RADIOIMMUNOTHERAPY – TRANSLATIONAL OPPORTUNITIES AND CHALLENGES

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RADIOIMMUNOTHERAPY – TRANSLATIONAL OPPORTUNITIES AND CHALLENGES

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Editorial: Radioimmunotherapy—Translational Opportunities and Challenges

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Keywords: translational reseach, immunotherapy, radiotherapy, radioimmunotherapy, vaccination, tumor stroma, immune checkpoint inhibitors

Editorial on the Research Topic

Radioimmunotherapy—Translational Opportunities and Challenges

It has become evident that radiotherapy has both, immune suppressive, and immune activating properties (1). This is why this important component of cancer treatment should be combined with immune therapies to shift the balance toward immune activation against tumor cells. During the last decade a manifold of pre-clinical work was put into investigation of combination of radiotherapy either with additional immune stimulants such as cytokines or vaccines or in combination with antibodies that target immune suppressive molecules such as immune checkpoint inhibitors. Luckily, some of these approaches are currently tested in clinical trials, high lightening the huge translational opportunities by examination of modes of action of radiotherapy in combination with immunotherapy; named in this special issue *radioimmunotherapy*. However, one has always to keep in mind that many challenges do still exist such as what is the best sequence and timing of joint applications, what are the best immunotherapy approaches, how to overcome tumor resistances, what about healthy tissue cytotoxicity, or which biomarkers or matrices of biomarkers are most beneficial for patients stratification, just to mention the most burning ones. The articles in this special issue grab many of these challenges.

Integration of radiotherapy in multimodal tumor treatments is not to be challenged since above half of the tumor patients do receive it during their diseases history. Further, it has been proven that locally applied radiotherapy does not destroy the immune system in a way that additional immunotherapy is not feasible. Voos et al. show that exposing cells of the adaptive immune system, namely T cells, to radiation even results in their $Ca2^{+}$-dependent activation. Furthermore, radiation-exposed T cells adhered better to endothelial cells (Voos et al.). Nevertheless, these features might impact both, toxic effects of radiation and a better T cell-mediated treatment response. The latter can be enhanced by immune activatory cytokines. This is the focus of the work of Palata et al. who review on the efficacy of combination treatments of radiotherapy with IL-2, IFN-alpha, TNF-alpha, GM-CSF, and immunocytokine-based approaches which are already tested in clinical trials. Additionally, work about IL-12 and IL-15-based immunotherapy approaches is presented. This again high lights the huge translational opportunities of radioimmunotherapies (Palata et al.). Besides cytokines, active stimulation of the immune system can be achieved by vaccination approaches. Seitz et al. demonstrate for the first time in pre-clinical model systems that radiotherapy can be combined

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with vaccination with syngeneic whole-tumor cell vaccine generated by high hydrostatic pressure, mimicking in cancer patients autologous vaccine from their own tumor cells. Radiotherapy thereby acts as an adjuvant for the vaccine that contains many tumor-associated antigens (adjuvanticity plus antigenicity) (Seitz et al.). The work of Liu et al. gives some additional hints that whole-body irradiation with low doses enhances the *in situ* vaccine effects of locally applied radiotherapy. This reflects the complexity and diversity of mechanisms of radiation-induced immune modulation. While low radiation doses mostly seem to enhance immune cell infiltration into tumors, higher doses do induce immunogenic cancer cell death, and create an immune stimulatory micro-environment for the attracted immune cells.

The change in the stromal compartments of tumors following radiation exposure have to be followed very detailed for future radioimmunotherapy optimization. Martinez-Zubiaurre et al. particularly summarize the time-dependence of stromal changes following radiation exposure. Only short windows of opportunities might exist for effective combination of radiotherapy with immune therapies (Martinez-Zubiaurre et al.). Sevenich summarizes the key features how to turn immunological "cold" into "hot" tumors and discusses an additional challenging fact about immune properties of different tumor entities. Particularly brain tumors have highly immune suppressive properties and are located at an immune privileged site. Nevertheless, immune cells do infiltrate brain tumors and distinct well-elaborated combinations of radiotherapy with immune therapy could be successful for primary and metastatic brain tumors (Sevenich). Buchwald et al. review about pre-clinical and clinical work dealing with radioimmunotherapy-induced immune responses against the primary, irradiated, and abscopal, non-irradiated, tumor masses. They stress that besides the tumor location, timing, dose, and fractionation strongly impacts anti-tumor immune responses. They focus on T cell exhaustion and on how radiotherapy should be combined with immune checkpoint-inhibitors such as antibodies targeting the PD-1/PD-L1 network in this context (Buchwald et al.). One has never to forget that classical tumor features such as hypoxic regions have to be taken into account, as these regions do also show immune suppressive features such as increased amounts of regulatory T cells and myeloid-derived suppressor cells and increased concentrations of TGF-beta. Eckert et al. stress that particularly patients with hypoxic tumors might therefore benefit from radioimmunotherapies.

Since multiple immune suppressive properties of tumors do exist, combined approaches that aim to both, activate tumor-reactive T cells, and neutralize exhausted T cells should be more efficient. Ostrand-Rosenberg et al. report about bispecific T cell engagers (BiTE) that activate and target cytotoxic T cells and natural killer T cells to kill PD-L1 expressing tumor cells. They further stress that additional combination with co-stimulatory sCD80 increases T cell-mediated anti-tumor immune responses and should be tested in the future in combination with radiotherapy (Ostrand-Rosenberg et al.). Another innovative approach of targeted stimulation of anti-tumor immune responses is the use of functionalized superparamagnetic iron oxide nanoparticles (SPIONs), as outlined by Janko et al. These nanoparticles have the great advantage to locally targeting the tumor by reducing side effects. Besides cytotoxic agents, immune modulatory molecules can be coupled to these particles and by application of an external mantic field, additional heating of the tumor is possible, again contributing to enhanced immunogenic features of the tumor (Janko et al.).

In all of the described approaches of combining radiotherapy with immune modulators, patient's stratification is of key importance. Here, immune contextures play a central role, besides genetic features of the tumor (e.g., tumor mutational burden) and viral pathogenic factors, since the latter seem to impact radiation sensitivity and antitumor immunity (2). Clinical data about association of viral polyomavirus load and $CD8^+$ T cell infiltration into Merkel cell carcinoma are presented by von der Grün et al. While high viral load was associated with worse overall survival (OS), high intratumoral $CD8^+$ T cell was associated with improved OS. Importantly, expression of immune suppressive PD-L1 was correlated with increased T cell infiltration. These clinical observations once more stress that multiple immune features do impact on efficient anti-tumor immune responses.

Radiotherapy in this context has functions as immune stimulator, immune suppressor, and as fine-tuner of immune responses. Let's go ahead with multimodal radioimmunotherapies for cancer. The knowledge about joint actions of radiotherapy and immunotherapy is increasing daily and the results of the ongoing clinical trials will help to further improve personalized radioimmunotherapies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Ionizing Radiation Induces Morphological Changes and Immunological Modulation of Jurkat Cells

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Impairment or stimulation of the immune system by ionizing radiation (IR) impacts on immune surveillance of tumor cells and non-malignant cells and can either foster therapy response or side effects/toxicities of radiation therapy. For a better understanding of the mechanisms by which IR modulates T-cell activation and alters functional properties of these immune cells, we exposed human immortalized Jurkat cells and peripheral blood lymphocytes (PBL) to X-ray doses between 0.1 and 5 Gy. This resulted in cellular responses, which are typically observed also in naïve T-lymphocytes in response of T-cell receptor immune stimulation or mitogens. These responses include oscillations of cytosolic Ca^{2+}, an upregulation of CD25 surface expression, interleukin-2 and interferon-γ synthesis, elevated expression of Ca^{2+} sensitive K^+ channels and an increase in cell diameter. The latter was sensitive to inhibition by the immunosuppressant cyclosporine A, Ca^{2+} buffer BAPTA-AM, and the CDK1-inhibitor RO3306, indicating the involvement of Ca^{2+}-dependent immune activation and radiation-induced cell cycle arrest. Furthermore, on a functional level, Jurkat and PBL cell adhesion to endothelial cells was increased upon radiation exposure and was highly dependent on an upregulation of integrin beta-1 expression and clustering. In conclusion, we here report that IR impacts on immune activation and functional properties of T-lymphocytes that may have implications in both toxic effects and treatment response to combined radiation and immune therapy in cancer patients.

Keywords: Jurkat cells, peripheral blood lymphocytes, x-ray triggered immune stimulation, T-cell adhesion, x-ray stimulated integrin-β clustering, radiation-induced increase in cell size

INTRODUCTION

Ionizing irradiation of eukaryotic cells elicits, in addition to DNA damage and damage responses, also non-targeted effects, which are mainly related to immune activation and immune functional properties (1, 2). An impairment or modulation of the latter has an impact on immune surveillance in both tumor cells and non-malignant cells. This fosters therapy response and unintentional side

effects/toxicities as well as an induction of secondary malignancies by radiation therapy (RT) (3, 4). Among the immune cell (sub) populations involved, infiltration of T-lymphocytes, especially cytotoxic CD8+ cells, emerge as valuable prognostic marker for treatment response following RT or multimodal chemoradiation therapy (5, 6) in line with a pro-inflammatory scenario (7, 8). By contrast, a hampered adhesion of peripheral blood lymphocytes (PBL) to the endothelium comprises a major mechanism of the anti-inflammatory effect of low-dose (<1 Gy) RT used in the clinical management of inflammatory and degenerative benign disorders for decades (9, 10).

We have recently reported that an increase of reactive oxygen species (ROS) following X-irradiation of A549 cancer and human embryonic kidney HEK293 cells with doses ≥1 Gy is not restricted to the nucleus but spreads throughout the cell including the cytosol (11). The increase in cytosolic ROS further triggers a Ca^{2+}-mediated signal transduction cascade and subsequent activation of Ca^{2+}-sensitive channels and membrane hyperpolarization (11, 12). Since a rise in ROS and a downstream triggering of Ca^{2+} signaling cascades may comprise a more general cell response to ionizing irradiation we hypothesize that comparable signaling cascades can be triggered in other types of cells, including immune cells. In line with that it is well established that Ca^{2+} signaling cascades play a crucial role in T-cell activation (13–16) and mediate downstream events like gene expression, entry into the cell cycle and T-cell effector functions. Notably, these signaling cascades can be short-circuited by elevating the concentration of free Ca^{2+} in the cytosol ($[Ca^{2+}]_{cyt}$) without employing receptor activation (17).

With this background information, we analyze here the effect of ionizing radiation (IR) with low (<2 Gy) and higher doses (≥2 Gy) on morphological changes, immune activation, adhesion properties, and ion channel expression of a leukemic Jurkat T-cell line and PBL. The Jurkat cell line has served for two decades as a valuable model for analyzing basic signaling events engaged in T-cell activation (17). Our data indicate that irradiation of Jurkat and PBL cells triggers a series of distinct cellular responses. These include an increase in cell diameter, augmented integrin β1-mediated adhesion to endothelial cells (ECs), CD25, interferon-γ (IFNγ), and interleukin (IL)-2 stimulation and modulation of Ca^{2+} sensitive K^+ channels.

MATERIALS AND METHODS

Cell Culture

Jurkat cells (ACC 282) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The human EC line EA.hy926 (Crl-2922) was established by fusion of human umbilical vein ECs and the adenocarcinoma epithelial cell line A549 (18) and was purchased from ATCC (LGC Standards, Wesel, Germany). Cells were either grown in RPMI 1640 medium (Jurkat), supplemented with 10% heat inactivated fetal calf serum (FCS; PAA, Cölbe, Germany) and 2 mM L-glutamine or in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany), supplemented with 10% FCS 50 U/ml penicillin and 5 µg/ml streptomycin (Sigma-Aldrich, Munich, Germany). PBL were isolated from buffy coats using density gradient centrifugation (Biochrom, Berlin, Germany). After centrifugation (40 min at 1,000 × *g*, RT) interphase cells were isolated, washed twice with PBS, and pelleted by centrifugation (300 × *g*, 10 min). For adhesion assays, Jurkat cells and PBL were biotinylated by incubation (15 min on ice) with a biotin-*N*-hydroxysuccinimid ester (NHS-biotin, 10 mg/ml, Sigma-Aldrich) and maintained in RPMI 1640 Medium with 20% FCS, 1% HEPES, and 1% penicillin/streptomycin prior to assays. PBL isolation was performed in a biolevel II laboratory with an institutional approval by the local governmental authority (Regierungspräsidium Darmstadt IV/F-45.1/jr-F 018164-23623/2017-Bio-30/17).

Determination of Cell Diameters

Cell diameters were measured with an EVE automatic cell counter (NanoEnTek, Seoul, South Korea). For cell diameter studies, a suitable protocol for Jurkat cells was established and all measurements were validated by visual inspection and if necessary corrected by hand using a personal computer based software. Viability was estimated by using trypan blue exclusion assays.

Cell Irradiation and Treatments

Cells were exposed to X-ray irradiation in cell culture flasks using an Isovolt 160 Titan E source with a voltage of 90 kV and 33.7 mA (GE Sensing & Inspection Technologies, Alzenau, Germany). Doses were delivered at a 30 cm source to probe distance with cell culture flasks placed on a 2 mm aluminum sheet. CDK1-inhibitor RO3306 (Axon Medchem, Groningen, Netherlands) was dissolved in DMSO at 14.2 mM and added to the cell culture medium in a final concentration of 3 µM. Cyclosporin A (Sigma-Aldrich) was dissolved in ddH_2O and added to the cell culture medium of non-irradiated control cells or directly after irradiation of cells in a concentration of 1 µM. The cell permeable Ca^{2+} buffer BAPTA-AM [*1,2-Bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl) este*r, Thermo Fisher] was added to the cell culture medium 30 min prior to cell irradiation at 50 µM and was removed immediately after irradiation. Phytohemagglutinin (PHA-L) was purchased from Biochrom (Berlin, Germany). Cells were treated for 48 h by adding PHA-L to the cell culture medium at a concentration of 7.2 µg/ml. To activate human T-cells ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator (Stem cell Technologies, Vancouver, BC, Canada) was added to the cell culture medium (25 µL per 1 mL of cell suspension) and maintained at 37°C and 5% CO_2 for 48 h. The $K_{Ca}2.2$-specific ion channel blocker Tamapin was purchased from Alomone Labs (Jerusalem, Israel) and dissolved in purified water and diluted in external solution for patch clamp experiments.

Immunofluorescence

Staining of IFNγ and IL-2 for Immune-Fluorescence Detection

4×10^5 Jurkat cells/ml were treated with either 25 µl/ml CD3/CD28/CD2 T-cell activator or irradiated with X-ray doses

between 0.1 and 5 Gy. After 48 h incubation at 37°C, 5% CO_2 the cell suspensions were washed with PBS at 400 × *g* for 5 min. Next, the cells were fixed for 30 min at room temperature in 4% paraformaldehyde (PFA) with 0.2% glutaraldehyde in PBS and permeabilized with 0.2% Triton X-100 solution. T-cell suspensions were washed in PBS, resuspended in PBS and primary antibodies for IFNγ (#14-7317-85, Thermo Fisher Scientific, Waltham, MA, USA) or IL-2 (#92381, Abcam, Cambridge, UK) were applied at a 1:2,500 dilution over night at 4°C on a shaker. Jurkat cells were subsequently washed with 0.05% Tween20 (in PBS) and incubated with anti-mouse Alexa488 secondary antibody (anti-mouse Alexa488 IgG, Thermo Fisher Scientific) in a dilution of 1:2,500 for 1 h at RT. Finally, stained cells were washed with 0.05% Tween20 (in PBS) and stored in PBS before analysis. For an analysis of IL-2 and IFNγ expression by immunostaining untreated control cells and cells irradiated with X-ray or treated with activator were imaged with the same microscope settings. For a quantitative analysis, a region of interest (ROI) was defined and fluorescence intensity was measured relative to the size of the ROI.

Integrin β1 and $K_{Ca}2.2$ Staining for Single Molecule Analysis

Cell fixation and antibody staining were performed as described earlier (19). In brief, Jurkat cells were fixed with a rapid and complete immobilization fixation protocol optimized for membrane proteins (20). Cells were incubated in 4% PFA supplemented with 0.2% glutaraldehyde for 1 h at 4°C followed by anti-integrin β1 (CD 29, Biozol Diagnostica, Eching, Germany) immunostaining with a directly fluorescent labeled antibody (Alexa 488). $K_{Ca}2.2$ channels were stained with KCNN2 antibody (PA5-41012, rabbit IgG, Thermo Fisher Scientific) as primary antibody and with an Alexa 488 labeled anti rabbit secondary antibody (Thermo Fisher). In both procedures an antibody dilution of 1:10,000 was used.

Western Immunoblotting

For Western blotting, cells were lysed in radio-immune precipitation assay buffer supplemented with protease inhibitors. Equal amounts of proteins (30 µg) as determined by a micro BCA-protein assay (Pierce, Rockford, IL, USA) were separated on 12% SDS polyacrylamide gels and transferred to a nitrocellulose membrane (Hybond C, Amersham, Freiburg, Germany). Membranes were next incubated with rabbit anti-CD25 antibodies (S-IL2R Oligo, Life Technologies, Darmstadt, Germany). This was, followed by an incubation with appropriate horseradish peroxidase-conjugated secondary antibodies (Southern Biotech, Birmingham, AL, USA). Next, membranes were developed by using an enhanced chemo luminescence detection system (ECL, Perkin Elmer, Waltham, MA, USA) and Odyssey Fc Imaging System (LI-COR, Bad Homburg, Germany). To confirm equal protein loading, membranes were in parallel probed with anti β-actin antibodies (Sigma-Aldrich). Individual bands were quantified using the Image Studio Version 5.2 (LI-COR).

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy was performed on a Leica TCS SP or SP5 II system (Leica Microsystems, Mannheim, Germany) equipped with a 63× water (HCX PL APO 63× NA 1.2 W CORR) and 63 × 1.4 oil UV objective (HCX PL APO lambda blue). Coverslips were cleaned using acetone followed by plasma cleaning in a plasma furnace (Zepto-B) from Diener electronic (Ebhausen, Germany). The external buffer used for microscopy contained (140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 5 mM Mannitol, 10 mM HEPES, 2 mM $CaCl_2$, pH 7.4). Plasma membranes were imaged with CellMaskOrange™ (Thermo Fisher Scientific) at a concentration of 0.5 µg/ml. Nuclei were stained with Hoechst (200 µg/ml) diluted 1:50 in external microscopy buffer or PBS; cells were stained for 10 min at 37°C. Subsequently, cells were washed twice and resuspended in microscopy buffer or PBS.

Ca^{2+} Imaging

The sensor Fluo-4 was loaded into Jurakt cells by incubating cells for 30 min in buffer (140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 5 mM Mannitol, 10 mM HEPES, 2 mM $CaCl_2$, pH 7.3) containing 1 µM Fluo-4 AM (Life technologies, Carlsbad, CA, USA) on coated glass coverslips (Ø 25 mm). The latter were prepared by cleaning in a plasma furnace (Zepto-B, Diener electronic GmbH, Ebhausen, Germany) and coating with one layer of PBS/5% BSA in a spincoater (PIN150, SPS Europe Spincoating, Putten, Netherlands). After the initial layer had dried, it was further coated with a layer of poly-L-lysine (molecular weight 75–150 kDa). Coating was essential to prevent spontaneous Ca^{2+} oscillations, which usually occur when Jurkat cells are settling on glass coverslips. The dye was subsequently removed by washing cells with dye free buffer. After irradiation, the cells were then transferred for imaging on a Leica TCS SP5 II confocal microscope (Leica, Heidelberg, Germany) with a HCX PL APO CS 40.0 × 1.30 OIL oil immersion lens. The dye was excited with a 488 nm argon laser and the emission sampled at 505–550 nm.

Single Molecule Microscopy and Data Analysis (SMD)

For SMD measurements a standard STORM buffer containing 100 mM MEA (β-mercapto ethylamine, pH 8.5, Sigma-Aldrich, St. Louis, MO, USA), 140 U catalase (Sigma-Aldrich, St. Louis, MO, USA, C3515), and 10 U glucose oxidase (Sigma-Aldrich, St. Louis, MO, USA, G0543) in Tris-buffer [50 mM Tris, 10 mM NaCl (both AppliChem, Darmstadt, Germany), pH 8] supplemented with 10% (w/v) glucose was used. All SMD measurements were performed with a custom built instrument. A detailed description of this setup and the data analysis of detected molecules were published elsewhere (19). In brief, editing of images was performed with Fiji software (version: 1.51h) (21). Single molecules were detected and filtered using the Thunder Storm plugin for Fiji (22). For the add-on data analysis, custom written software in MATLAB R2014b was used. Therefore, Ripley's K function cluster analysis (23, 24) was combined with a binary cluster map analysis based on the

publication of Owen et al. (25). With this add-on it is possible to (i) determine the number of molecules per ROI and (ii) the ratio of clustered/total signals. Detected single molecules are visualized as Gaussian rendered images. Here, a symmetric 2D Gaussian is drawn for every localized molecule with a SD equal to the localization uncertainty. The localized and rendered molecules are added sequentially leading to the final super-resolution image. To remove duplicates, molecules that convert to the positions within a distance of the uncertainty were removed.

