell death. This model, however, could not explain DNA damage response or cellular DNA repair. These factors were incorporated in the linear quadratic model, which is widely used in radiobiological research and clinical radiotherapy to describe radiation response of the tumor. In this model, the sensitivity of different tissue types to fractionation is determined by the α/β ratio which was developed to account for these variables. The product of the two Poisson escape probabilities for single-hit and double-hit inactivation events, respectively, describes cell death, in this model.[19]

$$S/S_o = e^{-\alpha D - \beta D^2}$$

S/So = surviving fraction, α = co-efficient that represents cell death from 'single hit' events, or lethal damage caused by a single incident particle, β = coefficient that represents cell death from 'multiple hit' events, or, cell death caused by the interaction of damage from various radiation tracks, that increases in proportion to the square of the dose (no repair), and D = dose. Coefficients α and β describe the cell's radiosensitivity. Cells with high α/β ratios have negligible DNA repair and undergo a relatively constant rate of cell killing with increasing dose, while those with a low α/β ratio exhibit a substantial curvature indicative of repair at lower doses (Figure 3).

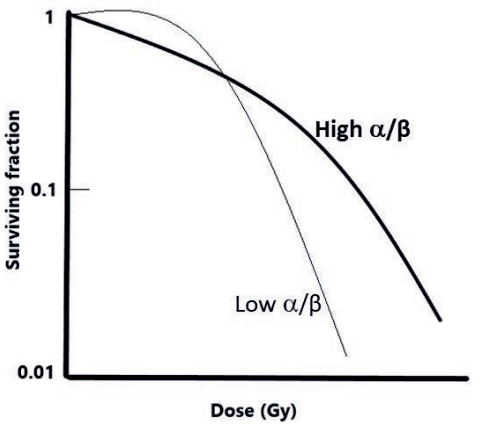

Figure 3: Cell survival curves for tissues that respond quickly or slowly. Early or quick responding tissues are rapidly proliferating cells such as skin, oral mucosa and bone marrow have high α/β ratios, (7–10 Gy). In contrast, tissues with slower cellular turnover, such as heart, lung or kidney have α/β ratios of 3–5 Gy and are slow or late responding (adapted from [4]).

The goal of radiotherapy is to expose the tumor with high enough radiation to trigger cell death while sparing nearby normal tissues from irradiation that can result in side effects. Radiotherapy is delivered in fractions to maintain this balance, and the basis of fractionation can be understood in terms of $5R^{20}$. These include (a) repair: sublethal damage is repaired in the time interval between two fractions (b) redistribution: progression of cells within the cell cycle from a relatively radioresistant to radiosensitive phase (c) reoxygenation: as tumor cells in the periphery are killed, surviving cells in the hypoxic core of the tumors are reoxygenated, increasing their sensitivity to further fractions (d) repopulation: active proliferation by some cells in response to cytotoxic insults; (e) the tissue's inherent radiosensitivity. Radiotherapy can cause various types of damage, including lethal damage, which leads to cell death, and sublethal damage, that can be repaired with appropriate recovery conditions. A higher dose per fraction of radiotherapy is likely to cause irreversible lethal damage, whereas a lower dose per fraction allows for recovery periods in between, allowing for repair. During radiation exposures, the most crucial factors are the homogeneity of cell populations and minimizing sublethal damage repair.

Table 1: TGF-β signalling, hybrid epithelial-mesenchymal phenotype and cancer stem cells contribute to radio resistance in breast cancer cells [3].

	Gene expression	Irradiated MCF 7 cells after recovery period	Irradiated MDA-MB-231 cells after recovery period
TGF-β1 isoforms	TGF-β1	↑	↑
	TGF-β2	↑↑↑	↑↑
	TGF-β3	↑↑	↑↑↑
TGF-β receptors	TGF-βR1	↑↑	↑↑↑
	TGF-βR2	↑↑	↑↑↑
TGF-β downstream transcription factors	Snail	↑↑	↑↑↑
	Zeb-1	↑↑	↑↑↑
	HMGA2	↑↑	↑↑↑
Epithelial genes	E-cadherin	↑↑	↑↑↑
	Occludin	↑↑	↑↑
	Desmoplankin	↑	↑↑↑
Mesenchymal genes	N-cadherin	↑↑↑	↑↑↑
Cancer stem cells	$CD44^{+}24^{-}$ cells	↑↑	↑↑
	ALDH activity	↑↑↑	↑↑↑
Stem cell transcription factors	Oct-4	↑↑↑	↑
	Sox-2	↑↑↑	↑↑↑
	Nanog	↑↑↑	↑↑↑

To better understand the repair of sublethal damage and the changes it can cause, we irradiated MCF7 and MDA-MB-231 breast cancer cell lines with a dose of 6 Gy and allowed to recover for 7-days. This dose and time were found to be optimal for recovery following initial standardisation experiments. We named these as D7-6G cells and conducted several experiments to better understand the changes happening in the surviving cells after ionizing radiation exposure and the repair of sublethal damage. D7-6G cells proliferate more due to an elevated expression of transforming growth factor isoforms (TGF)-β1, β2, and β3, their receptors TGF-βR1, and TGF-βR2 that was abrogated by treatment with a TGF-βRI inhibitor. This was also associated with an increase in the expression of TGF-β downstream transcription factors Snail, Zeb-1 and HMGA2. D7-6G cells from both breast cancer cell lines showed a hybrid epithelial-mesenchymal (E/M) phenotype with augmented migration and E/M marker expression. D7-6G cells expressed higher levels of cancer stem cell markers aldehyde dehydrogenase, Sox2, Oct4, and Nanog, and a higher proportion of $CD44^{+}CD24^{-}$ cells (Table 1). When subjected to a challenging dose of ionizing radiation, this was accompanied by radio resistance[3]. We also characterized the tumorigenesis capacity of these cells in SCID mice. SCID mice were subcutaneously injected with untreated (MCF-C) and D7-6G cells (MCF-R), and tumor development was tracked. MCF-R cells grew into larger tumors with a shorter latency period. When these tumors were analysed, they were found to have increased TGF-β signaling as characterised by elevated expression of the different isoforms (in serum and tumor tissues), their receptors, and their downstream transcription factors; Changes in migration and hybrid epithelial/mesenchymal phenotype were similar to *in vitro* experiments but more pronounced. Radio resistance following a challenge dose of radiation was also observed in these cells. Proteomic analysis of these tumors were carried out and data has been submitted in ProteomeXchange database with the identifier

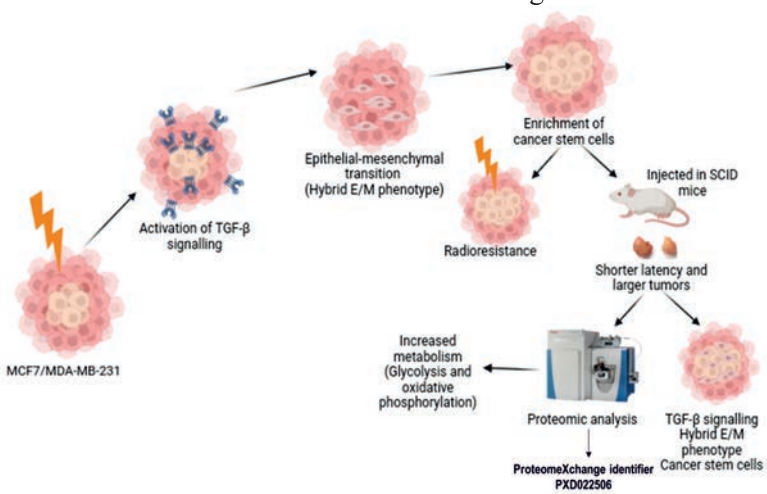

Figure 4: Role of TGF-β signalling in emergence of radio resistance phenotype in breast cancer.

PXD022506. Data analysis indicated enrichment of metabolism associated genes which was confirmed by increased oxygen consumption rate, extracellular acidification rate analysis as well as increased uptake of fluorescent analogue of glucose, 2-NBDG [2-N- (7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose. In summary, we demonstrated that enrichment of TGF-β signaling and increased metabolism contributes to radio resistance in breast cancer

cells following repair of sublethal DNA damage[2] (Figure 4). The effectiveness of radiotherapy depends upon the tumor size to be treated, with bigger tumors requiring a higher dose for local control. Conventional fractionation involves delivering a 1.8–2 Gy dose per fraction five times per week, amounting to a total dose of 9–10 Gy. Gross tumors require 70–80 Gy, and microscopic tumors require 45–50 Gy for tumor cell killing.