Patch Clamp Recordings

Membrane currents of cells were recorded in a whole cell configuration (26) using an EPC-9 amplifier (HEKA Electronics, Lambrecht, Germany). The pipette solution contained (in mM) 100 K-Aspartate, 40 KF, 5 KCl, 2 $MgCl_2$, 1.223 EGTA (1 μM free Ca^{2+}) or 2.62 EGTA (100 nM free Ca^{2+}), 1 $CaCl_2$, and 10 Hepes/KOH pH 7.4. Sorbitol was used to adjust the osmolarity to 285 mOsmol/kg. The extracellular solution contained (in mM) 130 Na-Asp, 4.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, and 10 Hepes/NaOH pH 7.4. Currents were elicited with a pulse protocol consisting of voltage steps from a holding voltage at −60 mV, to 800 ms long test pulses between −100 and +80 mV and a 200 ms long post pulse at −80 mV. Currents were recorded and data analyzed with an EPC-9 amplifier and Patchmaster Software (all from Heka Electronic).

Cell Cycle Analysis by Flow Cytometry

Flow cytometric analyses were performed after 48 h following irradiation with propidium iodide (PI) solution (4% PI stock, 0.5 mg/ml PI, 38 mM sodium citrate, pH 7), 5% RNAse A stock (RNAse A 5 mg/ml, Tris–HCl 10 mM, NaCl 15 mM, pH 7) using a BioRad S3 Cell Sorter and the FlowJo 10 software for analysis (FlowJo LLC). The percentage of cells in G_2/M phases was determined by single-parameter histograms of DNA content.

Cell Adhesion Assay

EA.hy926 EC were grown to 95% confluence and stimulated by the cytokine TNF-α (20 ng/ml, MiltenyiBiotec, Bergisch-Gladbach, Germany) at 4 h before the adhesion assay. Next, a total of $2–3 \times 10^5$ irradiated and biotinylated Jurkat cells or PBL were added and adhesion assays were performed for 30 min at 4 or 37°C under non-laminar shear stress as reported before (27). Next, adherent PBL or Jurkat cells were fixed with methanol, tagged with a streptavidin-Cy3 conjugate (Dianova, Hamburg, Germany) and counted using an Operetta High Content Screener (PerkinElmer, Waltham, MA, USA). The counts of a minimum of 160 selected fields per well were averaged as one data point.

CD25 Detection by Flow Cytometry

Surface expression of CD3 and CD25 was analyzed either on Jurkat cells harvested directly from cultures flasks or PBL isolated by density gradient centrifugation as described before. Next, cells were stained with fluorochrome-conjugated mAb targeting CD3 (CD3-PerCP-Cy5.5 clone SK7; Becton Dickinson, Heidelberg, Germany) and CD25 (BV510 Mouse anti human CD25 clone 2A3, Becton Dickinson) and subjected to multicolor flow cytometry using a CytoFlexS cytometer (Beckman Coulter, Krefeld, Germany). Data acquisition and analysis were accomplished with CytExpert Version 1.2 software (Beckman Coulter).

Taqman-Based Quantitative Real-Time PCR (qRT-PCR)

RNA was isolated at 24 h post irradiation or T-cell activation using the NucleoSpin Kit (Macherey-Nagel, Dueren, Germany) in combination with the QiaShredder Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Reverse transcription was performed with M-MLV reverse transcriptase (Promega, Mannheim, Germany) and random hexamers (Thermo Fisher Scientific). qRT-PCR was achieved with 20× Taqman Assays (Thermo Fisher Scientific) specific for IL-2 (Assay ID: Hs00174114_m1) or IFNγ (Assay ID: Hs00989291_m1) with the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific), ABsolute QPCR Mix, ROX (Thermo Fisher Scientific) and standard settings. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method relative to untreated controls with the housekeeping gene ribosomal protein L37A (RPL37A) as endogenous reference. For each data point, two independent experiments performed in triplicate were acquired and displayed as mean value + SD. The primer and probe sequences for RPL37A detection were as follows: RPL37A-fw 5′-TGTGGTTCCTGCATGAAGACA-3′, RPL37A-rev 5′-GTGACAGCGGAAGTGGTATTGTAC-3′, RPL37A probe: 5′ FAM-TGGCTGGCG GTG CCT. GGA-3′ TAMRA (28), manufactured by Eurofins Genomics (Ebersberg, Germany).

Statistical Analysis

Data are expressed as means ± SDs or SE of at least two independent experiments; number of biological replicates (n) or independent experiments (N) were denoted. Significance was estimated by using the Student's t-test and Microsoft Excel software. P values <0.05 (*), <0.01 (**), and <0.001 (***) are indicated in the figures.

RESULTS

Ionizing Irradiation Increases Cell Diameter of Jurkat Cells and PBL

Jurkat cells exhibit a narrow size distribution with a mean value of 10.1 ± 0.2 μm (**Figures 1A,B**). Forty-eight hours after a 5 Gy exposure the distribution widens and the mean value increases to 12.5 ± 0.5 μm. Comparable findings were also evident following irradiation with doses ranging between 0.1 and 10 Gy (**Figure 1C**) confirming a dose-dependent increase in the mean cell diameter (Δd). A fit of the plot with a logistic equation (Eq. 1)

$$f(x) = \frac{\Delta d_{\max}}{\left(\frac{D50}{x}\right)^k + 1} \quad (1)$$

where $\Delta d_{\max}$ is the maximal Δd increase, $D50$ the dose for half maximal increase and k the steepness of the curve, yields a $D50$

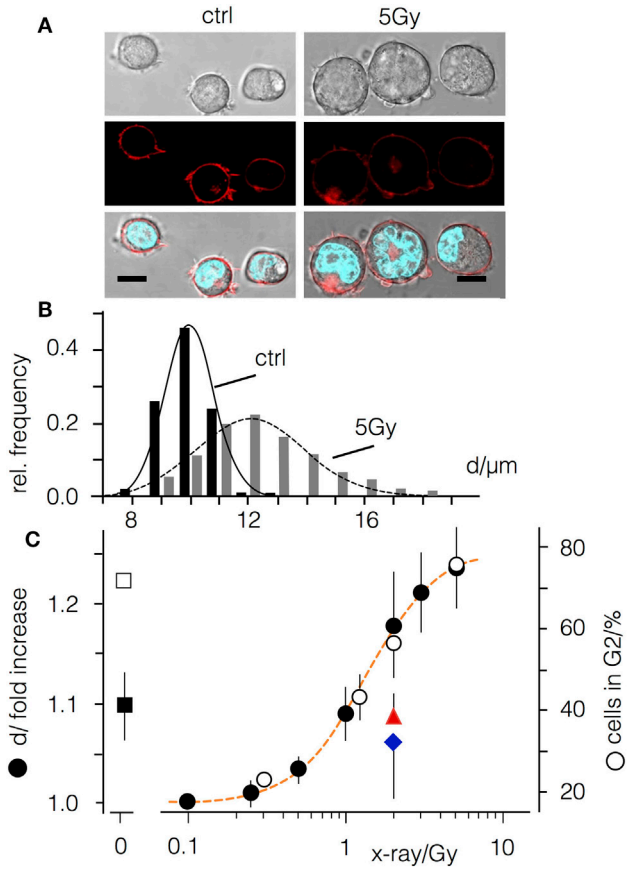

FIGURE 1 | Ionizing irradiation causes an increase in diameter of Jurkat cells and an arrest in G2. **(A)** Images of Jurkat cells before and 48 h after X-irradiation with 5 Gy. Top row: wide field image, central row: confocal section in equatorial plane with fluorescent plasma membrane label CellMaskOrange™. Lower row: overlay of wide field image with CellMaskOrange™ labeled plasma membrane and Hoechst labeled nucleus; scale bars 10 µm. **(B)** Cell diameter distribution of non-irradiated Jurkat cells (black bars) and cells irradiated with 5 Gy (gray bars). **(C)** Relative increase of cells in G2 phase of the cell cycle (open circles) and increase in cell diameter (d, closed symbols) in the absence (black circles) or presence of either 1 µM cyclosporine A (red triangle) or 50 µM BAPTA-AM (blue diamond). Square symbols show relative increase in cell diameter (closed symbol) and percentages of cells in G2 in non-irradiated cells treated with 3 µM CDK1-Inhibitor RO3306 (open symbols).

value of 1.34 Gy with a steepness of 2; the curve saturates at a maximal Δd of 23.5% for doses ≥5 Gy.

It has been shown that peripheral blood leukocytes increase in size in response to PHA-L immune stimulation (29). Accordingly, we next asked whether IR may increase cell diameter in a comparable manner. Indeed, as depicted in **Figures 2A–C** stimulation of PBL from healthy donors ($N = 3$) with PHA-L (30) resulted in a comparable increase in cell diameter. The size distribution of mock-treated and irradiated cells can be fitted by either a single Gaussian distribution confirming a uniform size with a mean diameter of 7.2 µm (mock treated) or by the sum of two Gaussian distributions. The two populations indicate that following PHA-L stimulation 45% of the cells have increased their mean size to 10.2 µm, while 48 h after irradiation with a dose of 5 Gy 32% of the cells exhibit an increased diameter (mean value at 9.7 µm).

Effect of Ionizing Irradiation on Cell Cycle Distribution in Jurkat Cells

Jurkat cells are deficient in p53 (31) and consequently an irradiation-induced arrest is restricted to the G2 phase of the cell cycle (**Figure 1C**), which is associated with an increase in the size of the cell nucleus (**Figure 1A**). The distribution of cells in G2 phase exhibits a similar dose-dependency as the increase in cell size (**Figure 1C**). To test whether these two parameters are related, Jurkat cells were treated with the CDK1-inhibitor RO3306. Incubation with RO3306 arrested 71% (±2.1%) of the cells in G2 phase (**Figure 1C**) but only resulted in a 9.6 ± 3% increase in cell diameter (**Figure 1C**). Irradiation with a dose of 5 Gy, by contrast, revealed a similar accumulation in the G2 phase (76.3 ± 6%) but with an increase in diameter of 24% (±4%) (**Figure 1C**).

Next, we co-treated irradiated Jurkat cells (2 Gy) with cyclosporine A or the Ca^{2+} buffer BAPTA-AM. We reasoned that immune suppression or blocking the Ca^{2+} signaling cascades may abolish the radiation-induced increase in cell diameter without affecting the G2 cell cycle arrest (**Figure 1**). Indeed, both treatments decreased the effect of irradiation (**Figure 1C**) with an increase of diameter in cyclosporine A treated cells of 10 ± 3%, as compared to 19 ± 5% in mock-treated controls. Notably the remaining value of 8% increase was comparable to the value induced by the CDK1-inhibitor (**Figure 1C**).

The sensitivity of the irradiation triggered morphological response of Jurkat cells to the Ca^{2+} buffer BAPTA-AM suggests that a Ca^{2+}-mediated signaling cascade is connecting the primary radiation stress and the morphological alteration. To test this prediction we loaded Jurkat cells with the Ca^{2+} sensitive dye Fluo-4 and imaged the concentration of free Ca^{2+} in the cytosol $[Ca^{2+}]_{cyt}$ in untreated cells and with 1.25 Gy irradiated cells. The representative recordings of the Fluo-4 fluorescence in **Figure 3** indicate that the signal remains constant in the majority of control cells but starts oscillating after a delay of about 30 min in most irradiated cells. 1 h after irradiation with 1.25 Gy, 67% of the treated cells exhibited oscillations in $[Ca^{2+}]_{cyt}$. In the respective control cells, only 7% exhibited an oscillation at this time point of recording. The results of these experiments confirm that ionizing irradiation triggers in Jurkat cells a Ca^{2+} signaling cascade, which is initiated only after a considerable delay.

To further analyze irradiation-induced morphological changes of Jurkat cells, we imaged them for 48 h after exposure to 1.25 Gy. As depicted in **Figure 4A**, non-treated cells were spherical with a small foot on the glass surface. This foot area became much larger in irradiated cells. This suggests that the inherent tendency of Jurkat cells to adhere to the glass surface was accelerated by ionizing irradiation. To further quantify adhesion on glass surface, we estimated the contact angle between the cell and the glass (**Figure 4A**). As shown in **Figure 4B**, irradiation triggers a significant ($P < 0.001$) decrease in the contact angle from

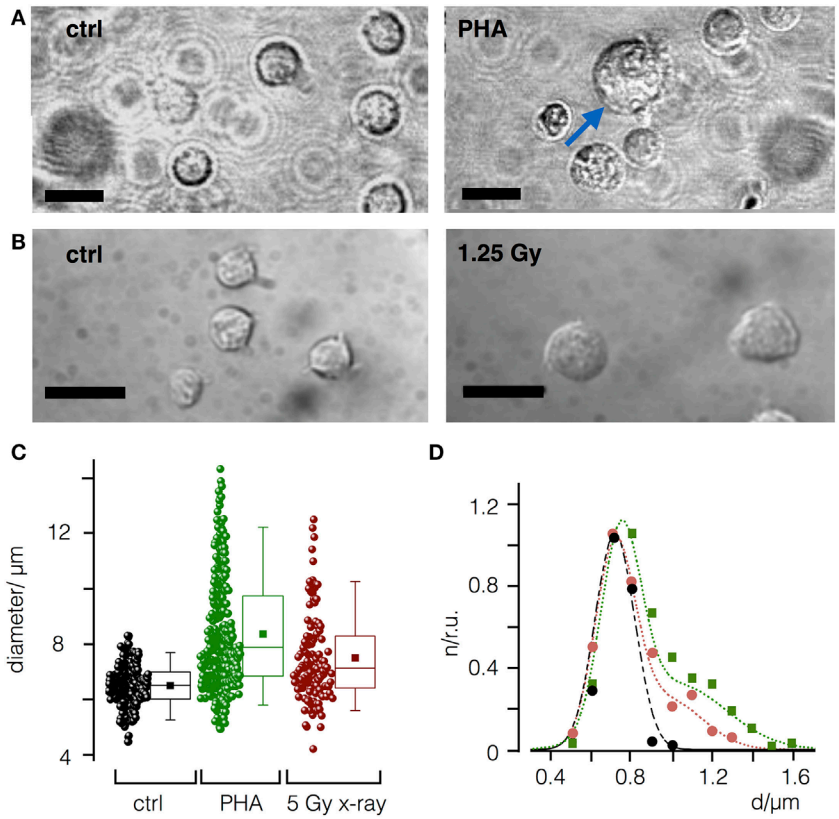

FIGURE 2 | Mitogen phytohemagglutinin (PHA-L) and ionizing irradiation cause an increase in diameter of naïve peripheral blood leukocytes (PBL). **(A)** Representative images of PBL before (control) and 48 h after treatment with PHA-L (7.2 µg/m). **(B)** Before and 48 h after irradiation with a dose of 5 Gy. Scale bars 10 µm. **(C)** Cell diameters of PBL control cells (ctrl) or PBL treated with PHA-L or 5 Gy. Each data point represents a single PBL with mean (filled square) and median value (line) as well as 25 and 75 percentile of data; whiskers indicate 5 and 95 limits of data. **(D)** Size distribution histogram of ctrl (black) and of cells treated with PHA-L (green) or 5 Gy (red) from **(B)**. Distribution was normalized to maximal value for each condition and fitted with single Gauss distribution (control, black line) or the sum of two Gaussians for T-cell activator (green line) or X-ray (red line) treated cells.

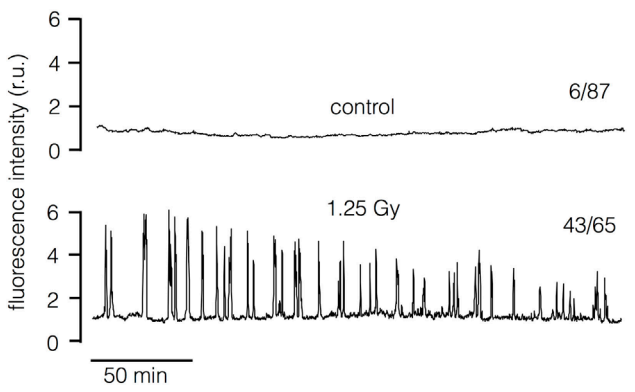

FIGURE 3 | Ionizing irradiation stimulates oscillations of cytosolic Ca^{2+} in Jurkat cells. Representative recordings of Fluo-4 fluorescence in untreated control cell (top) and in cell after exposure to 1.25 Gy x-Ray (bottom). The recordings start approximately 7 min after exposure to radiation. The numbers of cells (*n*) which show $[Ca^{2+}]_{cyt}$ oscillations 1 h after irradiation (1.25 Gy) or after sham treatment (control) with respect to total number of cells investigated (*N*) is reported at respective traces (*n*/*N*). Data are pooled from three independent experiments.

101.1° ± 17.7° in control cells to 78.3° ± 20.8° in irradiated cells. To test whether this effect is the consequence of cell adhesion, experiments were repeated on polyethylene glycol (PEG)-coated glass coverslips. **Figure 4C** illustrates that PEG coating increases the contact angle due to a decreased cell-surface adhesion. This tendency is strongly accelerated by X-irradiation of the cells (**Figures 4B,C**).

Next, to test whether ionizing irradiation also stimulates cell adhesion in a more physiological context, we performed an adhesion assay on EA.hy926 ECs. As depicted in **Figure 4D**, Jurkat cells or PBL irradiated with a dose of 1.25 Gy exhibited an elevated adhesion rate to EA.hy926 cells, which was most pronounced for both cell types after stimulation of the ECs with the pro-inflammatory cytokine TNF-α. Moreover, to analyze the involvement of integrin adhesion molecules, Jurkat cells or PBLs were incubated with recognition sequences Arg–Gly–Asp (RGD) peptides to compete for binding of endothelial-leukocyte adhesion molecules and vascular cell adhesion molecule receptors. Results presented in **Figure 4E** indicate a significant reduction of adhesion in the presence of the peptides, indicating a mechanistic impact of RGD motifs.

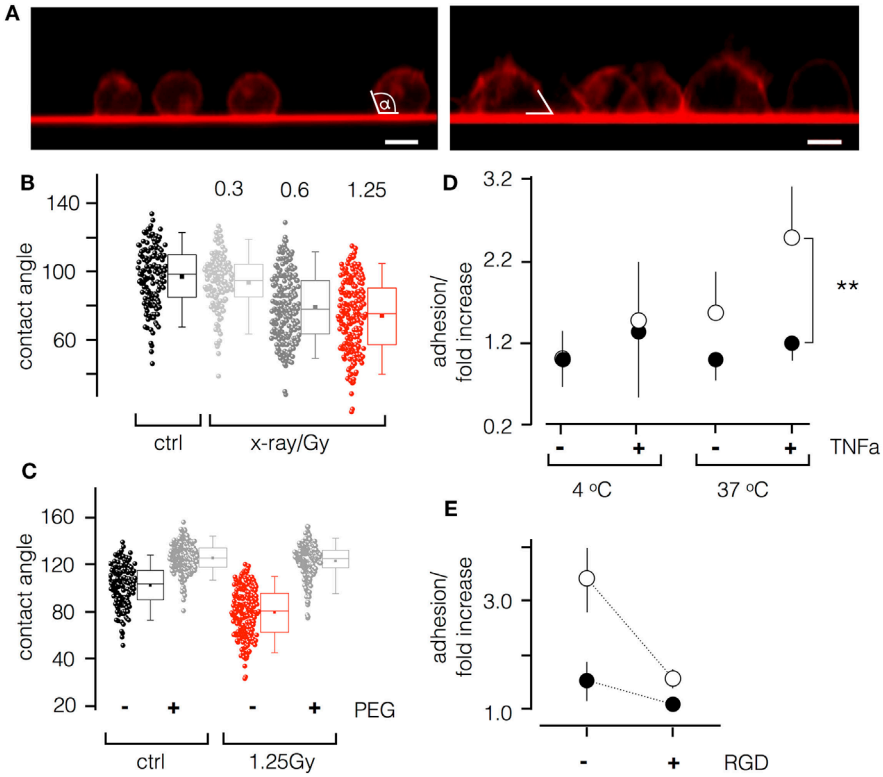

FIGURE 4 | Ionizing irradiation stimulates adhesion of Jurkat cells and peripheral blood lymphocytes (PBL). **(A)** Side view of Jurkat cells with fluorescent plasma membranes on glass surface. Confocal images of non-irradiated cells (left panel) and of cells 48 h after irradiation (1.25 Gy, right panel) were taken 10–15 min after incubating cells on red fluorescent glass cover slip. White lines indicate the contact angle between cell and glass surface. **(B)** Box plot of contact angles for un-irradiated cells and cells irradiated with increasing doses of X-ray. **(C)** Contact angles of un-irradiated (ctrl) and irradiated cells (1.25 Gy) on untreated (–) or polyethylene glycol (PEG) pretreated (+) cover slips. Data obtained as in **(B)**. Box plot symbols have the same meaning as in **Figure 2**. **(D)** Relative adhesion rates of Jurkat cells and PBL to endothelial cells (ECs). Non-irradiated (closed circles) or irradiated (1.25 Gy X-ray) Jurkat cells at 4 or 37°C with or without stimulation of ECs with TNF-α (20 ng/ml). Mean value ± SD ($N = 4$; $n = 12$). **(E)** Cells as in last column of **(D)** with or without 10 μM RGD peptide in incubation buffer.