1.c. Host characteristics: The radiation sensitivity of tissues varies based on their proliferative ability and the differentiation of the constituent cells. The following is the increasing order of radio sensitivity with the most sensitive cells being those that divide regularly with no differentiation > cells that divide regularly with some differentiation between divisions > cells that divide at regular intervals in response to a need > cells that do not divide regularly, are variably differentiated > cells that do not divide and are highly differentiated. Based on these features, the gut, skin, bone marrow, and mucosa are the quick or acute responding tissues, whereas brain, spinal cord, kidney, and lung are the slow or late responding tissues (Figure 3). The radio sensitive cells die a mitotic death and cells that never divide are radio resistant and require very large doses to kill them. Lymphocytes are an exception to both of these rules. They rarely divide, die through interphase death and are among the most sensitive mammalian cells[21].

2. Interaction between tumor and immune cells following radiotherapy: The interaction between the tumor and immune cells following radiotherapy involves both tumor and host characteristics and can be classified into four categories (Figure 5).

(a) microenvironment changes; (b) leukocyte infiltration; (c) DC activation and T cell priming; (d) expression of stress-induced immunogenic molecules on tumor cells.

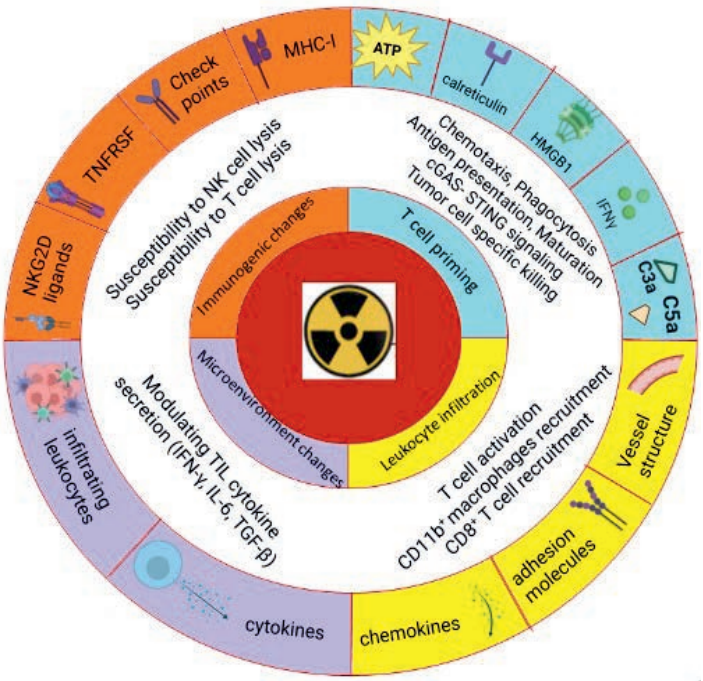

Figure 5: Radiotherapy induced changes in the immune system (adapted from[1]).

2.a. Microenvironment changes: The tumor microenvironment is the milieu in which the tumor cells coexist alongside lymphocytes, fibroblasts, the extracellular matrix, and the tumor vasculature. Depending on the stage of tumor cells and the type of invading immune cells, the tumor microenvironment can either enhance or inhibit the anti-tumor activities of immune cells. Radiotherapy affects tumor vasculature and endothelial cells in the microenvironment. High radiation doses impact endothelial cells, causing death, cell separation from the underlying basement membrane, and increased vascular permeability. This can result in hypoxia, which increases the expression of hypoxia-inducible factor-1 (HIF-1). HIF-1 overexpression can result in elevated levels of various chemokines and tumor-associated macrophages[22]. High doses of radiation can promote aggregation of platelets, formation of microthrombus, and enhanced adherence of inflammatory cells to endothelial cells inside the vasculature leading to extravasation and diapedesis into the perivascular space. These early effects are temporary and can be reversed 3–4 months after the completion of radiotherapy. The effect of radiotherapy on the microenvironment is determined by the type of tumor, their staging and grading, tumor vasculature, total dose and the number of fractions delivered. Endothelial cells proliferate rapidly and hence are extremely radiosensitive. Radiation-induced vascular damage causes tumor hypoxia as well as the leukocyte infiltration due to increased permeability, resulting in the release of several cytokines and chemokines. It has also been demonstrated that ionizing radiation increases the expression of the intercellular and vascular adhesion molecules ICAM-1 and VCAM-1[23].