The same types of adhesion assays were performed with PBL revealing a similar response of these cells to irradiation (Figure S1 in Supplementary Material). 1.25 Gy significantly augments adhesion to ECs in particular in TNF-α stimulated ECs; the response is comparable to that elicited by a T-cell activator. Also in these experiments, the Arg–Gly–Asp peptide caused a reduction of radiation-triggered adhesion suggesting an involvement of integrins.

To confirm an integrin-mediated adhesion, we imaged the integrin β1 subunit in non-irradiated and irradiated (1.25 Gy) Jurkat cells with single molecular resolution. Representative images in **Figure 5A** visualize a significant increase of integrin β1 molecules and cluster detection upon irradiation by quantitative evaluation (**Figures 5B,C**). Here, single molecules of integrin β1 are visualized as Gaussian rendered images. For this, the localized and rendered molecules are added sequentially resulting in a better visualization of regions with a higher density of signals. These regions, shown as white spots, are well known as integrin clusters (32). Quantitative analysis with the Ripley's K function supports the visual impression of an irradiation-induced increase in the density of clusters and number of integrin β1 molecules. In addition, size of the clusters is larger in irradiated cells as compared to control cells (**Figures 5B,C**).

Up to this point, the data so far supported the hypothesis that ionizing irradiation induces morphological changes and increases adhesion of Jurkat cells and PBL, which may resemble immune activation processes (33, 34). To further analyze the effect of X-irradiation on Jurkat cell activation, we monitored the surface expression of CD25 (IL-2 receptor alpha chain), and IL-2 and IFNγ response by FACS analyses and quantitative PCR, respectively. The results of these assays indicate a dose-dependent increase of CD25 expression by X irradiation in Jurkat cells while the number of CD25+ cells in PBL was not affected (**Figure 6A**). Quantitative analysis of IFNγ and IL-2 mRNA revealed an increased expression in Jurkat cells, most pronounced following a 5 Gy exposure (**Figures 6B,C**). By contrast, as compared to a huge activation level by the CD3/CD28/CD2 cocktail, we observed a low IL-2 (**Figure 6B**) or marginal radiation-dependent induction of IFNγ in native PBL (**Figure 6C**). Increased induction of either CD25, IL-2 or IFNγ in Jurkat cells was further confirmed by Western-Blot analyses and immuno-fluorescent detection and quantification (Figure S2 in Supplementary Material).

Finally, immune activation is reported to upregulate Ca^{2+} sensitive K^+ channels in immune cells for differentiation and activation (35). In Jurkat cells, the $K_{Ca}2.2$ (SK2) channel is activated

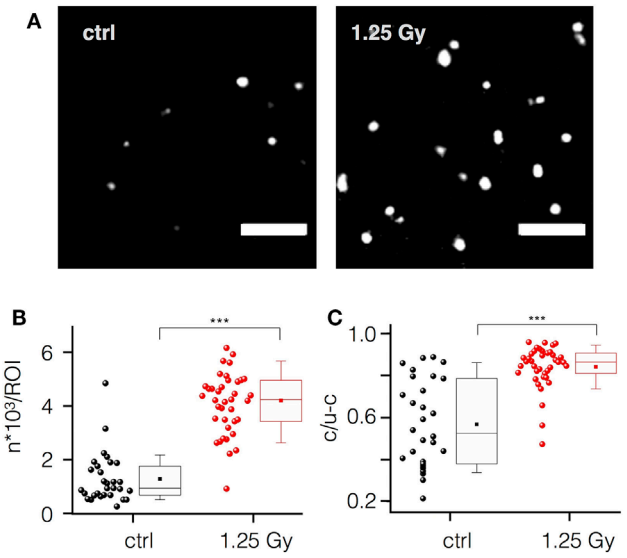

FIGURE 5 | Ionizing irradiation increases integrin β1 expression and clustering in Jurkat cells. **(A)** Localization of single integrin β1 molecules and their organization as clusters in plasma membrane areas of non-irradiated (control) and irradiated (1.25 Gy) Jurkat cells visualized by high-resolution microscopy (scale bar = 1 µm). **(B)** Mean number of molecules per region of interest (ROI) in control cells (black circles) and in irradiated cells (red circles). **(C)** Ratio of clustered versus non-clustered integrin β1 molecules in irradiated and non-irradiated cells. Each point in **(B,C)** represents data from one ROI of an image. Box plot symbols have the same meaning as in **Figure 2**.

by elevated $[Ca^{2+}]_{cyt}$ concentration and may serve as a target of an IR-induced Ca^{2+} signaling cascade. To examine the effect of IR on channel expression and activity, we analyzed channel currents 48 h after X-ray exposure where the increase in cell diameter and the expression of CD25 is most pronounced.

Current responses and corresponding current–voltage (I/V) relationships in mock and irradiated Jurkat cells are reported in **Figures 7A,B**. Hyperpolarizing voltage steps elicited only small currents in non-irradiated cells; voltages ≥−40 mV activated the outward rectifying Kv1.3 channel, which is prominently and constitutively expressed in Jurkat cells (36). During extended positive test pulses these channels were fully inactivated, resulting in a small background current I_b (**Figure 7C** inset). This small current includes, among others, the voltage independent small conductance K^+ channel $K_{Ca}2.2$. Subtraction of the latter from the peak current provides a measure for the Kv1.3 channel (I_{Kv}).

To evaluate the effect of IR on the relative contribution of $K_{Ca}2.2$ to the total current, we measured Jurkat cells under four different conditions: (i) mock-irradiated cells with low (≤100 nM) and (ii) high (1 µM) $[Ca^{2+}]_{cyt}$ as well as irradiated cells with (iii) low or (iv) high internal $[Ca^{2+}]_{cyt}$ (**Figure 7D**). Data given in **Figure 7** indicate that I_b/I_{kv} is not augmented by an elevation of $[Ca^{2+}]_{cyt}$. This situation is different in irradiated cells where a high $[Ca^{2+}]_{cyt}$ caused a significant increase in the relative conductance of I_b. An example for the currents and the corresponding I/V relation from an irradiated cell measured with high $[Ca^{2+}]_{cyt}$ is shown in **Figure 7**. The increase in I_b is most apparent in the elevated instantaneous activating inward current. To test whether this additional conductance includes $K_{Ca}2.2$ activity cells were treated with the scorpion toxin Tamapin (10 nM), a specific high affinity inhibitor (IC_{50} = 24 pM) of $K_{Ca}2.2$ channels (37). Treatment revealed a marginal 3.3 ± 0.7% (N = 3) inhibition of I_b in mock-irradiated control cells while Tamapin resulted in a 23 ± 10% inhibition of the respective current in irradiated cells.

To further test the IR triggered upregulation of $K_{Ca}2.2$ channels, their density in the plasma membrane was analyzed by high-resolution single molecule microscopy. The representative images in **Figures 7D,E** show that irradiation caused an elevated number of fluorescent signals in the plasma membrane of Jurkat cells after 48 h (**Figure 7E**). The mean number of fluorescent signals from immunostained $K_{Ca}2.2$ molecules was 1.3 times higher in irradiated cells compared to controls.

DISCUSSION

The relationship between IR and the activation or suppression of the immune system is considered complex and multifactorial. It strictly depends on the dose applied as well as on the type and differentiation status of the immune cell type investigated (2, 38). X-irradiation with single doses ≥2 Gy used in clinical oncological practice generally triggers activating (pro-inflammatory) functions to mediate toxic and/or immune stimulatory effects of RT (1, 3). Application of low-dose radiotherapy with single doses <1 Gy on the other hand is reported to mediate anti-inflammatory effects in a multitude of benign disorders (39, 40).

During the last decades, multiple efforts have been made to uncover the molecular events following radiation exposure and subsequent irradiation-triggered pathways including induction of an inflammatory response (41, 42). We have recently reported that an increase in ROS following X-irradiation with doses ≥1 Gy results in both nuclear and cytoplasmic detection in malignant cells (11, 12). An increase in cytosolic ROS further triggers a Ca^{2+}-mediated signal transduction cascade, which eventually activates Ca^{2+} sensitive K^+ channels and causes membrane hyperpolarization (11, 12). Moreover, upon contact with antigen presenting cells, mitogens or IR, T-lymphocytes respond with a rise in $[Ca^{2+}]_{cyt}$ (43–45). This elicits a multitude of responses, including protein expression, altered phosphorylation patterns, induction of transcription factors (13–16) and an increase in cell diameter (46). In this study, we observed that IR causes also in Jurkat cells a Ca^{2+} signaling cascade, which was not an immediate consequence of irradiation but triggered only after a considerable delay. The same treatment furthermore enhanced expression of the IL-2 receptor (CD25), and cytokines IFNγ and IL-2 at least in Jurkat cells, elevated levels of integrin β1-mediated cell adhesion, augmentation in the conductance of the Ca^{2+} sensitive $K_{Ca}2.2$ channel and a dose-dependent increase in cell diameter. Collectively, this indicates that IR presumably affects an immunological activation or modulation of these cells. In favor of the view that the increase in cell diameter is related to immune activation and Ca^{2+} dependent, we monitored a 50% reduction of the cell diameter increase upon treatment

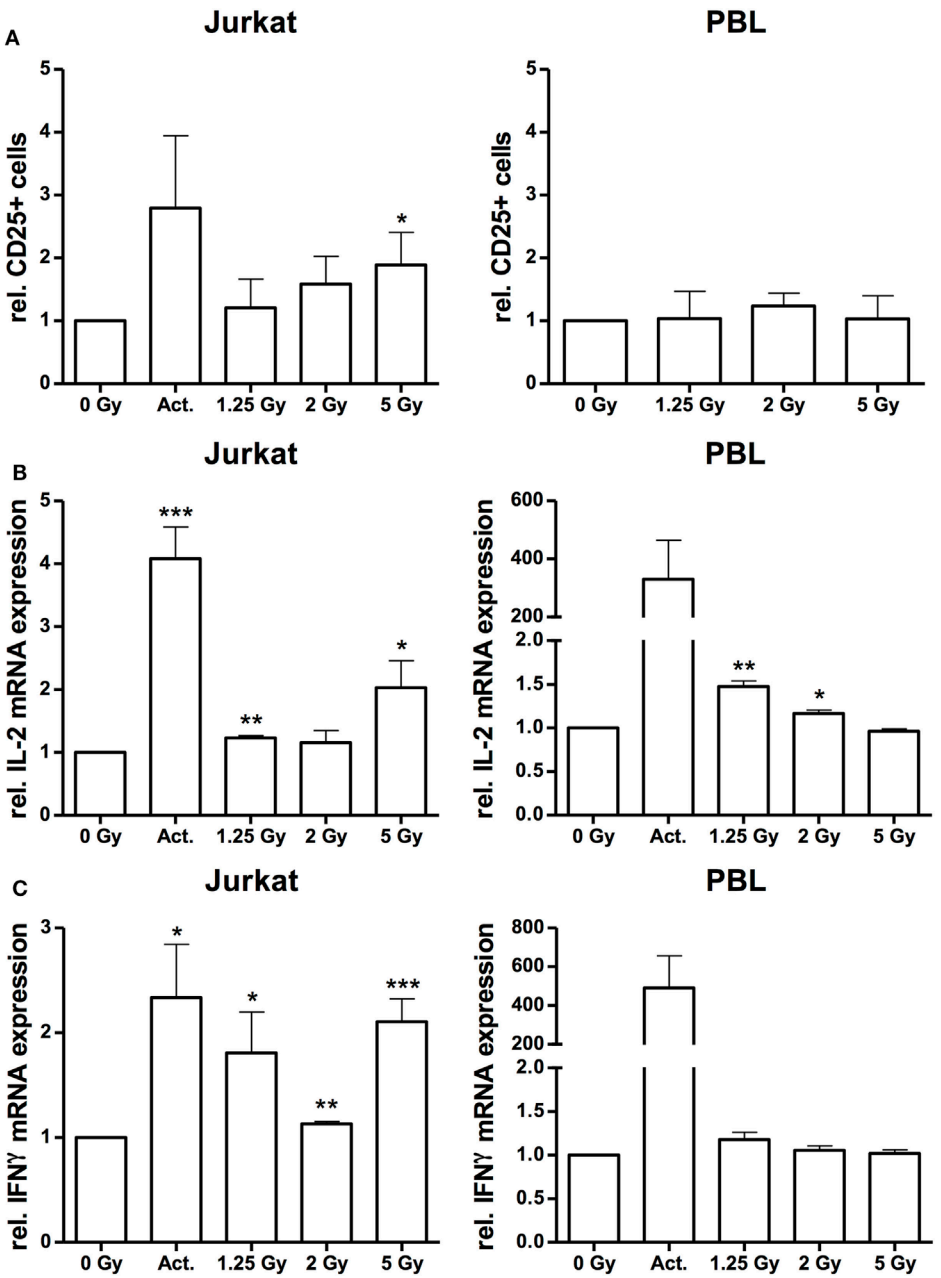

FIGURE 6 | Irradiation stimulates immune activation in Jurkat cells and peripheral blood lymphocytes (PBL). FACS analysis of CD25 surface expression on Jurkat cells and CD3-positive PBL **(A)** following irradiation with a dose of 1.25, 2, and 5 Gy. Stimulation with 25 µl/ml CD3/CD28/CD2 T-cell activator (Act.) in Jurkat cells or mock-irradiated cells served as controls ($N = 3$). In PBLs, an activator could not be applied due to inference with the CD3 stimulus. Quantification of interleukin (IL)-2 **(B)** and interferon-γ (IFNγ) **(C)** mRNA expression by quantitative real-time PCR in Jurkat cells and PBL at 24 h after irradiation with a dose of 1.25, 2, or 5 Gy. Stimulation with 25 µl/ml CD3/CD28/CD2 T-cell activator (Act.) or mock-irradiated cells served as controls ($N = 2$). Data are represented as mean + SD. Student's *t*-test compared activator-treated or irradiated cells with non-irradiated controls; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

with the immunosuppressant cyclosporine A or by buffering changes in $[Ca^{2+}]_{cyt}$ by BAPTA-AM. Moreover, we recognized an IR-induced G2 cell cycle arrest that correlated to the increase in cell diameter by an increase in the size of the nucleus. Consequently, the IR induced increase in cell diameter can be dissected at least in two components, a Ca^{2+}-mediated immune stimulation and a radiation-induced cell cycle arrest.

Adhesion of immune cells to the endothelium displays an initial step in inflammatory cascades and recruitment of T-lymphocytes from peripheral blood to tumor tissue sites (47). Here, we indicate that single doses of 1.25 Gy (48) increase Jurkat T-cell adhesion to Ea.hy926 ECs. The IR-induced increase in adhesion of Jurkat cells was significantly inhibited by addition of peptide comprising the three amino acids Arg–Gly–Asp

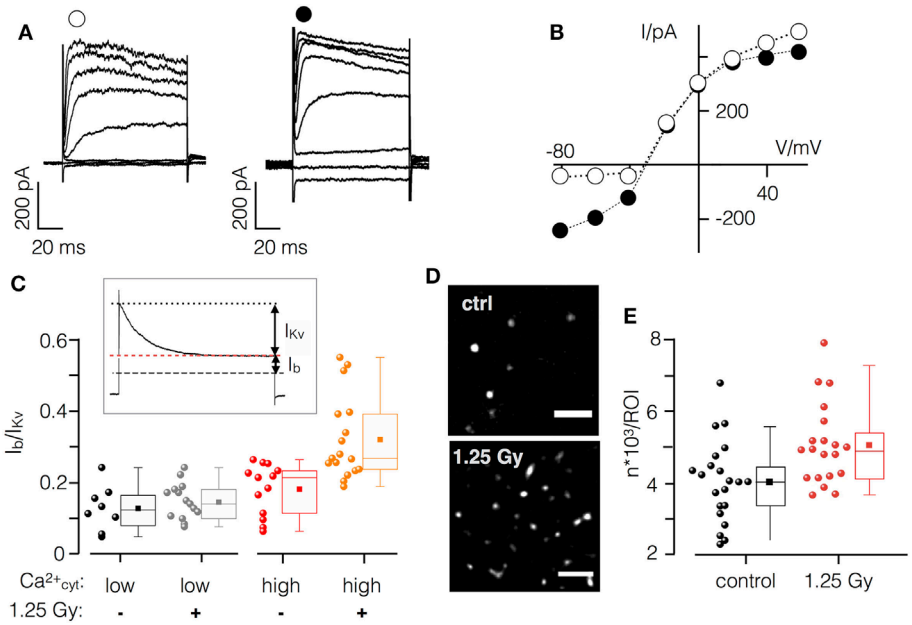

FIGURE 7 | X-ray irradiation activates Ca^{2+} sensitive K^+ channel in Jurkat cells. **(A)** Current responses of un-irradiated (left) and irradiate (right) Jurkat cell in whole cell configuration with high (1 μM) cytosolic Ca^{2+} to test voltages between −80 and +60 mV. **(B)** Peak current/voltage relation of cells in **(A)**. Symbols in **(A)** correspond to symbols in I/V plot. **(C)** Inset: during long clamp steps, the time-dependent Kv1.3 current inactivates (I_{Kv}) leaving the voltage-independent background current I_b. Ratio of I_b/I_{Kv} from non-irradiated (–) and irradiated (+) Jurkat cells (1.25 Gy) with low (<100 nM) or high (1 μM) $[Ca^{2+}]_{cyt}$. **(D)** Single molecule resolution images of $K_{Ca}2.2$ channels in plasma membrane of irradiated (1.25 Gy) and non-irradiated Jurkat cells (scale bar = 1 μm). **(E)** Mean number of $K_{Ca}2.2$ molecules in control cells (black symbols) and 48 h after irradiation (red symbols). Each circle represents an individual region of interest (ROI) of a single Jurkat cell. Box plot symbols have the same meaning as in **Figure 2**.

(RGD peptide) indicating a predominant involvement of integrin β1 adhesion molecules (49) but less pronounced for PBL. This may be attributed to the heterogeneity of cell populations in PBL suspensions with different sets of adhesion molecule expression. By combined immunostaining and high-resolution single molecule microscopy resolving an increased expression, we further confirmed clustering of integrin β1 molecules on Jurkat cells to contribute to the adhesion process. Although not detailed in the present investigation, the underlying mechanism(s) seem to be multifactorial. They may include radiation-induced activation of a variety of transcription factors like the immune relevant nuclear factor kappa B (50). The latter was recently reported to directly bind the integrin β1 promotor region in response to IR resulting in an upregulation of the subunit and modulation of invasiveness and radiation resistance (51).

The IR triggered altered adhesion properties may have different consequences: inflammatory IR responses can favor malignant cell invasion, providing a favorable environment for tumor promotion and metastasis (52–54) or secondary malignancies (55). By this, IR may alter cell phenotypes, which in turn contribute, directly or indirectly, to carcinogenesis. It may also affect the activity or abundance of tissue proteases, growth factors, cytokines and adhesion molecules, which are involved in tissue remodeling (56).

This study mainly focused on the established Jurkat model for analyzing immunological effects of IR but exemplary experiments were also performed on PBL from healthy blood donors indicating differences in CD25 surface detection, cytokine IFNγ and IL-2 expression, and integrin-mediated adhesion to ECs. There is compelling evidence that subpopulations of T cells may display differential radiation sensitivities. While T helper lymphocytes and cytotoxic T cells are characterized by a radiation sensitive phenotype, regulatory T cells, appear to be more radioresistant (38). Notably, by comparing the effects of IR on gene expression in CD4+ T lymphocytes and in Jurkat cells, Mori et al. reported on a predominat upregulation of p53 target genes in naïve CD4+ positive cells. By contrast, Jurkat leukemic cells with a non-functional p53 gene are characterized by alterations in a more limited set of genes belonging to the Rho GTPase and cytokine signaling pathways (57). Accordingly, one may assume that activation of CD25 expression and cytokine response in Jurkat versus PBL may arise from a differential (p53 dependent) gene activation.

More recently, however, it has further become evident that IR not only induces inflammatory reactions and unwanted, temporary immune suppression like leukopenia but is also capable of triggering specific anti-tumor immune responses. This occurs especially when IR is applied in multimodal settings in combination with checkpoint cytotoxic T-lymphocyte-associated protein 4 and programmed death PD-1 and its ligand PD-L1 inhibitors (1, 8). In line with that, distinct tumor infiltrating immune cells, most relevant cytotoxic CD8+ T-cells, predict the response to

radio(chemo)therapy in a multitude of tumor entities and display an essential prerequisite for successful radio-immune therapeutic strategies (4–6).

In summary, our findings indicate that IR in a clinically relevant dose may foster immune activation and functional properties of T-lymphocytes that may have implications for both toxic and cancer inducing effects of radiotherapy but also increases tumor response to combined RT and novel immune therapies in cancer patients and patients with non-malignant disorders.

AUTHOR CONTRIBUTIONS

PV, SF, FW, LB, SH, and DT performed experiments and analyzed data. PV, TM, CF, SH, FR, AM, and GT designed experiments and analyzed/interpreted data. PV, CF, AM, FR, and GT wrote the paper. All authors were critically revising the work and approved the final content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00922/full#supplementary-material.

FIGURE S1 | Ionizing irradiation stimulates adhesion of peripheral blood lymphocytes (PBL). Relative adhesion rates of PBL to endothelial cells. Cells were incubated at 4°C (open circles) or 37°C (closed circles) without (–) or with (+) TNF-α (20 ng/ml). Cell were further treated with CD3/CD28/CD2 T-cell activator (25 µl/ml), irradiated with 1.25 Gy X-ray in absence or presence of 10 µM RGD peptide in incubation buffer. All data were normalized to value measured for untreated cells at 4°C. Mean values ± SD ($n = 5$; $N = 3$). Student's *t*-test compared activator-treated and irradiated cells with non-irradiated controls with TNF-α, 37°C and irradiated cells with and without RGP peptide; $^{*}P < 0.05$, $^{**}P < 0.01$.

FIGURE S2 | Irradiation stimulates immune activation in Jurkat cells. Western immune blots **(A)** and quantification **(B)** of Jurkat cells 48 h after irradiation with a dose of 1.25 and 2 Gy using an anti-CD25 antibody. Mock-irradiated cells served as a control ($n = 3$). Immuno-fluorescent detection (scale bar = 25 µm) **(C)** and quantification **(D)** of interleukin-2 (IL-2) in control and Jurkat cells 48 h after irradiation with 2 Gy. Mean fluorescent intensity ± SD in region of interests (ROIs) in the cytoplasm of control cells and irradiated cells ($N = 2$; $n \geq 10$ cells). Immuno-fluorescent detection (scale bar = 25 µm) 10 **(E)** and quantification **(F)** of interferon-γ (IFNγ) in control and Jurkat cells 48 h after irradiation with X-ray doses between 0.5 and 5 Gy or treatment with 25 µl/ml CD3/CD28/CD2 T-cell activator. Mean fluorescent intensity ± SE in ROIs of control cells and irradiated cells ($N = 3$; $n \geq 70$ cells). Data in **(F)** were fitted by Eq. 1 yielding a D50 value of 1 Gy and a maximum increase in fluorescence of 2.

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REVIEW
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Radiation-Induced Transformation of Immunoregulatory Networks in the Tumor Stroma

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The implementation of novel cancer immunotherapies in the form of immune checkpoint blockers represents a major advancement in the treatment of cancer, and has renewed enthusiasm for identifying new ways to induce antitumor immune responses in patients. Despite the proven efficacy of neutralizing antibodies that target immune checkpoints in some refractory cancers, many patients do not experience therapeutic benefit, possibly owing to a lack of antitumor immune recognition, or to the presence of dominant immunosuppressive mechanisms in the tumor microenvironment (TME). Recent developments in this field have revealed that local radiotherapy (RT) can transform tumors into *in situ* vaccines, and may help to overcome some of the barriers to tumor-specific immune rejection. RT has the potential to ignite tumor immune recognition by generating immunogenic signals and releasing neoantigens, but the multiple immunosuppressive forces in the TME continue to represent important barriers to successful tumor rejection. In this article, we review the radiation-induced changes in the stromal compartments of tumors that could have an impact on tumor immune attack. Since different RT regimens are known to mediate strikingly different effects on the multifarious elements of the tumor stroma, special emphasis is given to different RT schedules, and the time after treatment at which the effects are measured. A better understanding of TME remodeling following specific RT regimens and the window of opportunity offered by RT will enable optimization of the design of novel treatment combinations.

Keywords: radiotherapy, tumor microenvironment, immunotherapy, tumor stroma, angiogenesis, extracellular matrix, mesenchymal cells, myeloid cells

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INTRODUCTION

Radiation therapy (RT), either used alone or combined with systemic therapies, is a cornerstone of cancer treatment. Technological improvements now enable precise delivery of large radiation doses to tumors, stimulating profound changes in RT treatment schedules for some cancers. The use of stereotactic body radiotherapy (SBRT), in which high-dose radiation is delivered with extreme precision in small numbers of fractions, is becoming increasingly widespread (1). RT impacts upon both tumor and host cells, exerting multiple effects beyond the simple destruction of malignant cells. In recent years, we have witnessed an increased awareness of the role played by the complex tumor microenvironment (TME) in the response to therapy (2, 3). Consequently, recent research has investigated the effects of radiation on tumor stroma elements such as fibroblasts, connective tissue, vasculature, or immune cells.

The field of cancer immunology has also witnessed tremendous progress, leading to the development of new therapies that do not target tumor cells but instead boost the host immune system to fight against malignancy. The clinical implementation of novel immunotherapies in the form of immune checkpoint inhibitors (ICIs) is becoming one of the greatest advancements in the history of cancer treatment (4). In responders, ICIs may induce long-lasting tumor regression, even in patients with multiple metastatic lesions (5). Recently, the immune contexture of the TME was introduced as a new concept that classifies tumors by quantifying immune cell densities, and may define the likelihood of responding to immunotherapy (6). Patients with lymphocyte-rich "hot" tumors have been seen to respond remarkably well to ICI with long-lasting tumor regression. Unfortunately, the majority of patients present with "cold" tumors, which may explain the relatively low response rates observed when ICI is given as monotherapy.

Radiotherapy has been proposed as a promising, readily available, non-toxic, and cost-effective partner to immunotherapy. The immune-stimulatory properties of RT have generated widespread interest based on preclinical and clinical observations that localized RT can induce regression of non-irradiated metastases (abscopal effects) (7). However, it remains to be determined whether radiotherapy is only an occasional enhancer of ICI effects or represents a true "game changer" (8). In addition, our understanding of how, and how often, radiotherapy can convert tumors from being unresponsive to responsive is limited. As a proof-of-principle, it was demonstrated more than 30 years ago that T-cells can contribute to radiation-induced tumor control, a phenomenon that adds to the direct killing of malignant cells (9, 10). Moreover, it has been shown that radiation is able to ignite adaptive antitumor immune responses through the induction of immunogenic cell death and the release of endogenous adjuvants from dying tumor cells (11, 12). Likewise, systemic antitumor responses after combined ICI and local RT have been demonstrated in some murine models (13–15). Nevertheless, abscopal effects of RT in the clinic remain rare, thus highlighting the need to better understand and address the obstacles to effective *in situ* tumor vaccination.

Numerous reports have demonstrated that the "*in situ* vaccination" effects of local radiotherapy are mediated through induction of immunogenic cancer cell death and the associated release of powerful danger signals, which are essential to recruit and activate dendritic cells (DCs) and mount an adaptive immune response. However, efficient immune rejection is often hindered by intrinsic barriers within the TME (16). For instance, migration of effectively primed T-cells into the tumor can be inhibited by the disorganized vasculature, high interstitial fluid pressure, and other mechano-biological and chemotactic signals. In addition, resident and recruited cells (and molecules) in the TME can impair the survival, activation, proliferation, and effector-function of cytotoxic T-cells. Given the importance of the multifactorial immunosuppressive forces encountered in the TME, in this review we focus on RT effects on stromal elements that may influence antitumor immune responses. Intentionally, we will not cover RT effects on the malignant component of tumors, which have been comprehensively reviewed by other authors in the past (17, 18).

In our view, insufficient consideration has been given to the divergent biological effects elicited either by different radiation regimens, or to the timing of key biological processes. Most preclinical studies exploring the immunogenic effects of RT (alone or in combination with immuno checkpoint blockers) have been limited to testing a single radiation dose or schedule at a single time point, despite the unquestionable fact that different radiation regimens induce markedly different cellular and tissue responses (2, 18). In addition, the numerous ongoing clinical trials exploring RT-IT combinations are not consistent with each other, and are largely designed based on empirical choices of radiation regimens instead of rational ones (19). Consequently, the outcomes are likely to be divergent and/or inconclusive, and may fail to demonstrate the ability of radiation to synergize with immunotherapy. In this review, therefore, we put special emphasis on describing effects associated with specific radiation regimens, and draw attention to the chronology of events. To avoid misinterpretation, we refer to radiation doses of 2 Gy or less as "low," doses of 4–10 Gy as "intermediate," and doses above 10 Gy as "high."

EFFECTS OF RT ON ECM REMODELING, CONDUCTIVITY, AND TISSUE STIFFNESS

Solid tumors generally display increased tissue stiffness and tensile strength compared to neighboring normal tissues. Tumor stiffening results from augmented deposition of interstitial extracellular matrix proteins, mainly collagen (fibers), but also hyaluronan, elastin, and fibronectin, along with a steadily increasing population of non-malignant and malignant cells. The mechanical forces mediated by these structural components (20) constitute physical barriers that hinder access and motility of blood-borne antitumor T-cells (21, 22), (therapeutic) antibodies (23), liposomes, and nanoparticle drugs (24), thereby greatly affecting immune surveillance and immunotherapy responses.

Dynamic RT Effects on ECM Remodeling

Based on the idea that depletion or reduction of intratumoral collagen can reduce solid stress and open up compressed blood and lymphatic vessels (25), several laboratories have demonstrated improved blood-borne drug delivery by reducing collagen content (25–27). Paradoxically, RT, despite being a well-known trigger of fibrotic tissue reactions (28–31), has been shown to augment tumor penetration by "large" macromolecules such as monoclonal antibodies (32–34), and also liposomes, and nanoparticles (35–39), enhancing the passive processes of enhanced permeability and retention (40). The clue to understanding this paradox is time. Obviously, temporal aspects of drug/antibody administration versus RT delivery are of utmost importance in achieving optimal responses. The limited time-frame for using RT to improve drug distribution was highlighted by Jain et al. (29), who measured the effects of ionizing radiation (IR) (1 × 10 Gy) on tumor hydraulic conductivity, hyaluronan, and collagen type-I in colon adenocarcinoma xenograft tumors. They found unchanged

collagen levels 24 h post-RT, but 4 days later hydraulic conductivity was decreased (12-fold) while collagen-I levels were elevated. Lower radiation doses may not induce such fibrotic reactions. In a preclinical study by Appelbe et al., quantification of collagen in xenograft tumors excised 17 days post-RT revealed increased collagen-I staining after high (1 × 15 Gy) but not low or moderate radiation doses (2 and 5 Gy) (38).

Enhanced intra- and inter-molecular cross-linking of collagen and elastin fibers is another factor directly affecting tissue stiffness. The enzyme lysyl oxidase (LOX), which initiates cross-linking in the extracellular space, is elevated in response to hypoxic microenvironments and various cytokines (41, 42), and is associated with metastasis and poor survival in breast and head-and-neck cancer (43). Inhibition of LOX activity decreased levels of fibrillar collagen, increased tumor infiltration of macrophages and neutrophils, eliminated metastases in models of orthotopic breast (43) and transgenic pancreatic cancer (44), and enhanced drug delivery in a PDAC tumor model (44). Of note, IR promotes secretion of LOX from several tumor cell lines in a time- and dose-dependent manner (45). Shen et al. analyzed conditioned medium from lung tumor cells collected 16–20 h after exposure to single RT doses (2, 5, or 10 Gy), and observed increased secretion of both active LOX enzyme and inactive LOX pro-enzyme, with 10 Gy increasing LOX secretion 15-fold. Histological quantification in irradiated lung tumor xenografts revealed no change after 24 h, but prominent changes in LOX were observed 48 h post-RT for the two regimens examined (1 × 10 Gy) and (2 × 10 Gy). Moreover, LOX blood serum levels 48 h post-RT were doubled in mice that received (2 × 10 Gy) compared to the group receiving (1 × 10 Gy) (45). Others have collected murine lung tissue 2, 4, 8, and 20 weeks after thoracic radiotherapy (5 × 6 Gy), and found elevated LOX expression and activity at every time point (46). *Time* post-RT is clearly an important factor to consider.

The Role of Transforming Growth Factor Beta (TGF-β)

Radiation-induced fibrotic reactions are initiated and sustained by a cascade of pro-inflammatory cytokines, which are released hours to days after radiation exposure (28). TGF-β—a master switch for the fibrotic program (47)—stimulates collagen production and functions as a chemoattractant for fibroblasts, with the capacity to reprogram fibroblasts into tumor-promoting and *fibrosis-associated* myofibroblasts (48). Rube et al. irradiated the thoracic region of fibrosis-sensitive mice and examined temporal aspects of TGF-β expression. They found a dose-dependent induction of TGF-β in lung tissue: a single dose of 12 Gy triggered TGF-β release that peaked after 12 h, whereas 6 Gy released minor amounts of TGF-β (49). In a similar experiment, Finkelstein et al. found upregulated TGF-β during 14 days (50). In line with the notion that TGF-β is critical for radiation-induced fibrosis, blocking TGF-β reduces the fibrosis induced by high-dose RT in animal models (51, 52). In a mouse model of mammary carcinoma, Liu et al. blocked TGF-β and found decreased collagen content and normalized tumor interstitial matrix, which improved drug uptake and decreased tumor growth (25). Besides the well-known immune-suppressive functions exerted on inflammatory and immune cells, TGF-β modulates ECM deposition and tissue stiffness, thus exerting both direct and indirect immunoregulatory effects. TGF-β could therefore represent a major obstacle to radiotherapy-induced antitumor immunity, which may be overcome by TGF-β neutralizing antibodies (53). TGF-α may also be involved in radiation-induced lung injury, as elevated tissue levels of TGF-α (46) post-RT have been demonstrated.

Dynamic Effects of RT on Proteases of the ECM

Connective tissue homeostasis is tightly controlled by the balanced expression of proteases and their inhibitors. Matrix metalloproteinases (MMPs) and their endogenous inhibitors, TIMPs, are key matrix regulators. Studies *in vitro* and *in vivo* have demonstrated radiation-induced alterations in protease activity, which may lead to increased tumor invasion (54, 55). In particular, transient and dose-dependent upregulation of extracellular MMP-2 and MMP-9 have been observed in irradiated cell lines derived from pancreatic cancer (54), glioma (56), lung cancer (57, 58), melanoma (59), fibrosarcoma (55), and hepatocarcinoma (60).

Transient upregulation of various MMPs in response to IR has been characterized in many experimental settings. Speake et al. analyzed conditioned medium from a fibrosarcoma cell line (55), and demonstrated pro-MMP-2 and pro-MMP-9 levels to peak at 24 and 48 h post-RT, respectively, whereas others found MMP-2 secreted by lung tumor cells to peak at 12 h (58) or 24 h (57) post-RT. Co-culture systems—exemplified by glial and endothelial cells (ECs)—are also responsive to RT, with MMP-2 and MMP-9 levels being markedly elevated 72 h after irradiation (61). Stromal cells also contribute to release of proteases into the TME. Human lung tumor fibroblasts respond to single-high radiation doses (18 Gy), by reducing secretion of MMP-1 when measured 5 days post-irradiation, whereas MMP3 levels are enhanced and MMP2 unchanged at the same time point (62).

In an animal model of Lewis lung carcinoma, serial measurement of urinary MMP-2 revealed increasing levels during tumor growth, but reduced levels 6 days post-RT (2 × 20 Gy) (63). At the clinical level, Susskind et al. measured plasma levels of MMP-9 and TIMP-1 in lung and breast cancer patients and observed very high levels before initiation of fractionated radiotherapy (66 Gy, 2.0 Gy/fx), a sharp decline in MMP-9 levels within 10 days of completion of RT, but no change in TIMP-1 levels (64). The latter finding is in line with results from irradiated human lung tumor fibroblasts (62). IR also affects membrane-associated metalloproteinases (or ADAMs). McRobb et al. found that a single dose of 20 Gy to brain microvascular ECs downregulated the alpha secretase ADAM10, with concomitant upregulation of ADAM10 target proteins at the cell surface (65). Another study by Sharma et al. revealed that radiotherapy activates ADAM17 in non-small cell lung cancer (NSCLC), inducing shedding of multiple survival factors, growth factor pathway activation, and IR-induced treatment resistance (66).

Collectively, these studies underscore the importance of tissue stiffness on drug uptake and immune cell infiltration. Lessons learned from the field of drug delivery indicate that RT can be used to transiently reduce intratumoral interstitial pressure and increase vascular permeability. However, the effects of RT are temporary and only provide a window of opportunity during the first day(s) after the radiation insult. By contrast, prolonged exposure to multiple fractions of RT seems to induce matrix deposition, long-term fibrotic reactions, and increased stiffness. A summary of radiation-induced effects on ECM remodeling and tissue stiffness is presented in **Figure 1**.

EFFECTS OF RT ON TUMOR VASCULATURE AND LYMPHATIC VESSELS

Trafficking of newly activated antigen-specific T-cells is dysfunctional in cancers. Tortuous and leaky vessels hinder transit and extravasation of leukocytes into tumors; an imbalance of pro- and anti-angiogenic factors in solid tumors contributes to such vascular aberrations. The tumor vasculature is also a recognized obstacle to therapeutic access, and both preclinical and clinical studies have shown that vascular normalization can augment drug delivery in tumors. Such approaches may also enhance antitumor immunity.

Dynamic RT Effects on Tumor Vessels

Effects of RT on blood endothelial cells (BECs) are highly dependent on total dose and fraction-size, as well as tumor stage-location-type and maturation stage of vessels. High-dose RT (≥10 Gy) is more likely to induce EC death (67) and tumor vessel collapse (68, 69), whereas at low doses (≤2 Gy), BEC survival is promoted through miRNA upregulation (70) with enhanced EC migration and angiogenesis (71). There is some evidence that intermediate doses (4–10 Gy) may induce tumor vessel normalization and vessel dilation, reducing vascular leakage and increasing tumor oxygenation (72, 73). Scheduling must also be taken into consideration if combination strategies are to be optimized. Kabacik and Raj found that endothelial permeability to macromolecules of various sizes increased in a radiation dose-dependent manner, and involved ADAM10 activation and cleavage of VE-cadherin junctions (74). Park et al. measured vascular permeability in the skin of C3H-mice exposed to local irradiation (2, 15, or 50 Gy), and found that it peaked 24 h post-IR, followed by a gradual decrease to baseline over the next 3–10 days. Of note, the extent and duration of

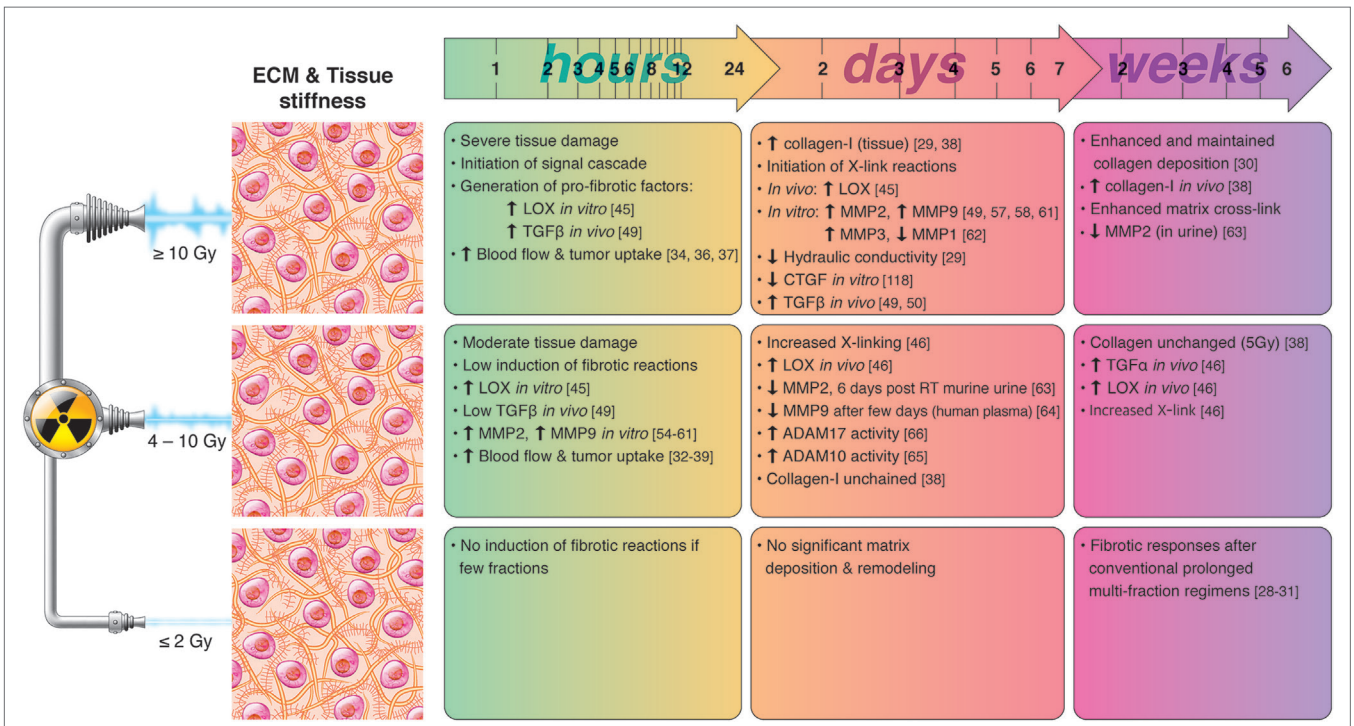

FIGURE 1 | Chronological effects observed on ECM remodeling agents and tissue stiffness after RT delivered at different dose per fraction. *The figure is a compilation of observations registered in different experimental models, comprising primary cell cultures (in vitro), cell lines, animal (mainly mice) models, and clinical observations, on effects of RT given at different dose/fraction. Radiation schemes range from single fractions to oligo (daily) fractions and prolonged multifractionated regimens. The vast majority of preclinical observations comprise RT regimens of one or few fractions irrespective of the radiation dose. In clinical settings, RT protocols comprising moderate or high doses are always applied in one or few fractions*. Although some inconsistencies may exist between studies, it is generally observed that small doses in few fractions do not ignite substantial changes in ECM composition and tissue stiffness, whereas both medium and high RT doses exert measurable changes on matrix deposition and tissue stiffness in a dose- and time-dependent manner. RT mediated pro-fibrotic effects and matrix stiffness may become apparent several days post-irradiation and may last for weeks and months after RT.

vascular permeabilization was dose-dependent (75). Kalofonos et al. also measured vascular permeability and vascular volume of irradiated (1 × 4 Gy) colon adenocarcinoma xenografts (34), and observed increased vascular permeability 24 h post-RT, but no differences between treated and control tumors at 72 h. Appelbe et al. (38) applied an intermediate radiation dose (5 Gy) to mammary adenocarcinoma xenografts, with drug administration before and after RT, and demonstrated 1.2- to 3.3-fold enhancement of probe accumulation in tumors. In addition, they observed maintained vascular integrity during the first 2 days post-RT, even at doses up to 15 Gy. They concluded that intermediate to high doses of radiation—insufficient to achieve tumor control—are sufficient to enhance drug delivery, independent of endothelial integrity. Other authors have also observed that low to intermediate RT doses (≤5 Gy) can stimulate angiogenesis (71) and/or vasculogenesis (76) in ECs. Hallahan et al. measured microvascular blood flow in irradiated murine hind-limb tumors just before and 24 h after RT, and found that a single-low dose of 2 or 3 Gy increased tumor blood flow 24 h post-RT, whereas 6 Gy markedly reduced blood flow (77). Others have observed that a single dose of 8 Gy causes minimal damage to microvessels and the EC lining (78), with a modest 4.3% reduction in perfusion (4 h post-RT). Kolesnick et al. have previously suggested a threshold dose of (1 × 10 Gy) for induction of apoptosis in ECs (79).