2.b. Leukocyte infiltration: Radiation-induced damage to the endothelial system leading to leukocyte infiltration or extravasation is believed to be mainly responsible for the systemic inflammatory response, which requires the interaction of the integrin molecules on their surface with endothelial cell adhesion molecules ICAM-1 and VCAM-1. Several pro-inflammatory cytokines like tumor necrosis factor (TNF)-α, and interleukins (IL)-6, IL-17, chemokines, prostanoids, and significant amounts of reactive oxygen and nitrogen species (ROS and RNS) can be produced by these infiltrating leukocytes. All of these mediators contribute to amplifying and sustaining the inflammatory cascade. They can induce DNA damage as well as inhibit DNA repair mechanisms, resulting in genetic instability. Some of the cytokines, like TNF receptor-associated death-inducing ligand or TRAIL, can induce apoptosis of cancer cells. Sometimes, these cytokines can also be responsible for immune evasion and increased tumor growth and progression.

2.c. Dendritic cell (DC) activation and T cell priming: Regulated cell death induced by various stresses can be either immunogenic cell death or tolerogenic cell death (TCD). Immunogenic cell death (ICD) results in anti-tumor immunity mainly due to the release or exposure of damage-associated molecular patterns (DAMPs) that include the release of ATP, heat shock proteins, or the nuclear protein HMGB1, and calreticulin exposure on the surface of dying cells. Ionizing radiation has been shown to induce ICD, and this is influenced by the dose, type, and fractionation of the radiation exposure. ICD can be induced by certain chemotherapeutics and targeted anticancer agents as well. Tumor cells can undergo cell death by many different mechanisms, like apoptosis, necrosis, secondary necrosis, necroptosis, autophagy, ferroptosis, pyroptosis, and NETosis (cell death of neutrophils by forming neutrophil extracellular traps). Apoptosis or autophagy can induce TCD or ICD whereas

other modes of cell death predominantly induce ICD. The ability of the dying tumor cell to launch an adaptive immune response with the establishment of a memory response depends on three crucial characteristics of the dying cell. These are antigenicity, adjuvanticity, and the inflammatory microenvironment (Table 2). The antigenicity is due to the development of neo-epitopes that arise as a result of increased mutation burden or protein post-translational modifications. The absence of antigens results in an inflammatory response. In the presence of antigenicity, adaptive immune responses can be initiated only if accompanied by adjuvanticity (contributed by DAMPs, calreticulin exposure, release of ATP, HMGB1, Hsp70), the absence of which results in tolerance. The effectiveness of primed T cells to cause cytotoxicity in tumor cells is ultimately dependent on the permissive microenvironment[24].

Table 2: Different forms of cell death and their immunological consequences

RCD	-	+	+	+	+
Antigenicity	-	-	+	+	+
Adjuvanticity	-	+	-	+	+
Microenvironment	+/-	+/-	+/-	-	+
Effect	silent	Inflammation	Tolerance	Priming	Execution

For the dying tumor cells to activate an immune response, six signals are required: (1) the cell death event; (2) coordinated release of DAMPS; (3) recognition, phagocytosis, and processing of dying cells by the antigen-presenting cells (APC) followed by their activation and antigen presentation; (4) antigen recognition by the T cell through T cell receptors and peptide associated major histocompatibility complex (MHC) proteins; (5) engagement with co-stimulatory molecules on dendritic cells (DCs) for effective activation of T cells; (6) presence of cytokines that induce T cell differentiation, such as IL-12 or IFN-γ[25]. All these steps are important for an effective immune response (Figure 6). We have studied the effect of different regimens of localized irradiation of the tumor on the status of dendritic and T cells. Localized radiation (2 Gy X 5 days) increased the expression of markers CD40, CD80, and MHC II on both splenic as well as bone marrow-derived DC (Table 3) in mice bearing 4T1 mammary carcinoma. This was associated with increased secretion of IL-12 by bone marrow derived DC (BMDC)[10]. Hypofractionation with localized exposure of 8 Gy X 3 days also altered splenic $CD11c^+$ and $CD11b^+$ cells (Figure 7). When splenic $CD11c^+$ cells were purified, they were found to express elevated levels of the co-stimulatory molecules CD40, CD80, and MHC II. However, this schedule of radiation also increased Treg or immunosuppressive cells in the tumor and increased $CD4^+$ T cells and $NKp46^+$ NK cells in the spleen (Figure 7). To answer the question of whether ICD induced by 2 Gy (dose used for a single fraction in radiotherapy) can activate DC, mice were exposed to 2 Gy whole body irradiation, followed by evaluation of co-stimulatory molecule expression on splenic DC as well as IL-12 secretion and phagocytosis. NK cell function after this dose was evaluated as well. As seen in Figure 8, a single whole-body exposure to 2 Gy also increased co-stimulatory marker expression in splenic DC, secretion of IL-12, and activation of T and NK cells. This was also recapitulated in bone marrow derived DC (BMDC) differentiated from progenitors irradiated *in vitro* or those derived from whole-body irradiated mice with

increased expression of markers CD80, CD40, CD86, and MHC II[10] (Table 3). But the optimum dose for this increase was different, with DC derived from 1 Gy *in vitro* irradiated progenitors showing an increase, whereas in WBI progenitors, exposure to 2 Gy showed an increase (Table 3).

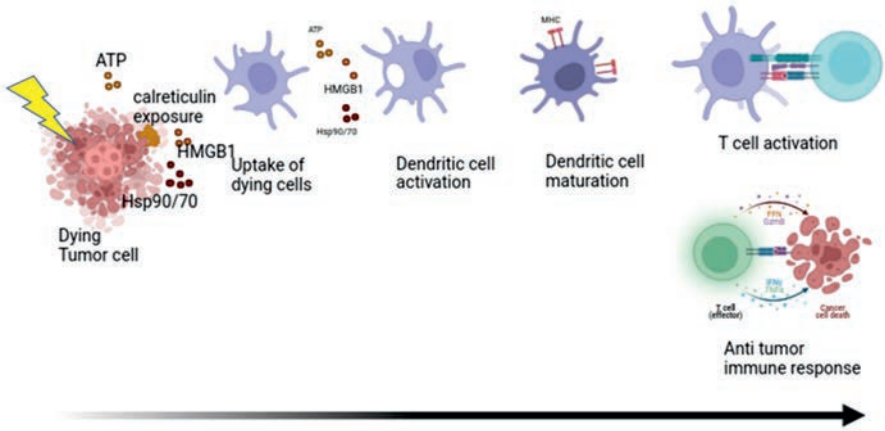

Figure 6: Immunogenic cell death of tumors resulting in activation of DC and T cells (adapted from[9]). Radiation exposure causes dying tumor cells to display significantly more tumor associated antigens on their surface and to release DAMPs. Dendritic cells take up these antigens resulting in their activation. Presence of T_{H1} cytokines like TNF-α results in their maturation. Mature DC migrate to draining lymph node and activate cells. These activated T cells migrate from the lymph node to tumor microenvironment and causes tumor specific killing.

Table 3: Effect of localised radiotherapy (2 Gy X 5 days) on the expression of various markers in splenic and bone marrow derived DC (Values are percent positive cells by flow cytometry for the respective marker and IL-12 is in pg /ml).

	No tumor mice	Tumor bearing mice (TBM)	TBM exposed to localized radiation (2Gy X 5 days)
Splenic DC			
CD40	38±2	23±2#	35±4*
CD80	23±3	14±3#	19±5
MHC II	45±2	25±3#	44±2*
Bone marrow derived DC			
CD40	30±3	17±2#	29±4*
CD80	31±3	19±3#	33±2*
MHC II	19±2	13±4#	16±2*
IL-12	290±15	65±5#	185±20*