While inconsistencies in the preclinical literature persist, accumulating evidences indicate that the main response of quiescent BECs to IR is induction of premature senescence rather than apoptosis (80). Panganiban et al. found that 10 Gy induced accelerated senescence in the majority of pulmonary artery ECs (87%, 120 h post-IR), but only residual levels of apoptosis (81). Moreover, at doses above 8 Gy, 99% of the ECs were alive but not competent to form colonies. Oh et al. irradiated bovine aortic ECs (5, 10, and 15 Gy) and observed increasing numbers of large, flattened senescent-like cells at higher doses, with a twofold increase in average cell surface area after 15 versus 10 Gy (67). Massive cell death appeared 2–5 weeks after 15 Gy, whereas 5 Gy induced only transient morphological disturbances. Others have also demonstrated radiation-induced senescence in BECs (82–84), with long-lasting DNA damage responses and durable nuclear foci formation (82, 84). Of note, the extent and duration of senescence in various types of BECs after different radiation doses corresponds with radiation-induced senescence in lymphatic endothelial cells (LECs) (85) and cancer-associated fibroblasts (CAFs) (62).

In general, extensive endothelial damage after doses above 10 Gy causes reduced vascular flow, which impairs effector T-cells recruitment to the tumor, and exacerbates the hypoxia-driven immunosuppressive environment. Hypofractionated regimens using doses per fraction below 10 Gy might induce sufficient cancer cell death without exacerbating hypoxia and immunosuppression.

RT Effects on Cell Adhesion Molecules in ECs

Dysfunctional extravasation of leukocytes into tumors because of structural abnormalities of vessels is exacerbated by changes in the adhesive properties of tumor ECs. Reduced expression of E-selectin may lead to impaired lymphocyte recruitment. Other adhesion receptors such as ICAM-1, ICAM-2, and VCAM, which facilitate integrin-mediated extravasation, are often poorly expressed by tumor-associated ECs.

Radiation exposure is known to alter the expression of cell adhesion molecules on ECs. Hallahan and colleagues irradiated human umbilical endothelial cells (HUVECs) and observed induced expression of both E-selectin and ICAM-1 in a dose- and time-dependent manner (86). Threshold doses of 1 and 5 Gy for induction of E-selectin and ICAM-1, respectively, were observed, however, VCAM-1 and P-selectin surface expression were apparently unaffected by IR. Similarly, Gaugler et al. (87) irradiated cultured HUVECs and observed upregulation of ICAM-1 but not VCAM-1 after various doses of IR (2, 5, and 10 Gy). Others exposed epidermal keratinocytes and dermal microvascular ECs to 6 Gy, and found that IR triggered surface expression of ICAM-1 on these cells within 24 h, independent of *de novo* protein synthesis (88). At sub-lethal doses, IR may enhance expression of certain cell adhesion molecules in ECs and thereby contribute to leukocyte homing and immune recognition.

Recruitment of Endothelial Progenitors Following RT

Vasculogenesis, the formation of new blood vessels by recruitment of bone marrow-derived endothelial precursor cells (BMDCs), is a major mechanism for vessel repair and tumor regrowth after RT (89). Several laboratories have demonstrated radiation-induced recruitment of proangiogenic myeloid BMDCs into tumors, orchestrated by chemotactic SDF-1-CXCR4 signaling. In an intracranial xenograft model of glioblastoma (GBM), Kioi et al. found that whole brain irradiation (8 or 15 Gy) triggered dose-dependent recruitment of BMDCs into tumors (90). Interestingly, BMDC levels were only slightly elevated from control levels after 8 Gy, but more than doubled after 15 Gy. However, BMDC influx and/or retention after 15 Gy was efficiently blocked by AMD3100, an inhibitor of the SDF-1/CXCR4 axis. In this study, AMD3100 was administered on the day of irradiation, with continued infusion over the following 21 days. Kozin et al. exploited the same concept in breast and lung tumor xenografts, and found that combined AMD3100 and local irradiation significantly delayed tumor growth, but only when the drug was applied immediately after local irradiation (91). In their model, drug administration 5 days post-IR was ineffective. Hence, radiation-induced recruitment of BMDCs into tumors was suggested to be a rapid process (91). Altogether, results from preclinical studies indicate that a single large dose of local irradiation may trigger two waves of BMDCs influx (92): one shortly after exposure (3–5 days) (91) and a second delayed response (associated with hypoxia) after about 2 weeks (90). Accumulated knowledge coming mainly from preclinical models supports the notion that recruitment of bone marrow precursors is the main mechanism behind tumor neovascularization following RT, and that the effect is proportional to the radiation dose. Importantly, this process seems to be activated immediately after radiation exposure and completed within few days after tissue damage.

RT Effects on Pericytes

Pericyte coverage is also abnormal in tumor vessels; pericytes appear to be loosely associated with vessels and with poorly developed basal lamina, therefore contributing to increased leakiness. Increased VEGFA in the TME may hinder pericyte function and survival by suppressing PDGFRβ signaling. Pericytes from tissues such as the liver may also exert direct immunomodulatory effects by expressing negative co-stimulatory molecules (93) or, as in malignant glioma, by secretion of paracrine immunosuppressive signals, including PGE2, TGFβ, and NO (94).

The effects of radiation on pericytes have scarcely been investigated. In a xenograft model of neuroblastoma, tumor blood volume measurements 6 h post-RT were reduced by 63 and 24% after 12 and 2 Gy, respectively. Histopathological examination revealed a significant loss of EC at 6 and 12 h, and an additional loss of both mature and immature pericytes at 72 h (95). However, high-dose RT is postulated to enhance recruitment of mesenchymal stem cells to the TME, which could promote pericyte recovery and tumor recurrence. In a xenograft study by Wang et al. (96), bone marrow mesenchymal precursors were observed to home into tumors and transform into pericytes following (1 × 14 Gy) irradiation in an SDF-1 and PDGF-B-depending manner. Fractionated irradiation of murine prostate TRAMP-C1 tumors at intermediate doses (15 × 4 Gy) resulted in reduced microvascular density but increased tumor perfusion, associated with dilated vessels tightly connected to BM-derived pericytes (97). In a similar manner, Lewis lung carcinoma-bearing mice treated with high-dose RT (1 × 12 Gy) or (3 × 12 Gy) exhibited reduced microvessel density but increased perfusion, reduced hypoxia, and increased pericyte coverage (98).

Collectively, these studies suggest that irradiating tumors with both intermediate and high doses results in decreased microvascular density but increased perfusion due to dilation of surviving vessels and increased pericyte coverage, taking place some days after RT.

RT Effects on Lymphangiogenesis

Lymphatic vessels constitute a transport route for both antitumor immune cells and metastatic spread of tumor cells. However, the disorganized lymphatic system that is characteristic of solid tumors can lead to impaired fluid flow and increased interstitial pressure (99). LECs may also hinder antitumor immunity by cross-presentation of tumor antigens in a VEGF-C-dependent manner (100). In addition, the lymphatic drainage of tumor antigens may affect antitumor immunity by promoting a tolerogenic environment in sentinel lymph nodes (100).

Despite the fact that lymph nodes and vessels are often included in the irradiated field in clinical practice, relatively few studies have explored the effects of IR on LEC integrity and function. An array of studies have documented that, contrary to blood vessels, high doses of RT (>10 Gy) do not affect lymphatic vessel integrity (101–103). In skin biopsies from breast cancer patients, similar numbers of lymphatic vessels were observed in irradiated and non-irradiated sites (103). Sung et al. examined responses to high-dose radiation on LECs in the small intestine of adult and embryonic mice and in peri-tumoral areas of mice, and concluded that intestinal and peri-tumoral LECs are highly resistant to radiation-induced apoptosis (102). In fact, LECs are likely to respond to IR by the induction of stress-induced cellular senescence. Avraham et al. exposed cultures of dermal LECs to single doses of 4, 8, or 12 Gy and found that (4 days post-IR) senescence was triggered in 53, 64, and 74% of the cell population, respectively (85). The same study revealed a minor 8% apoptosis-induction in LECs upon (1 × 15 Gy). A recent study by Rodriguez-Ruiz et al., which utilized cultures of primary human LECs as well as mouse transplanted tumors and pre- and post-RT patient samples (104), revealed a radiation-dose and time-dependent induction of ICAM-1 and VCAM-1 surface expression on LYVE-1+ LECs. The maximum effect was observed at 20 Gy and persisted for more than 8 days. The authors proposed that such an effect may mediate enhanced adherence of T-lymphocytes on irradiated LECs.

Few reports studying normal tissue reactions to radiotherapy propose that IR at high doses may induce impairment of the lymphatic vasculature (105). However, most studies highlight the radioresistant nature of LECs and the beneficial effects of RT on induction of adhesion molecules that favor T-cell recruitment and extravasation. A summary of radiation-induced effects on tumor vasculature and hypoxias is presented in **Figure 2**.

MESENCHYMAL CELLS, RADIATION, AND IMMUNITY

RT Effects on CAFs

Immunomodulation is one of the best-characterized tumor regulatory mechanisms exerted by CAF. In general, CAFs are considered to promote an immunosuppressive TME. However, new evidence suggests that such effects may be specific for certain CAF subsets, and may depend on temporal and contextual factors (106, 107). Through secretion of a plethora of cytokines, chemokines, proteases, and proangiogenic factors, CAFs may exert both direct and indirect effects on tumor immunity. Direct effects on effector memory T-cells are mediated *via* secretion of potent immunoregulators such as TGFβ, PGE2, TSLP, interleukin (IL)-6, IL-8, or nitric oxide (16). In addition, CAFs may mediate indirect effects by expression of ECM molecules that attenuate antitumor immunity, such as tenascin-C, galectin-3, or thrombospondin-1, by participating in ECM synthesis and turnover, or by exerting an impact on tumor angiogenesis (108). Moreover, CAFs express cytokines and chemokines that support the recruitment and maintenance of immunosuppressive myeloid cells, promote the polarization of macrophages toward the M2-phenotype, and interfere with maturation of DCs (109). In the context of RT, CAFs are considered to be very radioresistant (62, 110–112), however, exposure to IR is able to induce cellular senescence in fibroblasts, especially at doses above 12 Gy (62). In xenograft models, senescent fibroblasts co-transplanted with cancer cells have been found to increase tumorigenicity. A recent preclinical study by Li and colleagues (113) demonstrated radiation (1 × 4 Gy) to enhance the tumor-promoting effects of CAFs, an effect that was associated with increased expression of

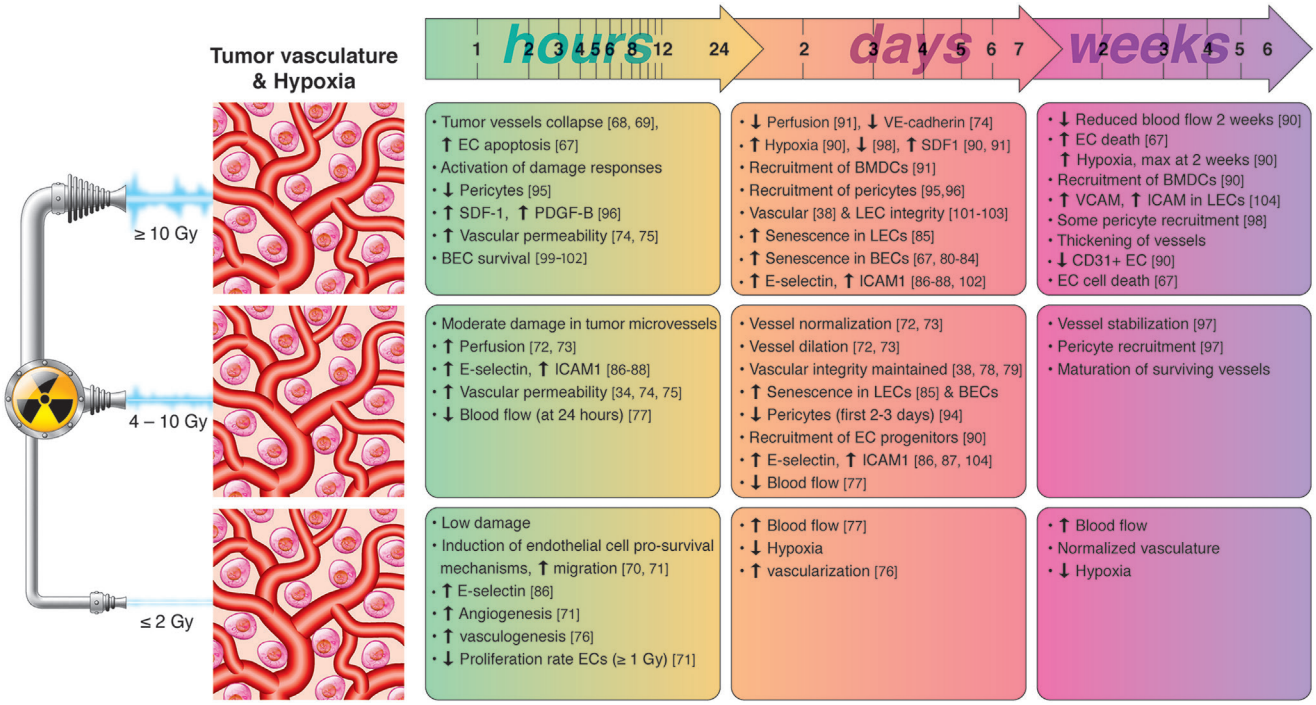

FIGURE 2 | Chronological effects observed on tumor vasculature and hypoxia after RT delivered at different dose per fraction. *The figure is a compilation of observations registered in different experimental models, comprising cell cultures (in vitro), animal (mainly mice) models, and clinical observations, on effects of RT given at different dose/fraction. Radiation schemes range from single fractions to oligo (daily) fractions and prolonged multifractionated regimens. The vast majority of preclinical observations comprise RT regimens of one or few fractions irrespective of the radiation dose. In clinical settings, RT protocols comprising moderate or high doses are always applied in one or few fractions.* Although some inconsistencies may exist between studies, it is generally observed that small doses in few fractions promotes endothelial cell (EC) survival and increased intratumoral blood flow, whereas high-dose RT induces EC apoptosis, hypoxia-response elements and the recruitment of EC progenitors during the first days post-RT. Medium radiation doses, given in one or few fractions, induce moderate damage in tumor blood vessels, promotes the dilation and normalization of existing vessels, pericyte recruitment, and the expression of cell adhesion molecules. Lymphatic endothelial cells (LECs) are more radioresistant than blood vessels but may enter into premature senescence already after moderate radiation doses.

CXCL12. However, the overall tumor regulatory properties of senescent or irradiated fibroblasts remain controversial, as other studies have observed no impact of (high-dose) irradiation on the tumor enhancing effects of fibroblasts, or even loss of pro-malignant properties (114–116).

The immunoregulatory phenotype of irradiated fibroblasts is less well characterized, since most *in vivo* studies have been conducted on immunocompromised animals. A recent *in vitro* study revealed that primary lung CAFs maintain their immunosuppressive phenotype after exposure to both high (1 × 18 Gy) and low (4 × 2 Gy) radiation doses (117). On the other hand, high dose IR (1 × 18 Gy) has been shown to alter the secretory profile of CAFs and the expression of factors that could exert immunomodulatory effects, directly or indirectly (118). Multiplex protein analyses on conditioned medium collected from irradiated human lung CAFs from five different donors with NSCLC revealed that single-high dose RT (1 × 18 Gy) leads to a prominent (38%) and significant reduction of SDF-1 and threefold reduction in macrophage inhibitory factor (118). Besides their direct paracrine effects on inflammatory and immune cells, CAFs may influence tumor immune responses indirectly by mediating ECM remodeling. As indicated earlier, CAFs are major contributors of desmoplastic reactions in tumors and thus could exert indirect effects on tumor immune infiltration by regulating tissue stiffness and interstitial fluid pressure. One recent study has compared levels of αSMA expressing CAFs in tumor specimens from colorectal cancer patients receiving neoadjuvant radio(chemo)therapy (45 Gy in 25 fractions) before and after treatment (119). Results from this study revealed increased amounts of αSMA expressing myofibroblasts and connective tissue post-therapy. Connective tissue growth factor (CTGF) is also mitogenic and chemotactic for fibroblasts, and stimulates synthesis of collagen-1 (33) and fibronectin (34). In response to IR (1 × 18 Gy), secreted levels of CTGF from human lung CAFs are reduced 3.5-fold compared to controls, suggesting that exposure to ablative radiation doses may exert anti-fibrotic effects on CAFs (118). However, in an animal model, *ex vivo* irradiated CAFs (1 × 18 Gy) co-implanted with A549 tumor cells induced tumors with similar extents of collagen deposition and inflammatory cell infiltration as tumors established with non-irradiated CAFs (116).

Recognizing that we still lack knowledge on the effects mediated by irradiated CAFs in the tumor context, and that different CAF subtypes may respond differently to IR, overall the existing literature indicates that CAFs are likely to survive radiation insults and that high-dose irradiation could exert beneficial effects in relation to CAF-mediated tumor immune regulation.

A summary of radiation-induced effects on fibroblasts and immuno-regulation is presented in **Figure 3**.

Effects of RT on Mesenchymal Stromal Cells (MSCs)

Tissue damage provoked by RT triggers the recruitment of MSCs from distant reservoirs such as bone marrow or adipose tissue. Recruited MSCs post-treatment have been associated with both pro- and antitumorigenic effects. The migration and differentiation potential of MSCs were characterized in a Lewis lung carcinoma and malignant melanoma-bearing recipient mice treated with (SB)RT, 14 Gy/1 fraction (96). Recruitment of circulating MSCs was promoted by secretion of SDF-1 and PDGF-B from irradiated tumor cells. In this study, it was proposed that engaged MSCs transform into pericytes to promote tumor vasculogenesis and tumor regrowth. On the contrary, irradiated MSCs may be a source of antitumor cytokines that decrease the proliferative activity and induce apoptosis of tumor cells (120). In the study by de Araújo Farias et al. (121), *in vivo* administration of unirradiated mesenchymal cells together with radiation lead to an increased efficacy of radiotherapy. In a separate study, tumor irradiation was shown to enhance the tumor tropism of adoptively transferred human umbilical cord blood-derived mesenchymal stem cells in an IL-8-dependent manner (122). Enhanced therapeutic effects were associated to TRAIL delivered by MSCs.

The effects of RT delivered in low-dose multifraction schedules on MSCs can be more unpredictable. MSCs recruitment may start already after the first cycles of radiation, however, IR, even when delivered at low doses, can have profound effects on the biology of MSCs. In a recent *in vitro* study, bone marrow-derived MSCs isolated from normal adults were irradiated with 2 Gy twice daily for consecutive 3 days (123). Irradiated MSCs showed much lower proliferative and differentiation potential, and induced clonal cytogenetic abnormalities of MSCs. Likewise, when isolated MSCs were irradiated with 2 Gy alpha particles or X-rays, adverse effects were observed on the vitality, functionality, and stemness of MSCs (124).