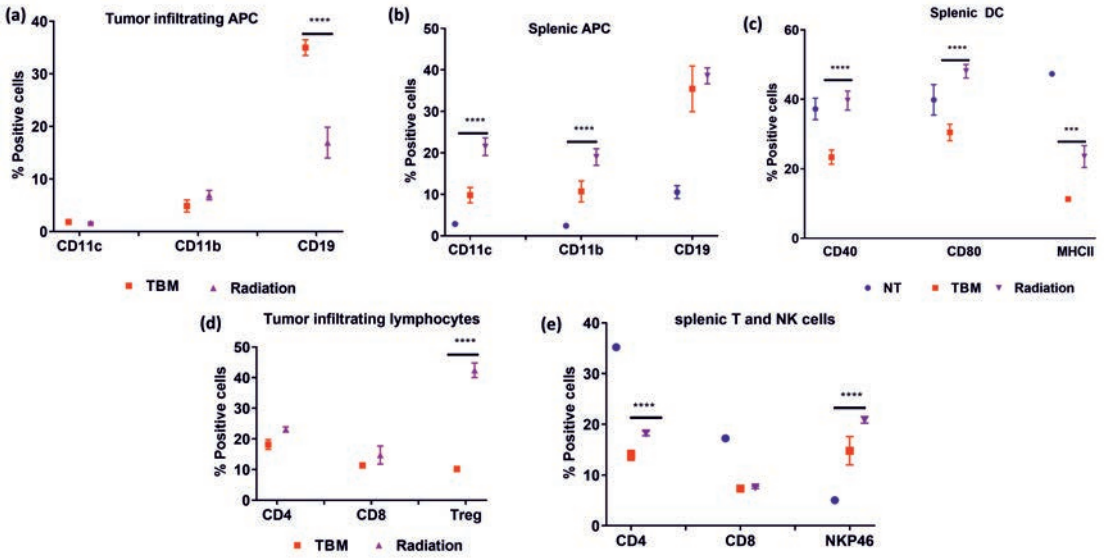

Figure 7: Effect of localised hypofractionated radiotherapy (8 Gy X 3 days) on various immune cells in spleen and tumor (a) proportion of tumor infiltrating antigen presenting cells (APC) (b) proportion of different antigen presenting cells (APC) in spleen (c) expression of co-stimulatory molecules in splenic DC. (d) proportion of tumor infiltrating lymphocytes (e) percentage of splenic T and NK cells.

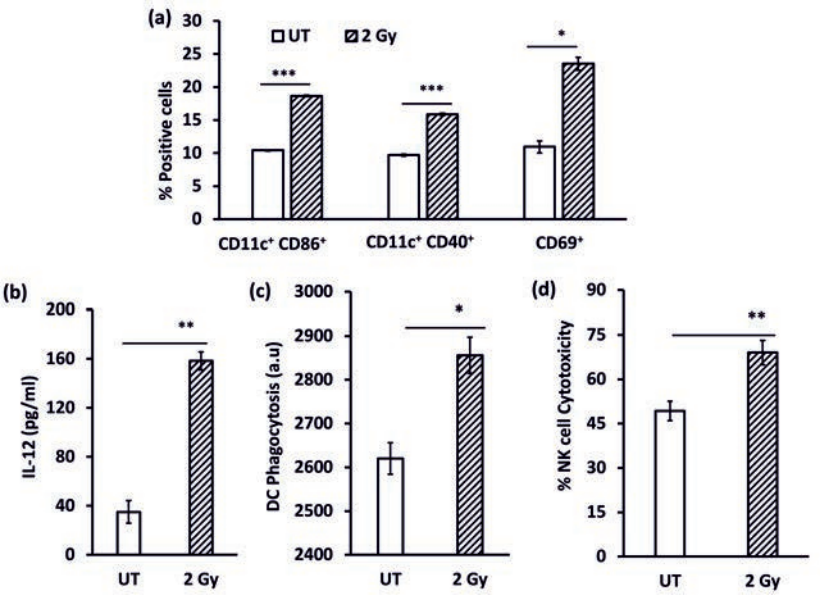

Figure 8: Effect 2 Gy whole body irradiation on the expression of splenic DC and NK cell function. Spleen cells from control and irradiated mice were cultured for 48 h with IL-2 followed by assessment of (a) CD86, CD40 in DC and total $CD69^+$ cells (b) secretion of IL-12 (c) phagocytosis of *E.coli* bioparticles (d) NK cell cytotoxicity against target YAC target cells

To understand the role of radiation-induced immunogenic cell death, the cells undergoing apoptosis in the presence and absence of GM-CSF were evaluated. In the absence of GM-CSF, there was a time-dependent increase in apoptotic cells, but there was only an increase at 24 h and no further increase in the presence of GM-CSF. This gave us the clue that radiation-induced apoptotic cells were increasing the immunogenicity of dendritic cells. To confirm this increase in DC immunogenicity was indeed induced by apoptotic bodies, they were depleted using annexin-V-conjugated magnetic beads, 24 h after irradiation of progenitor cells. Table 3 shows that the increase in expression of various co-stimulatory molecules by the irradiated progenitors was abrogated if apoptotic bodies were depleted 24 h after irradiation. Further, the signaling mechanisms by which these apoptotic bodies activate DC were also studied. The data shown in Table 4 demonstrate an increase in pSTAT5 and the cDC-specific transcription factor Zbtb46 in DC derived from irradiated progenitors. This was abrogated by the depletion of apoptotic bodies (AB), indicating that ionizing radiation-induced activation of DC was through apoptotic bodies/pSTAT5/Zbtb46-mediated signaling[10].

Table 4: Expression of in vitro and whole body- radiation of bone marrow progenitor cells on the expression of various markers in DC. (Values are percent positive cells by flow cytometry for the respective marker and IL-12 is in pg /ml). CP: control progenitors; IP-irradiated progenitors.

	DC-CP	DC-IP (1 Gy)	DC-IP (2 Gy)
In vitro			
CD40	40±4	54±4*	51±5*
CD80	35±6	48±2*	39±9
CD86	52±6	66±4*	55±4
MHC II	20±2	32±2*	38±5
IL-12	225±13	445±18*	189±14
Whole body irradiation			
CD40	30±3	36±1*	60±5**
MHC II	27±2	29±3	41±2**

In addition to immunogenic cell death-mediated signaling, DNA from a dying cell or damaged mitochondria is known to activate the cGAS-STING signaling pathway, resulting in Type I interferon production. Exposure to radiation can directly cause DNA damage or indirectly through the production of reactive oxygen species. These events can result in the accumulation of cytosolic DNA, which triggers the cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS)/interferon stimulating factors (STING) – type I interferon-signaling pathway[26, 27]. Though cGAS-STING signaling initiates innate immune responses, it has also been shown to activate $CD8^+$ cytotoxic T cell-mediated cancer cell killing.

Table 5: Depletion of apoptotic bodies (AB) abrogate radiation induced pSTAT5/Zbtb46 signalling in dendritic cells. (Values are percent positive cells by flow cytometry for the respective marker).

	DC (CP)	DC (IP)	DC (IP)-AB depleted
pSTAT3	53±4	27±2#	58±2*
pSTAT5	38±4	48±3*	30±2*
Zbtb46	58±3	80±4*	67±4*

2.d. Expression of stress induced immunogenic molecules on tumor cells: Radiation induces cell killing at high doses and changes the expression of several genes in surviving cells. Cancer cells have many adaptations to ensure immune evasion and cell survival. Some of these changes include the downregulation of NKG2D ligands to avoid being recognized by natural killer (NK) cells. NK cells are important regulators of immune surveillance and express NKG2D receptors, the engagement of which is essential for triggering downstream pathways that lead to tumor cell killing.

Binding of NKG2D ligands activates the cytotoxic potential of NK cells. Humans express two classes of NKG2D ligands: (i) MHC Class I polypeptide-related sequences A and B (MICA and MICB, respectively) and (ii) UL16-binding proteins (ULBP1–6). Our analysis of MICA and ULBP1 in MCF7, A549, MDA-MB-231, and U937, revealed varying basal level expression amongst these cell lines [28]. MCF-7 cells had the lowest basal level expression, and exposure to radiation increased the expression of MICA (Figure 9). Similar effects have been described by other investigators in different cell lines[29].

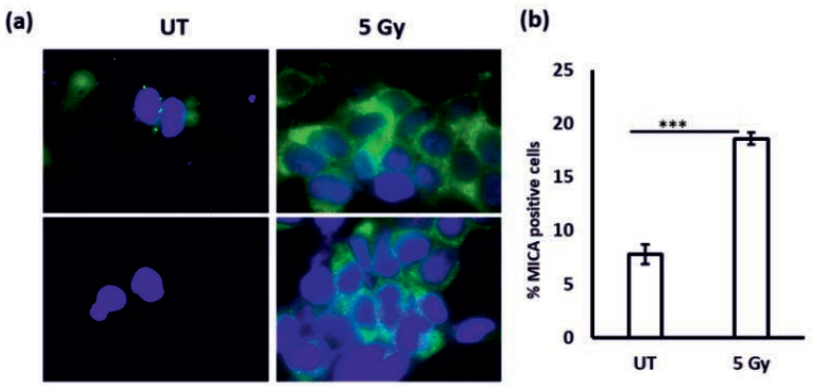

Figure 9: Expression of MICA in MCF7 cells following exposure to a dose 5 Gy. (a) representative fluorescence images (b) flow cytometric estimation of MICA positive cells.