Collectively, efforts in this field have shown that RT, especially when delivered at high doses, triggers the recruitment of progenitor mesenchymal cells into the irradiated tumors, and that such recruitment could exert both tumor-promoting or tumor-inhibiting effects. Considering the demonstrated immunoregulatory potential of MSCs, recruited MSCs following RT could play an important role on immunomodulation,

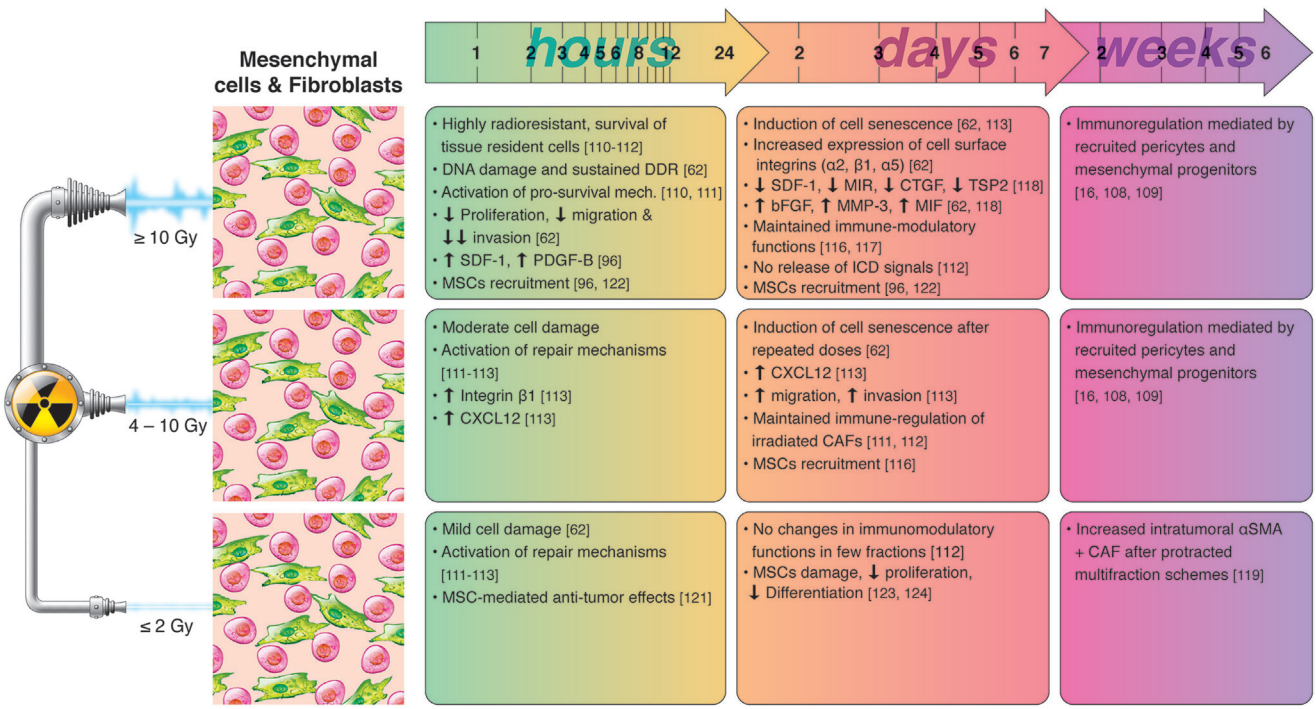

FIGURE 3 | Chronological effects observed on cancer-associated fibroblasts (CAFs) and mesenchymal cells after RT delivered at different dose per fraction. *The figure is a compilation of observations registered in different experimental models, comprising primary cell cultures (in vitro), cell lines, animal (mainly mice) models, and clinical observations, on effects of RT given at different dose/fraction*. Studies on RT effects on CAFs are scarce. It is well demonstrated that tumor fibroblasts are quite radioresistant and may survive even ablative doses of radiation. Small doses may not change importantly the phenotype and functions of CAFs. At high doses, CAFs become senescent within few days post-irradiation, less motile, and less proliferative. High RT doses may substantially alter the secretory profile of CAFs characterized by reduced levels of SDF-1, MIR, or connective tissue growth factor (CTGF), and increased levels of bFGF, MMP3, or macrophage inhibitory factor (MIF), observed within few days post-RT. Of relevance, *in vitro* observations reveal that the immunosuppressive functions of cultured CAFs are preserved after exposure to high and low RT doses. In a long run, mesenchymal progenitors and pericytes recruited after medium and high doses may contribute importantly to immunoregulations, to prevent the onset of autoimmune reactions.

however, this particular hypothesis remains to be demonstrated experimentally.

EFFECT OF LOCAL RADIATION ON INFLAMMATORY CELLS

Myeloid-derived cells are an important part of the TME, both numerically and functionally, and play central roles in regulating tumor vasculature and antitumor immune responses. Myeloid cells arise from a common myeloid progenitor that, upon differentiation, gives rise to various cell types including tumor-associated macrophages (TAMs), DCs, polymorphonuclear neutrophils, and myeloid-derived suppressor cells (MDSCs). Myeloid cells in tumors may exist in various differentiation stages, and possess a susceptible immunomodulatory phenotype that can be influenced by radiation.

Radiation-mediated changes on myeloid cells include killing of tumor-associated pools, recruitment of circulating progenitors, repolarization, and reorganization (125). Of note, bone marrow-derived cell recruitment following RT involves mainly SDF-1/CXCR4–7, CCL2/CCR2–4, and colony-stimulating factor-1 (CSF-1)/CSF-1R pathways. Observed effects seem to depend on radiation regimens and the timing post-RT, however, pre-existing tumor microenvironmental parameters such as hypoxia, necrosis, pH, stroma composition, and cytokine milieu may all influence tumor leukocyte composition following RT.

RT Effects on Macrophages

Tumor-associated macrophages are considered to be relatively radioresistant because of their well-developed anti-oxidative machinery. However, IR is able to affect both phenotype and recruitment of TAMs. Globally, data generated in different tumor types and using different RT regimens indicate that high doses (10–30 Gy)—either as single dose or oligo-fractioned (≤3×)—trigger recruitment of CD11b+ myeloid cells and reprogramming of macrophages toward the tumor-promoting M2-phenotype (126, 127). Interestingly, selective ablation of CD11b+ or CD18+ cells (128), or blockage of the SDF-1/CXCR4 or CSF-1/CSF-1R pathways prevents accumulation of myeloid cells/macrophages and improves antitumor immune response and the overall response to IR (90, 129). Of importance, upregulation of the M2-gene signature has been observed within few days of irradiation and may last for several weeks or even longer (130, 131). In the TRAMP-C1 prostate cancer model, a single fraction of 25 Gy or 15 fractions of 4 Gy induced the M2-genes COX2 and Arg-1 within few days (126). On the contrary, intermediate radiation doses (2–5 Gy) given in few fractions have been reported to repolarize macrophages from M2- to the pro-immunogenic M1-phenotype *in vitro* and *in vivo*. Non-polarized, monocyte-derived macrophages established in cultures shifted toward the M1-phenotype after daily (5 × 2 Gy) radiation schemes (132). Doses of 5–10 Gy have been shown to increase nitric oxide synthase and decrease M2-phenotypic traits (133). *In vivo* experiments have mainly utilized small doses. Klug and colleagues demonstrated that single fractions of (0.5–2.0) Gy polarize macrophages toward the iNOS + M1-phenotype (134), whereas whole body irradiation with a single dose of 2 Gy caused CD11+ peritoneal macrophages to repolarize into the M1-phenotype. In another study, induction of the M1-phenotype in tumors after local IR (1 × 2 Gy) was only possible in combination with CD8+ T-cell transfer (134). Upon M1 repolarization, the resulting iNOS expression appears to be responsible for vascular normalization, T-cell recruitment and activation, and finally tumor rejection. Of note, very low radiation doses (under 1 Gy) may favor the M2-phenotype of TAMs, as evidenced by *in vitro* culture experiments performed with different macrophage sources (135–137).

In summary, the accumulated knowledge in this area postulates that high-dose irradiation or moderate doses in multiple fractions facilitate the recruitment and reprogramming of macrophages with immunosuppressive functions, and that medium and low-dose radiation (down to 1 Gy) in single or few fractions may elicit immune-stimulatory macrophages that could help to unlock barriers to immunotherapy responses.

RT Effects on MDSCs

As with macrophages, local radiation is able to mobilize other myelomonocytic CD11b+ cells with immunosuppressive functions in tumors. MDSCs have the unique ability to radioprotect tumor cells through expression of high levels of Arginase-I, with subsequent depletion of L-arginine from the microenvironment, a common mechanism behind T-cell and macrophage inhibition (138). Many and varying effects of radiation on mobilization and function of MSDCs have been reported and are likely to be influenced by the pre-existing systemic and local immune contexture. As described for macrophages, several studies in murine models have reported increased recruitment of MDSCs after high-dose RT. In a glioma model, high-dose radiation (1 × 15 Gy) induced more marked recruitment of CD11b+ myeloid cells than lower doses (1 × 8 Gy) (90). In addition, selective inhibition of CSF-1/CSFR-1 signaling was observed to improve the efficacy of RT by reducing recruitment of immunosuppressive MDSCs (129). Low radiation doses may exert different effects. Whereas human subjects treated with protracted RT regimens show elevated CSF-1 in peripheral blood, analyses of immune cell composition in peripheral blood of patients receiving fractionated chemoradiotherapy often reveal a reduction in both MDSCs and Tregs in relation to effector T-cells after treatment (139–142). A study comparing intratumoral infiltration of immunocytes pre- and post-neoadjuvant chemoradiotherapy in rectal cancer specimens demonstrated significant elevation of CD8+ and CD4+ T-cells post-treatment whereas MDSC, Tregs, and expression of co-inhibitory receptors remained stable (143). Similarly, ablative radiotherapy (1 × 30 Gy) has been shown to increase CD8+ cells and decrease MDSC in the TME of CT26 and MC38 murine tumors, whereas fractionated radiation did not trigger such strong lymphocytic responses (144).

RT Effects on DCs

Dendritic cells can be divided into several subsets with specialized functions, and are key intermediaries between the innate and

the adaptive immune systems. However, very few studies have documented the effects of RT on DC subsets and their roles in immune regulation.

Previous work have shown that DCs are relatively resistant to IR and exhibited limited changes in response to high-dose irradiation, such as upregulation of CD80 and reduced levels of IL-12 but not IL-10 (145). The effect of IR on phagocytosis and antigen presentation in DCs appears to depend on radiation dose and DC maturation state. For instance, 5 Gy gamma irradiation downregulated expression of co-stimulatory receptors CD80/CD86 on immature derived DCs but not on mature DCs (146). In a different study, CD86 expression was increased in immature but decreased in mature DCs after 30 Gy, while other markers remained unaffected (145). Of interest, in the former study, irradiation impaired the stimulatory effects of both mature and immature DCs on proliferation of allogeneic T-cells (145). Although *in vitro* studies suggest that IR compromises the stimulatory activities of DCs, *in vivo* models demonstrate that IR at intermediate radiation doses (5 × 8.5 Gy) enhances the ability of DCs to capture tumor antigens, and promotes DCs migration to lymph nodes in a toll-like receptor-dependent manner (147, 148). A number of studies have demonstrated increased presentation of tumor antigens by DCs in the tumor-draining lymph nodes after RT. For example, in B16-OVA and B16-SIY melanoma models, single radiation doses (15–25 Gy) or five fractions of 3 Gy increased the number of antigen-presenting cells cross-presenting tumor-specific antigens, which correlated with increased priming of antitumor T-cell responses (149, 150). It is important to note that *in vivo* effects mediated by recruited "non-irradiated" DCs may explain the discrepancies between *in vitro* and *in vivo* observations.

Of importance, IR effects on DCs can also differ between murine and human systems. At a dose of 0.2 Gy, ϒ-irradiation increased surface expression of CD80, CD86, MHC-class I and II receptors in murine DCs, but inhibited their capacity for antigen uptake. In addition, this low-dose IR suppressed IL-12 production and increased IL-10, implying a shift to immune tolerance (151). On the other hand, low-dose radiation under 1 Gy did not affect surface markers or cytokine production in either immature or mature human DCs, and had no influence on the capacity of DCs to stimulate T-cell proliferation (152).

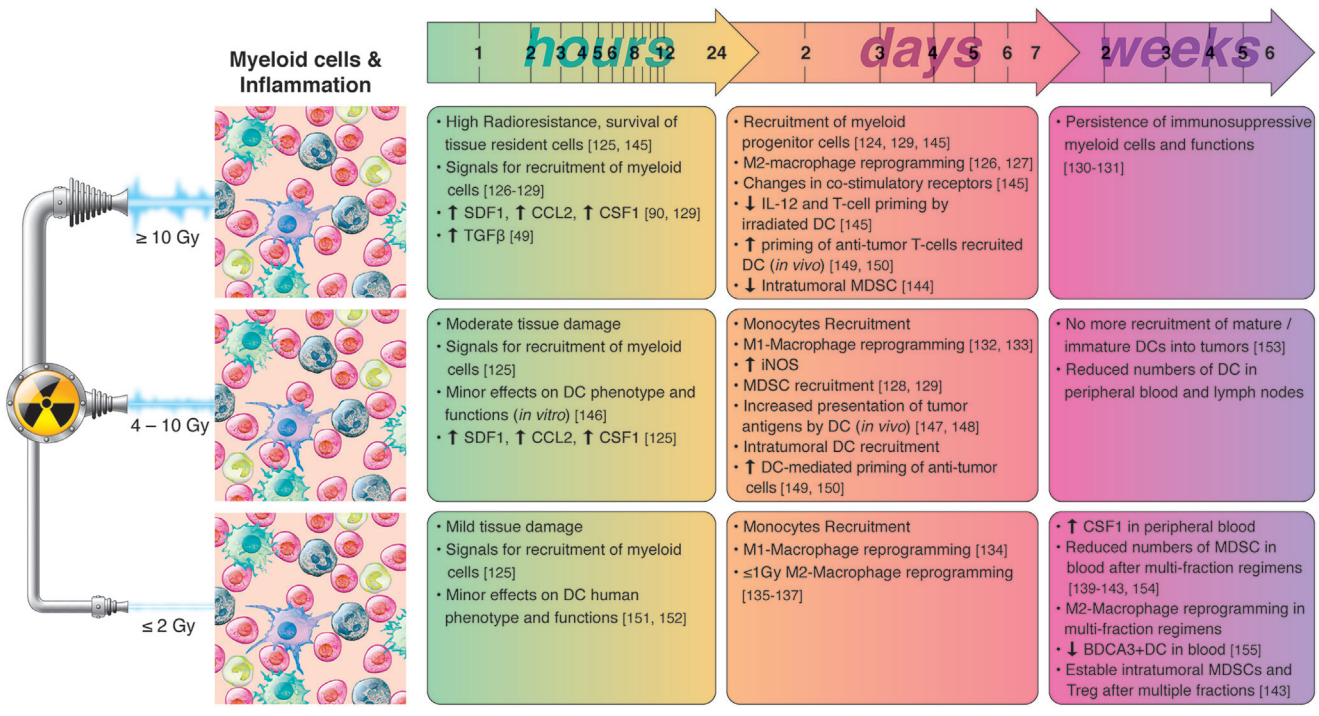

FIGURE 4 | Chronological effects observed on myeloid cells and inflammation after RT delivered at different dose per fraction. *The figure is a compilation of observations registered in different experimental models, comprising primary cell cultures (in vitro), cell lines, animal (mainly mice) models, and clinical observations, on effects of RT given at different dose/fraction. Radiation schemes range from single fractions to oligo (daily) fractions and prolonged multifractionated regimens. The vast majority of preclinical observations, using cell cultures and animals, comprise RT regimens of one or few fractions irrespective of the radiation dose. In clinical settings, RT protocols comprising moderate or high doses are always applied in one or few fractionations*. In small doses, RT do not affect substantially the phenotype and function of dendritic cells (DCs). However, such doses have been shown to promote M1-polarization of macrophages and monocyte recruitment. The severe tissue damage provoked by high radiation doses induces the rapid release of chemotactic molecules such as CCL2, colony-stimulating factor-1 (CSF1) and SDF-1, and the recruitment of myeloid cells. High radiation doses induce M2 polarization of macrophages, impair the immune-stimulatory functions of tissue resident DCs, and activates de recruitment of myeloid-derived suppressor cell (MDSC). Medium radiation doses also trigger the recruitment of myeloid cells to tumors, however, immune-activating effects preponderate, characterized by M1-polarization of macrophages, and increased presentation of tumor antigens by DC. The mobilization of myeloid cells following RT cease after the first week(s), and it is normal to observe reduced numbers of MDSC, monocytes, or DCs in peripheral blood when prolonged multifraction regimens are completed.

Different radiation schedules may influence DC function and recruitment in different ways. In a murine melanoma study testing intratumoral DC vaccination, it was demonstrated that (5 × 8.5 Gy) enhanced the ability of DCs to capture tumor antigens without inducing enhanced DC maturation, but improving cross-priming of T-cells (147). Hypofractionated RT has been shown to recruit and activate DCs, however, this effect maybe time-restricted. In a recent preclinical study using colon cancer as a model, MHC-II positive DC recruitment into tumors was observed only between days 5 and 10 after the first radiation dose (153). In patients, conventional low-dose multifraction regimens may have detrimental effects on DCs. In head-and-neck cancer patients, neoadjuvant treatment was associated with a general decrease of tumor infiltrating DCs in intraepithelial compartments as assessed by IHC (154). In a study from Liu et al., authors found a significant decrease of BDCA3+ DCs, the immune-stimulatory variant, in the blood of patients treated with conventional radiotherapy (155).

The majority of *in vitro* studies indicate that moderate and high radiation doses are able to inhibit antigen presentation capacity and production of Th1 cytokines by DCs. However, *in vivo* studies seem to reflect opposite effects. DCs responses to RT can be very divergent between hypofractionated (SBRT) or multifraction regimens. To understand the contradictory observations published in this area, it is utterly important to consider the difference between tumor-associated DC pools that become irradiated during treatment (normally occurring during long-lasting conventional RT) versus non-irradiated DCs that infiltrate tumors after treatment (possibly occurring in SBRT strategies).

A summary of radiation-induced effects on myeloid cells and inflammation is presented in **Figure 4**.

CONCLUDING REMARKS

A considerable number of ongoing clinical trials are aiming at improving the efficacy of immune checkpoint blockers by local radiotherapy. Mounting evidences reveal that RT may prime and/or induce tumor-specific adaptive immune responses through the induction of immunogenic cell death the release of tumor-specific antigens and danger signals, and the ignition of an inflammatory cascade. However, it is still uncertain whether RT can be used effectively to enhance the effects of immunotherapeutic drugs in clinical settings. In fact, radiation may promote immunosuppressive reactions in several ways, such as upregulation of co-regulatory molecules PD-L1 and PD-L2 (156, 157), transient potentiation of hypoxia, or by recruiting and reprogramming of immunosuppressive myeloid cells. Treatment outcomes will ultimately depend on the net effect of pro-immunogenic and anti-immunogenic signals, and will be heavily dependent on pre-existing host and tumor factors. Moreover, even after defining optimal RT regimens for combinatory treatments, numerous physical and functional barriers to immune attack must be overcome to achieve clinical benefit. These include immunosuppressive elements in the stromal components of non-irradiated metastasis, and antigenic heterogeneity at different metastatic sites.

The effects of radiation on the multifactorial elements of the TME may be tumor type and tumor stage specific, may be influenced by the pre-existing tissue contexture, and are likely to be highly dependent on the treatment protocol. In this review, we have attempted to gather existing knowledge on the potential effects exerted by different radiation schemes in the compartments of the tumor stroma that may modulate antitumor immunity. Published studies range from *in vitro* experiments to preclinical *in vivo* models and clinical observations. Despite intense endeavors, most of the existing preclinical reports are limited to exploring effects of a single radiation dose or regimen. The treatment outcomes reported could be equally influenced by experimental variables such as the intrinsic immunogenicity and/or radiosensitivity of the tumor cells, the immune competence of the host, implantation site, and tumor stage. Thus, information gathered from preclinical studies should not be interpreted as universal dogmas or generalizable evidences with direct applicability in the clinics. Also, knowledge from clinical studies is limited because of the inherent restrictions associated with the clinical protocols, where, for example, immunological effects are normally measured from peripheral blood samples and only rarely in the irradiated tissues. Conclusion about the relative effects of different radiation schemes on immune activation can only be made by performing systematic comparisons using the same tumor model.

Although the existing knowledge is fragmented, model-specific and in some cases inconsistent, some key patterns emerge. In general, high-dose RT, given as single dose or in few fractions, results in severe tissue damage, increased tumor cell death, and enhanced release of tumor-associated antigens and related danger signals. However, high-dose RT also seems to activate mechanisms that counterbalance these potentially overwhelming immune reactions. Thus, downstream effects associated with high-dose RT comprise substantial damage to tumor vasculature, transient potentiation of hypoxia, increased fibrosis and interstitial pressure, recruitment and reprogramming of immunosuppressive myeloid cells, and release of signals that favor Th2 pathways. On the contrary, low-dose radiation protocols (2 Gy/fraction and below) are often followed by a number of immune adjuvant effects comprising normalization of tumor vasculature, enhanced expression of cell adhesion molecules, increased perfusion, decreased interstitial fluid pressure and reprogramming of tumor infiltrating macrophages into the antitumorigenic M1-phenotype. However, low-dose RT may not be very effective in boosting the generation of tumor-associated antigens and danger signals. Furthermore, the conventional clinical protocols based on multifraction regimens applied over several weeks may exert detrimental effects on recruited DCs and effector T-cells, thus hampering the establishment of tumor-specific immune responses. Intermediate radiation dose protocols seem to reproduce many of the positive effects observed with low radiation dose protocols, including vessel normalization and transient induction of pro-inflammatory environments. Hypofractionated regimens comprising doses per fraction below 10 Gy might generate meaningful levels of cancer cell death without exacerbating hypoxia and immunosuppression. However, to achieve responses that can synergize with immunotherapies, it is of the utmost importance

to consider time and treatment sequence. In many instances, immune adjuvant effects occur within hours of RT treatment, and may be maintained for only a few days before the favorable circumstances are changed or lost. In such circumstances, radiation should perhaps be applied in reduced number of fractions, concomitant with or immediately after administration of the immunotherapeutic drug has begun.

For the future, we encourage clinicians and scientists to use existing knowledge to design clinical trials for assessing the overall clinical benefit of radiation combinations, and employ rational choices of dose, fractionation, treatment sequence, and timing. In parallel, further mechanistic studies are needed to understand how dose and fractionation influence the effects of RT on the pre-existing TME. There is a need to systematize protocols and knowledge by designing comparative studies of different RT-schemes using unmodified and immune competent animal models. The use of radiotherapy as a partner for immunotherapy is an exciting and revolutionary concept, but much remain to be learned before its true clinical potential is realized.

AUTHOR CONTRIBUTIONS

IMZ and TH contributed equally to the initial conception, the development and the writing of the article. AJC has critically revised the manuscript for important intellectual content and has approved the final version to be published.

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REVIEW
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Radiation, Immune Checkpoint Blockade and the Abscopal Effect: A Critical Review on Timing, Dose and Fractionation

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The combination of radiation and immunotherapy is currently an exciting avenue of pre-clinical and clinical investigation. The synergy between these two treatment modalities has the potential to expand the role of radiation from a purely local therapy, to a role in advanced and metastatic disease. Tumor regression outside of the irradiated field, known as the abscopal effect, is a recognized phenomenon mediated by lymphocytes and enhanced by checkpoint blockade. In this review, we summarize the known mechanistic data behind the immunostimulatory effects of radiation and how this is enhanced by immunotherapy. We also provide pre-clinical data supporting specific radiation timing and optimal dose/fractionation for induction of a robust anti-tumor immune response with or without checkpoint blockade. Importantly, these data are placed in a larger context of understanding T-cell exhaustion and the impact of immunotherapy on this phenotype. We also include relevant pre-clinical studies done in non-tumor systems. We discuss the published clinical trials and briefly summarize salient case reports evaluating the abscopal effect. Much of the data discussed here remains at the preliminary stage, and a number of interesting avenues of research remain under investigation.

Keywords: radiation, immunotherapy, checkpoint blockade, abscopal effect, PD-1, PD-L1

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INTRODUCTION

Traditionally, radiation therapy (RT) is considered a local form of cancer treatment with an "in-field" anti-tumor effect. RT has been used to treat localized malignancies with curative intent or to palliate painful, bleeding or otherwise problematic metastases. Over time radiation delivery has changed (2-D vs. 3-D vs. IMRT), however, the basic philosophy focused on controlling local disease has persisted. Interestingly, in patients with multiple lesions, tumor regression occurs, although rarely, outside the RT field. This is known as an abscopal effect or "ab"- away from, "scopus"–target. This was first described by Mole et al. (1) with over 46 cases of a RT-induced abscopal effect subsequently documented including a prominent report from Memorial Sloan Kettering (2, 3). Patients with several distinct cancer histologies and across a range of ages have benefited from this phenomenon. The abscopal response is now being interrogated with increasing vigor with the goal

of improved therapeutic outcomes for metastatic cancer patients, especially in combination with emerging immunotherapy agents (3).

T-cell checkpoints (CTLA-4, PD-1) are cell surface molecules which prevent T-cell activation or reinvigoration following chronic antigen exposure (4–6). Inhibiting these T-cell checkpoints leads to greater anti-tumor T-cell activity. Checkpoint inhibitors are now the most frequently prescribed immunotherapy and have shown great promise in many different malignancies (7–11). Interestingly, the relatively rare abscopal effect has been observed with increasing frequency as checkpoint inhibitors are being given in close temporal proximity or concurrently with RT (12). There are many questions that remain unanswered regarding the safety, efficacy, optimal dose/fractionation and timing of immune-checkpoint inhibitors in combination with RT. Here we present several mechanisms responsible for the abscopal effect and summarize relevant basic science findings, clinical trials, and clinical case reports. We also provide data which may inform optimization of RT dose, fractionation and timing of administration of immune-checkpoint blockade/immuno-modulators in order to maximize the RT-induced abscopal effect.

RADIATION AND THE IMMUNE SYSTEM

Classically, RT was thought to be immunosuppressive due to the exquisite radio-sensitivity of leukocytes; but, more recently, data has shown that RT can enhance various components of the antigen processing and presentation pathway (13–15). Reits et al. demonstrated *in vitro* and *in vivo,* a dose dependent increase in cell-surface MHC-I levels in response to RT in a transcription independent manner (16). This is thought to be due to an increased intracellular peptide pool from both increased protein translation and increased protein degradation leading to a larger epitope repertoire to be presented following tumor cell death.

Liberation of antigens and increased MHC-I expression alone, however, would not be sufficient for effective anti-tumor T-cell priming. For this, maturation of antigen presenting cells (APCs) is necessary. APC maturation involves, in addition to MHC-I and II upregulation, increased expression of costimulatory ligands B7-1, B7-2 as well as cytokine production important for T-cell proliferation and phenotypic skewing (17). This can occur via APC pathogen recognition receptor (PRR) ligation by non-self-derived adjuvants, pathogen-associated molecular patterns (PAMPs), or endogenous damage associated molecular patterns (DAMPs) (18). Importantly, RT can induce immunogenic cell death (ICD), which, in contrast to apoptosis, releases tumor cell contents, including DAMPs, in a disorganized fashion which can be highly pro-inflammatory. In the context of RT induced ICD, DAMPs include high-motility group box 1 (HMGB1), heat shock protein 70 (HSP 70), GP96 and calreticulin membrane exposure (19–21). Calreticulin, an endoplasmic reticulum resident molecular chaperone, can stimulate phagocytosis of cancer cells by dendritic cells (22) while HMBG1, a critical chromatin protein, promotes antigen presentation (23). Radiation-induced calrecticulin exposure increases T-cell mediated tumor lysis, and in the presence of a calreticulin-blocking peptide this effect was abrogated (24). Wang et al. have shown that RT, over a wide dose range, induced HMGB1 extracellular release and cytoplasmic translocation in a dose and time-dependent manner (25). The subsequent HMGB1 mediated APC maturation is TLR-4 dependent (26). An integral role for APCs in anti-tumor T-cell priming and the abscopal effect was shown in a bilateral syngeneic mouse model of breast cancer wherein immunoadjuvant treatment with FMS-like tyrosine kinase receptor 3 ligand (FLT3L), which promote DC development and bone marrow egress (27), resulted in growth delay in an irradiated flank tumor as well as the untreated, contralateral tumor (28). Together these data support an intimate relationship between an anti-tumor immune response and RT mediated tumor cell killing.

RADIATION SEQUENCING WITH IMMUNOTHERAPY

How does this immunogenic antigen bolus released by RT and presented by APCs synergize with checkpoint inhibitors to enhance the anti-tumor immune response, and how does this inform the sequencing of these two treatment modalities? Two candidate mechanisms to explain this synergy are proposed: (1) neo-antigens released in response to RT may act in concert with anti-PD-1 immunotherapy to only reinvigorate exhausted intratumoral CD8 T-cells, or (2) RT may stimulate proliferation and differentiation of naïve T-cells in response to liberated neo-antigens while anti-PD-1 may potentiate naïve T-cell activation in addition to reinvigorating exhausted T-cells. Each mechanism leads to a more robust immune response, but would result in a different response amplitude and carries different implications for combined modality therapy. If the immunogenic effect arises from naïve T-cell proliferation and activation, very close sequencing of RT and anti-PD-1 will be required for anti-PD-1 to potentiate early T-cell activation. Whereas, if the reinvigoration of exhausted T-cells is the dominant mechanism, this temporal overlap may be less critical and the effect would be additive rather than synergistic. Current evidence suggests that RT acts primarily to stimulate proliferation and differentiation of naïve T based on a broadening of the T-cell receptor repertoire post-RT although this may reflect an expansion of low frequency exhausted clones (29). These two mechanisms described are not mutually exclusive, however, pre-clinical tumor data has demonstrated that initiating anti-PD-L1 7 days following RT was inferior to starting on either the first or the last day (30). These data support (2), however, a deeper understanding of the underlying mechanism can be found in models of acute viral infections.

The Armstrong strain of lymphocytic choriomeningitis virus (LCMV) is a well characterized system for studying acute T-cell responses and naïve T-cell differentiation (31). In a recently published study, it was demonstrated that exposure to anti-PD-L1 during early T-cell differentiation to an acute Armstrong infection impacts T-cell effector function (32). The authors showed that the acute T-cell response is inhibited by endogenous

PD-1 activity, but that anti-PD-L1 during initial T-cell activation increases granzyme B expression in virus-specific CD8 T-cells, resulting in faster clearance of infection (32). While the role of PD-1 in mediating CD8 T-cell reinvigoration in chronic infection is well established (33), this finding supports previous reports by Barber et al. that showed in acute infection of PD-L1$^{-/-}$ mice with LCMV resulted in a heightened CD8 T-cell response. Furthermore, both found the CD8 T-cell response was also improved in chronic infection characterized by T-cell exhaustion (6). Together, these data support close sequencing of RT and checkpoint blockade as late administration of anti-PD-L1 reinvigorates exhausted T-cells without the added benefit of influencing initial T-cell activation and differentiation. Data to directly support these findings in a tumor model combining RT and checkpoint blockade is still lacking.

The kinetics of T-cell tumor infiltration following RT also helps inform the sequencing and timing of anti-PD-1/L1 administration. Following tumor irradiation with 12 Gy on 2 consecutive days, it was shown that overall leukocyte and CD8 T-cell frequencies peak at 5 days post-RT and then gradually decline to pre-RT levels (34). Five-days post-RT also reflects the highest effector to Treg ratio suggesting an ideal time point for checkpoint blockade. These data further support RT dosing with hypofractionation in a limited number of fractions as additional fractions may ablate recently infiltrated lymphocytes. The work by Frey et al. reinforce these findings (35). They showed that following 5 Gy × 2 fractions, CD8 T-cells peak at day 8 and decline significantly by day 9 while Treg have a bimodal peak on days 8 and 10 (35). Together these studies suggest that while the exact T-cell tumor infiltration kinetics may vary depending on the murine model and RT dose, close sequencing of checkpoint blockade following RT should be the goal to take advantage of the peak in tumor effector CD8 T-cells.

Sequencing Depends on the Checkpoint Agent

Optimal RT and immunotherapy sequencing may also depend on the immuno-modulatory agent utilized. As articulated earlier, anti-PD-L1 appears to have the greatest synergy with RT when administered concurrently (30). In contrast, Young et al. have shown data in support of pre-treating with a TGF-β inhibitor in a mouse model of multiple different cancer types including colorectal cancer (36). TGF-β is a factor critical for Treg differentiation, and it is capable of impairing CD8 T-cell effector function. Using a small molecule inhibitor of TGF-β, the authors found an increase in intra-tumoral activated CD8 T-cells and fewer CD4 Treg. For the colorectal cancer experiments, mice were treated with 20 Gy × 1 fraction 7 days after the initiation of the anti-TGF-β therapy. They demonstrated improved survival in mice pre-treated with anti-TGF-β and RT compared to RT alone.

More recently, the same group directly compared the sequencing of two different immuno-modulatory agents relative to RT. In this pre-clinical study, they first evaluated whether administering a CTLA-4 antagonist 7 days prior, 1 day following or 5 days following RT (20 Gy × 1 fraction) changed outcomes. The best outcomes were observed when anti-CTLA-4 was delivered before RT. Interestingly, they showed that all mice that cleared the tumors were resistant to re-challenge with the same cell line at 100 days, suggesting the development of T-cell memory. The group then tested sequencing of anti-OX40. OX40, a secondary co-stimulatory molecule expressed by activated T-cells, was stimulated with the same schedule as anti-CTLA-4 and the highest percent survival was seen in the 1 day post-RT group (37). The authors concluded that the effect of sequencing is dependent on the mechanism of the immunotherapy being used. Given that anti-CTLA-4 may act on naïve T-cells and Treg (38) and anti-PD-1 acts on newly activated and exhausted T-cells (6, 32, 39), these differences in optimal timing are not surprising.

We propose that ideally anti-PD-1/L1 and RT should be given concurrently but that if not RT should precede the administration of checkpoint blockade. RT delivered to the tumor following anti-PD-1/L1 may obliterate the recently infiltrated and reinvigorated T-cell response (**Figure 1**). In contrast, if RT is delivered before anti-PD-1/L1, RT stimulated naïve T-cell differentiation will synergize with checkpoint blockade and RT induced T-cell death of anti-PD-1/L1 reinvigorated T-cells may be avoided (**Figure 2**).

RT DOSE, FRACTIONATION AND THE IMMUNE RESPONSE

Varying dose and fractionation of RT in combination with immunotherapy and evaluating the anti-tumor immune response is an active area of investigation. Recent experiments from Morisada et al. in which primary tumor and abscopal tumor control rates were measured in a syngeneic mouse model of head and neck squamous cell carcinoma (SCC) following high-dose hypofractionated (8 Gy × 2) or low-dose daily fractionated (2 Gy × 10) RT in combination with concurrent anti-PD-1 showed that daily fractionated RT preserved peripheral and tumor-infiltrating CD8 T-cell accumulation and activation, reduced peripheral and tumor granulocytic myeloid derived suppressor cell (gMDSC) accumulation and did not impact Treg (40). Similarly, Type I IFN levels and expression of IFN-responsive MHC class I and PD-L1 was greater in those subjected to the daily low-dose fractionated regimen, and primary and abscopal tumor control improved when combined with anti-PD-1. Importantly, the local and abscopal effects appears to be similar for different hypofractionated regimens with similar biological equivalent dose (BED) (3 × 9.18 Gy in 3 or 5 days or 5 × 6.43 Gy in 10 days) (41).

Investigators have tested different total doses and fractionation schemes in a variety of pre-clinical models to maximize the abscopal effect (**Table 1**). Mice engrafted with the B16 melanoma cell line were treated with 15 Gy × 1 or 5 Gy × 3 fractions. The single fraction dose increased antigen availability and the number of tumor specific T-cells secreting IFN-γ in the tumor-draining lymph node to a larger extent than fractionated RT (42). They also showed that tumors receiving 15 Gy had greater infiltration of APCs and CD8 T-cells compared to 5 Gy × 3. To determine the dose for optimal tumor and immunologic response, Schaue et al. conducted a single fraction dose escalation

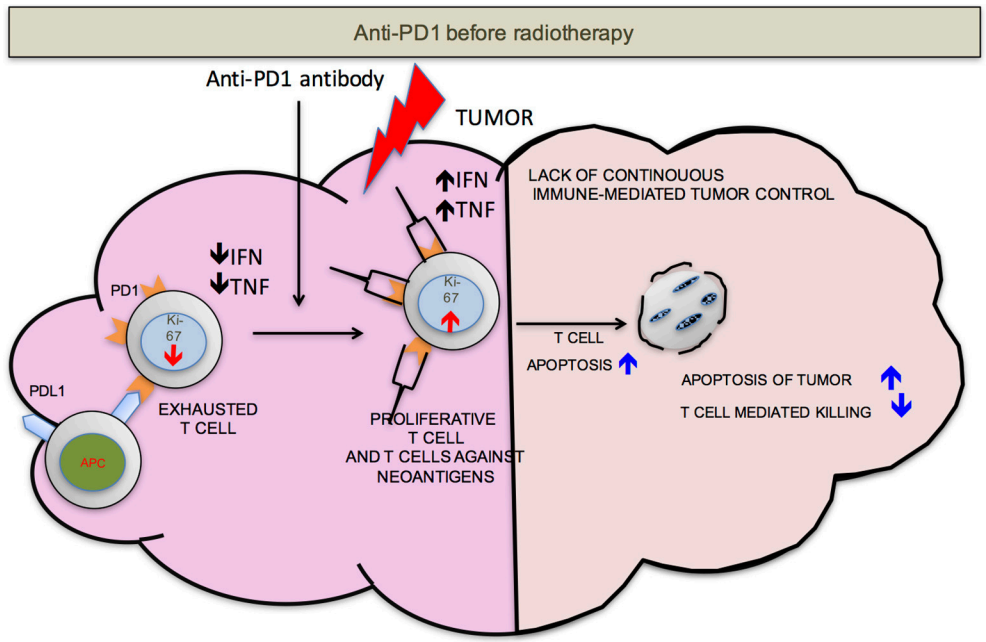

FIGURE 1 | PD-1 before RT. Once the anti-PD-1 antibody is infused into the host, the exhausted cells start proliferating as detected by increase in Ki-67, become functionally active (increased production of IFN-γ and TNF-α) and traffic to the tumor from secondary lymphoid organs. Radiation delivered to the tumor at this point may be detrimental to the anti-tumor responses due to radiation induced T-cell apoptosis. This may compromise control.

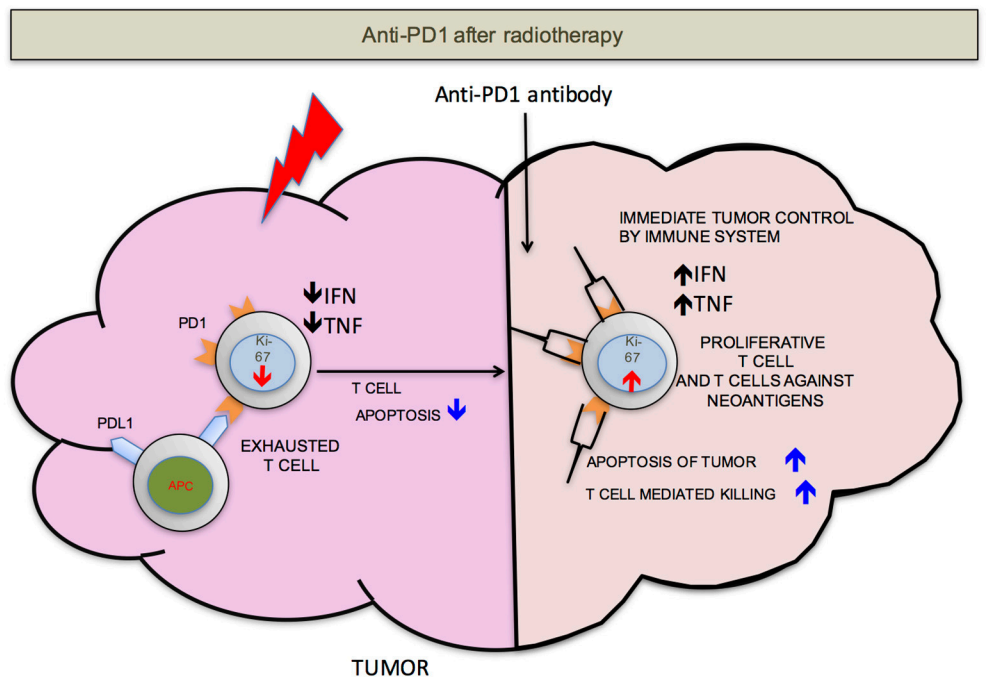

FIGURE 2 | PD-1 after RT. If the radiation is delivered before anti-PD-1 therapy, there will be less T-cells apoptosis. T-cells that infiltrate after radiation mediated neo-antigens release have a better chance to mount an effective anti-tumor response. For simplicity, PD-L1 is shown on only antigen presenting cells (APCs).

study with doses from 5 to 15 Gy and demonstrated that doses of 7.5 Gy and above are immuno-stimulatory, defined by an increased number of tumor-reactive T-cells (43). However, at high dose, 15 Gy × 1, there was an increase in the splenic Treg fraction. They showed that if they instead fractionated the 15 Gy into 2–5 fractions, fewer Treg and more effector T-cells were

TABLE 1 | Studies on the effect of RT dose and fractionation on immune effect.