Conclusion: The relationship between tumor and immune cells is complicated and depends on the stage of the tumor and immune infiltration. This is a dynamic process that can range from anti-tumor to pro-tumor effects. This is known as cancer immunoediting, with phases of elimination in the early stages of cancer with predominantly anti-tumor effects; followed by equilibrium; and escape in the late stages of cancer with mostly pro-tumor effects[30]. As described, ionizing radiation impacts the immune cells also in addition to the tumor cells. Since the composition and function of the immune cells against cancer is variable depending on the stage of the cancer, the interaction of the tumor and immune cells in the microenvironment is also very complex. In addition to this, different immune cells differ in their radiosensitivity, with lymphocytes (T cells, B cells, and NK cells) being the most radiosensitive, followed by monocytes, macrophages, and dendritic cells[31, 32]. The radiosensitivity of T cells also depends on their state of activation, with naive resting cells being more radiosensitive than activated T cells[31]. In the elimination phase, cell types of both the innate (NK cells, dendritic cells, and macrophages) and adaptive ($CD4^+$ and $CD8^+$ T cells) immune systems are involved. The involvement of B cells is not completely understood, but they are also believed to exert anti-tumor effects in the elimination phase. The radiosensitivity of each of these cell types is different. In the equilibrium phase, tumor cells that are more resistant to the immune attack emerge, and in the escape phase, the tumor cells present fewer antigens and actively evade the immune system. Immunosuppressive cells such as T regulatory cells and myeloid-derived suppressor cells, which are radioresistant, are now present in the microenvironment. Therefore, though radiation may enhance tumor immunogenicity by inducing immunogenic cell death, the release of tumor neoantigens, and danger signals, this relationship between radiotherapy, tumor, and immune system is very complex and delicate and can vary depending on the radiation dose or fractionation regimen[33]. The induction of a DNA damage response after radiation also increases the expression of immunosuppressive proteins such as PD-1 and CTLA-4[34].

Future directions: To understand the nuances of their interaction at different stages of cancer, a more detailed investigation of how different types of immune microenvironments respond to radiotherapy is required. This is made more difficult by patients' varying baselines in terms of the immune tumor microenvironment and intrinsic radiosensitivity. It is therefore critical to understand how the systemic and immune tumor microenvironments influence patient responses to radiotherapy and should be included as criteria to assess efficacy of radiotherapy (Figure 10). This knowledge will be helpful in developing optimal therapeutic combinations that can harness this relationship between radiation, tumor, and immune system.

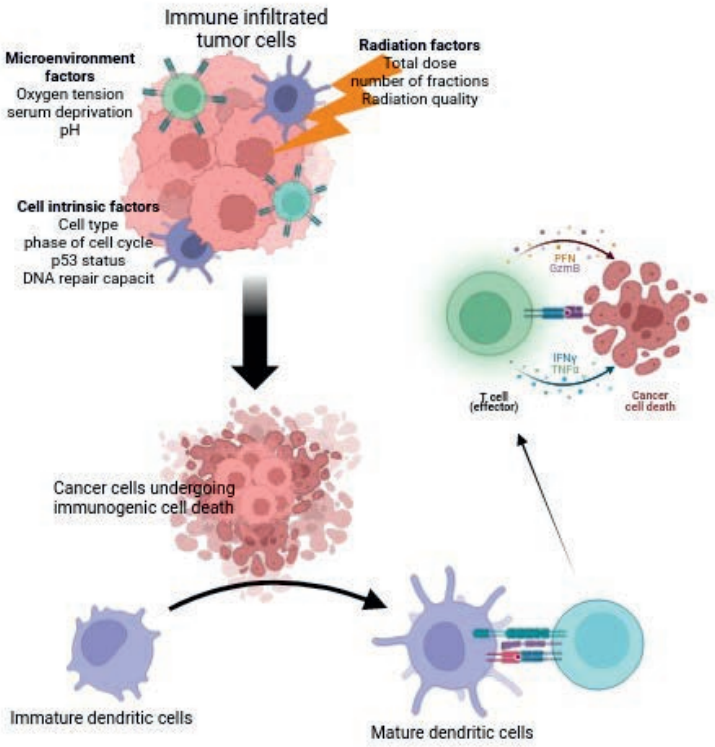

Figure 10: Inclusion of immunogenic cell death induced by radiotherapy and immune function status as criteria to assess efficacy of radiotherapy.

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