Disease and animals	RT dose and fractionation	Findings	References
Mice with OVA-expressing B16-F0 tumors	15 Gy × 1–3 fx	Single fx increased antigen availability and the number of T-cells secreting IFN- γ in the tumor draining LN to a larger extent that fractionated RT	(42)
Mice with B16-OVA	0–15 Gy × 1 fx; 15 Gy × 2, 3, or 5 fx	For single fx dose, tumor control increase with dose of RT. For 15 Gy, administration in 2 fx gave the best tumor control and tumor immunity.	(43)
Mice with TSA	20 Gy × 1, 8 Gy × 3, 6 Gy × 5	Abscopal effect occurred only in mice treated with the combination of immunotherapy and fractionated RT	(44)
Human SW480 colorectal tumor cells, *in vitro*	2 Gy × 5, 5 Gy × 3, 15 Gy × 1	Fractionated RT resulted in higher expression of IL-12p70, IL-8, IL-6, and TNF-α.	(45)

identified in the spleens with an optimal dose fractionation of 7.5 Gy × 2. The authors do not offer a clear mechanism for the increased splenic Treg frequency at higher dose or whether this was mirrored in the tumor, but it may depend on the immunologic milieu generated by high dose fractions. Dewan et al. investigated dose and fractionation in a murine syngeneic breast cancer cell line subcutaneously injected at two distinct sites to assess the abscopal response. RT was delivered to one tumor site in 3 different regimens (20 Gy × 1, 8 Gy × 3, or 6 Gy × 5) with or without anti-CTLA-4 (44). The primary site and the secondary site were then monitored for response. They found that a significant abscopal effect was only induced when RT was administered with anti-CTLA-4 in either of the fractionated regimens. They concluded that a single dose, despite, or perhaps because of its size, was insufficient to induce an abscopal effect. These data taken together suggest that there is an optimal range (typically high dose per fraction) for the abscopal effect induction which is further supported by the new data concerning the cGAS-STING pathway.

The importance of the cGAS-STING pathway on the anti-tumor immune response stimulated by both radiation and anti-PD1/L1 has now been established. cGAS (cGAMP synthase), a sensor of cytosolic DNA, a PAMP, catalyzes the formation of second messenger cGAMP which induces type I interferons via the adaptor protein STING. It was shown that cGAS –deficient mice bearing a B16 melanoma had a reduced response to anti-PD-L1 treatment relative to wild-type controls (46). In the cGAS knockout mice there was a decrease in the number of tumor specific CD4 and CD8 T-cells relative to wild-type anti-PD-L1 treated. The effect of anti-PD-L1 blockade was enhanced by intramuscular injections of cGAMP (cGAS product). In the RT context, it has been previously shown that type I interferons induced by RT are important for mediating the anti-tumor immune response (47). Deng at al. demonstrated that the STING signaling axis is activated in DCs, and cGAS is essential for the sensing by the DC of irradiated-tumor cell derived dsDNA. Additionally, they showed that STING promotes an anti-tumor CD8 T-cell response with an increased frequency of IFN-γ^+ CD8 T-cells in the tumor-draining lymph node. Interestingly, there appears to be a link between radiation dose per fraction, the cGAS STING axis and radiation's synergy with immunotherapy. The exonuclease, TREX1, is upregulated by an RT dose per fraction greater than 10–12 Gy, and its expression degrades cytosolic dsDNA. This leads to a decreased synergy between radiation and immunotherapy (48, 49). This pathway is now a focus of ongoing and active investigation.

The upregulation of checkpoint molecules, the target of anti-PD-L1, can be induced in the tumor following RT, and the magnitude and kinetics of the induction may vary by dose and fractionation. 10 Gy in 5 fractions has been shown to robustly upregulate PD-L1 on CT26 tumors with a peak at Day 3 post-RT completion (30). In another elegant study, Derer et al. investigated the impact of RT, chemotherapy, and chemoRT on PD-L1 expression in a variety of murine tumor cell lines and found that standard fractionation and hypofractionated RT led to significant increases of PD-L1 expression in both melanoma and glioblastoma cell lines (50). *In vivo,* fractionated RT with dacarbazine induced PD-L1 expression on B16-F10 tumors, but not RT alone. In the context of human rectal cancer, Lim et al. evaluated pre chemoRT biopsies and post-chemoRT surgical specimens for expression of PD-L1 (51). The chemoRT regimen consisted of 50.4 Gy of radiation in 28 fractions with concurrent 5-fluorouracil and capecitabine. They found that PD-L1 is induced on tumor cells following chemoRT. Interestingly, if they then divided patients into 4 PD-L1 groups based on their biopsy and surgical expression levels, they showed that patients with high levels on biopsy and surgical specimens had the shortest overall survival. Importantly, however, patients that went from low to high levels of PD-L1 did not have shorter survival times suggesting that the PD-L1 induction by chemoRT is not deleterious and may provide an additional opportunity for checkpoint blockade.

On occasion, it is difficult or impractical to deliver this higher dose per fraction ideal for eliciting an anti-tumor immune response. Under these circumstances, the RT may be delivered by irradiating a fractional tumor volume, thereby reducing adverse effects. Using a 3-dimensional lattice radiation therapy (LRT) system, we have shown in a preclinical abscopal model that 20% volume irradiation (delivered to two 10% volumes) of the tumor resulted in significant growth delay in both the irradiated and unirradiated tumors (52). These abscopal effects were mediated by the down-modulation of T_H2 functions and induction of robust IFN-γ and T_H1 response in addition to increased T-cell infiltration and expression of TRAIL in the irradiated and unirradiated tumors (52). Interestingly, significant radiation-induced abscopal effects were observed in two of seven patients where only the hypoxic region of the tumor was irradiated with a single fraction of high dose radiation

(53, 54). Immunomodulatory effects of the treatment were not assessed. These studies suggest that by partial irradiation of tumor volumes, high doses of radiation can be delivered with enhanced immunomodulatory potential, however, more studies are required to examine these novel approaches.

In summary, optimal radiation dose appears to be somewhere between 8 and 10 Gy per fraction in 1–3 fractions, and appears to be critical to an effective anti-tumor response. Although CD8 T-cells may be present in a tumor prior to RT, they may be downregulated by PD-L1-PD-1 mediated immune exhaustion, may not be able to find tumor cells that they were activated against, or there may be an immune suppressive environment induced by multiple cell types. An ideal radiation dose will induce tumor cell mitotic catastrophe (IDC), release tumor neo-antigens and endogenous adjuvants, increase APC maturation and antigen presentation, increase CD8 T-cell proliferation and migration to the tumor, and lead to effective anti-tumor response. A sub-optimal radiation dose may be effective in activating CD8 T-cells, but will fail to achieve standard of care treatment goals such as local control. An excessively high dose will induce tumor cell death and improve local control, but may also damage normal tissue and tumor vasculature with the added disadvantage of inducing widespread CD8 T-cell apoptosis, compromising immune priming, distant control, and the opportunity for induction of the abscopal effect (**Figure 3**) (55).

CLINICAL DATA

Dose, Fractionation and Sequencing

The earliest and best trial evaluating different RT doses and immunotherapy was published in 2012. This phase I trial combined three different doses of stereotactic body radiotherapy (SBRT) and IL-2 where tumor and immune responses were evaluated in patients with metastatic melanoma or RCC. Patients received one of three regimens: SBRT 20 Gy × 1, 2, or 3 fractions on a Monday, Wednesday, and Friday schedule, followed by

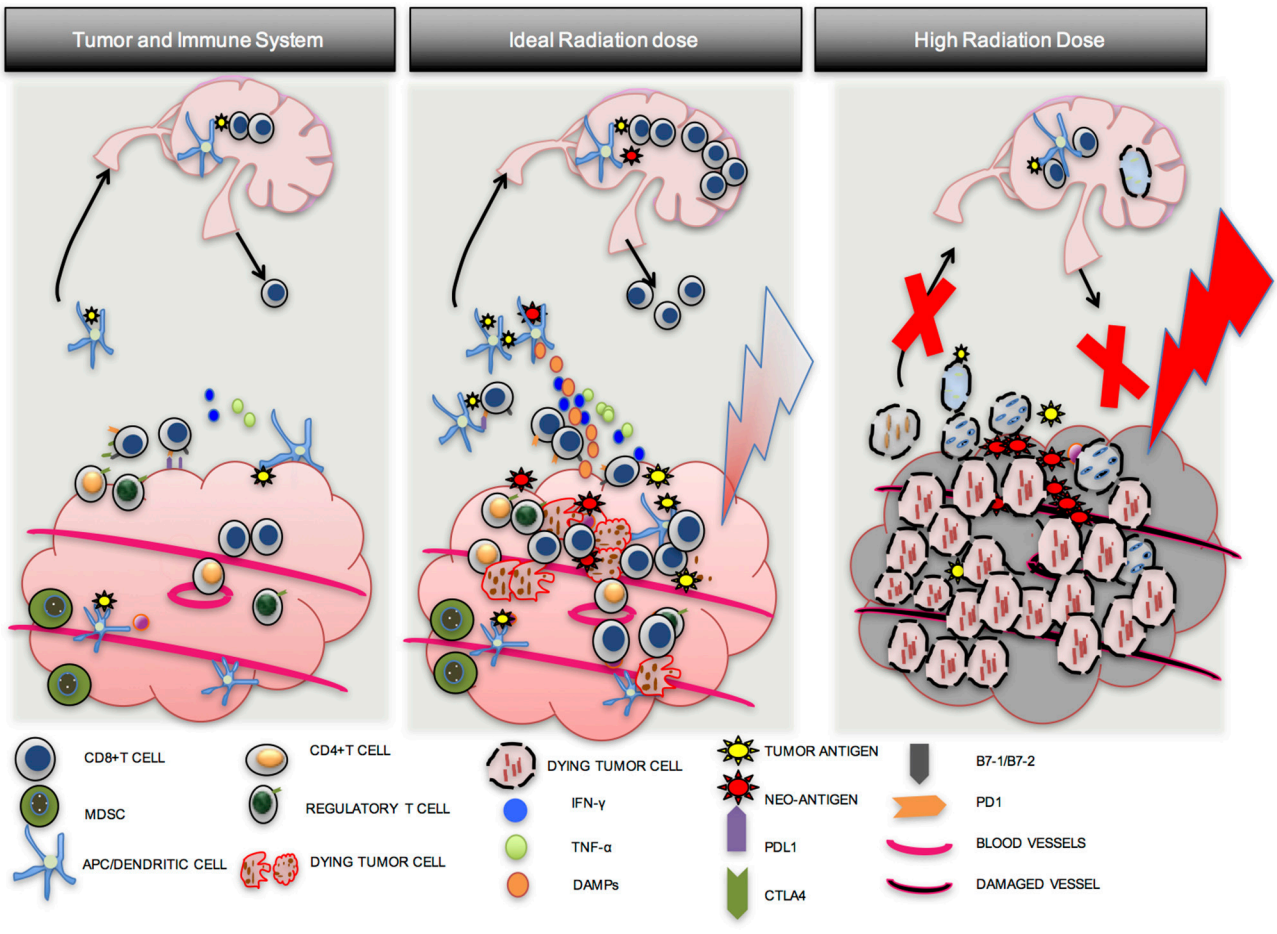

FIGURE 3 | Importance of optimal radiation dose. A number of factors lead to the establishment of tumors in a host. Although CD8 T cells may be present in the tumor, they are exhausted, may not be able to find tumor cells that they were activated against, or there may be an immune suppressive environment induced by multiple cell types (1st Column). An ideal dose of radiation will induce inflammatory tumor cell death and activate an anti-tumor T-cell response via APC maturation (2nd Column). A high dose of radiation may induce tumor cell death but may also damage blood vessels and induce more CD8 T cell apoptosis. Local control from the direct effects of RT may be good, but effective immune priming and distant control may be compromised (3rd Column).

high dose IL-2 (600,000 IU) on the following Monday (72 h after completion of RT). The authors observed an objective response of 66% (8 of 12 patients with a complete or partial response) as measured in the Response Evaluation Criteria in Solid Tumors (RECIST). Additionally, the responding patients had a higher frequency of proliferating effector memory CD4 and CD8 T-cells without a difference in the frequency of proliferating Treg (56). They concluded that SBRT and IL-2 could be administered safely. Interestingly, they did not demonstrate a relationship between SBRT dose and overall response, however, the very small number of patients in this trial and the use of IL-2 allow limited conclusions to be drawn. Additionally, as described previously, the specific immuno-modulatory drug administered with RT is expected to influence optimal timing as well as dose. Finally, it is also well known that different tumor histologies have different radiosensitivities (57), therefore, it is conceivable that the optimal dose for antigen release and immunologic activation is tumor specific. Despite these limitations, this is a landmark trial with one patient with widely metastatic disease achieving PET complete response—an abscopal effect.

More recent studies have confirmed the safety of combination checkpoint blockade and RT without supporting a specific RT dose or RT/checkpoint sequencing (58, 59). An exciting study out of the University of Chicago showed an increased immune score (median expression level of normalized pre-selected genes) in the irradiated metastasis correlated with a greater change in the unirradiated lesion (60). The dose used varied from 30 Gy in 3 fractions to 50 Gy in 5 fractions determined by anatomic site with anti-PD-1 given every 3 weeks and initiated within 7 days after the final SBRT fraction. Additional data suggests synergy between RT and anti-CTLA-4 or anti-PD-1 in metastatic castration resistant prostate cancer and advanced non-small cell lung cancer (NSCLC), respectively (61, 62). The majority of on-going clinical trials prescribe concurrent administration of immunomodulatory agents and RT guided by the preclinical data (63). Of note, the recently published PACIFIC trial of stage III NSCLC demonstrated an overall survival benefit to adjuvant durvalumab (anti-PD-L1) following chemoradiation (64). As NSCLC has a very high rate of distant failure, this suggests that durvalumab improved local control as well as the micrometastatic disease (abscopal effect). There is now an actively enrolling trial evaluating the benefit of concurrent durvalumab with chemoradiation in stage III NSCLC (NCT03519971).

Data from the brain metastasis literature also supports close sequencing of RT and checkpoint blockade, although most of these data evaluate local control rather than an abscopal response. In one of the larger retrospective analyses, 75 melanoma patients with 566 brain metastases were evaluated. They received SRS and immune checkpoint therapy between 2007 and 2015 at Yale University (65). SRS was given in a single fraction to a median of 20 Gy (range, 12–24 Gy). Seventy-two percent of patients received anti-CTLA-4 and 28% received anti-PD-L1. Fifty-five percent of lesions were treated with concurrent SRS and immunotherapy (SRS administered within 4 weeks of immunotherapy). It was shown that, compared to non-concurrent treatment, concurrent use of immunotherapy and SRS resulted in a significant greater median percent reduction

TABLE 2 | A selection of clinical trials and case reports that evaluated immune-stimulatory effects of RT.

Disease and patients	RT doses	IO	Sequence	Immune effects	Toxicity	References
12 patients with Stage IV melanoma or renal cell carcinoma	SBRT, 1–3 fx, 20 Gy/fx	IL-2	IO given 3 days after RT	8 (66%) patients achieved CR ($n = 1$) or PR ($n = 7$)	No DLTs attributable to SBRT	(56)
41 patients with metastatic solid tumors	35 Gy in 10 fx	GM-CSF	IO started during second week of RT	Abscopal effects occurred in 19 (46%) patients	13 Grade 3 and 1 grade 4 adverse events attributable to RT or IT	(14)
Patient with metastatic solid tumors	SBRT, 3–5 fx, 10–15 Gy per Fx	Pembro	IO given within 7 days after final SBRT	Correlation between immune score in irradiated tumor and size decrease in unirradiated tumor	3 Grade 3 pneumonitis, 2 Grade 3 colitis, 1 Grade 3 hepatitis	(60)
HCC patient treated to a large cranial lesion	30 Gy	None	N/A	Abscopal effect was observed in primary and un-irradiated bone lesions after 10 months	Not reported	(68)
NSCLC patient with bone and adrenal metastases	2 Gy × 30 fx and 26 Gy × 1 fx to 2 different lung lesions	None	N/A	Abscopal effect was noted in bone and adrenal metastases after 12 months	Not reported	(69)
Follicular lymphoma patient	36 Gy in 26 days to paraaortic and pelvic lymph nodes	None	N/A	Abscopal effect was observed in liver, spleen, axillary lymph nodes	Not reported	(70)
Patient with metastatic RCC	20 Gy in 10 fx to the right kidney	None	N/A	Abscopal effect was observed in paratracheal nodes and bilateral pulmonary nodules	Not reported	(71)
Patient with metastatic melanoma	28.5 Gy in 3 fx to paraspinal mass	None	N/A	Abscopal effect occurred in the right hilar and splenic lesions	Not reported	(3)

RT, radiotherapy; SBRT, stereotactic body radiotherapy; IO, immunotherapy; HCC, hepatocellular carcinoma; RCC, renal cell carcinoma.

in lesion volume. Another study of 46 patients with metastatic melanoma who received ipilimumab and SRS found that patients treated with SRS during or before ipilimumab had higher overall survival and less regional recurrence suggesting an abscopal response compared to those treated with SRS after ipilimumab (66).

In totality, these data as consistent with preclinical results and our model (**Figures 1**, **2**) that the concurrent use of RT and immunotherapy, results in a more pronounced treatment response.

Data supporting concurrent administration with RT for agents other than checkpoint inhibitors has also been evaluated. Forty-One patients with metastatic solid tumors treated with concurrent RT and granulocyte-macrophage colony-stimulating factor (GM-CSF) had stable or progressing metastatic solid tumors with at least three measurable metastatic sites and were on single chemotherapy or hormonal therapy (14, 67). Of the 41 patients, the most common tumor types were non-small cell lung cancer (44%) and breast cancer (34%). Two metastatic lesions were sequentially treated in each patient. Each lesion received 35 Gy of RT in ten fractions in two consecutive weeks, with daily subcutaneous GM-CSF injections lasting for 2 weeks starting during the second week of RT. The same process was repeated for the second metastatic lesion. Abscopal response (here defined as at least 30% decrease in the longest dimension of the best responding lesion) was observed in 19 (46%) patients. This is despite a non-optimal protracted regimen of 35 Gy in 10 fractions. Fourteen grade 3 or 4 toxicities attributable to either RT or immunotherapy were observed, with fatigue being the most common.

Finally, to date, at least 46 RT-induced abscopal effect case reports have been published from 1969 to 2014 (2). **Table 2** displays a selection of representative studies. Histologies that have demonstrated abscopal effects include hepatocellular carcinoma, adenocarcinoma of the lung and esophagus, medullary thyroid carcinoma, Merkel cell carcinoma, follicular lymphoma, lymphocytic lymphoma, Hodgkin's lymphoma, CLL, renal cell carcinoma, and melanoma. Of the reported cases, the median age was 64 years (range: 28–83), the median RT dose was 31 Gy (range: 0.45–60.75), and the median dose per fraction was 3 Gy. The median time to an abscopal effect was 2 months (range: 0–24 months) and the median time to progression was 6 months (range: 0.7–14 months). Of these 46 published cases, only five patients had immunotherapy during treatment, four of which were melanoma patients. Therefore, relying on currently published case reports to guide timing of RT with immunotherapy is difficult. However, what can be gleaned from these case reports is that the abscopal effect does occur in multiple different cancer histologies.

DISCUSSION

Many of the topics addressed in this review remain areas of active inquiry with a number of smaller checkpoint and RT studies having been published. Our lab is also investigating questions of fractionation and timing. Although there appears to be a consensus that hypo-fractionation is superior to conventional fractionation, the optimal dose for an abscopal or local immune response may depend on tumor histology and non-synonymous mutation burden due to varying radio-sensitivities and neo-antigen load (72). Additionally, the optimal interaction may also vary with the specific immunotherapy administered as CTLA-4 and PD-1/PD-L1 antagonists have distinct and non-redundant mechanisms. These numerous variables add complexity to any proposed clinical trial design.

We recommend including different fractionation schemes in any proposed immunotherapy and radiation clinical trials and suggest potentially varying the fractionation schemes from one tumor histology to another. These data suggest that a dose per fraction of close to 10 Gy with 1–3 fractions is likely optimal for abscopal effect induction. Importantly, a dose and fractionation regimen optimized for a robust local response may be expected to differ from that optimized for a distant abscopal response and additional data are needed to elucidate these likely tumor-specific thresholds.

We also encourage further investigation involving the sequencing of radiation and immunotherapy. Evidence presented here suggests immunotherapy should be initiated at the start of radiation when employing single or high dose per fraction RT as this is the time when a bolus of neo-antigens is released, followed later by more limited T-cell epitope availability. Conventional fractionation may instead lead to a steady release of tumor antigens throughout treatment and the exact point of immunotherapy initiation may be less critical, although earlier initiation of immunotherapy is likely to remain superior. Finally, the mechanism of radiation and immunotherapy for T-cell activation is specific (29), and understanding why close sequencing rather than more remote administration of immunotherapy improves control in several contexts is an important avenue of investigation.

CONCLUSION

The synergy between RT and immunotherapy has now definitively entered the mainstream. A deep and clear mechanistic understanding of RT's immune system stimulation and its synergy with immunotherapy affirms the value in pursuing and expanding this avenue of research. There are, however, still many unanswered questions in the optimization of the abscopal response including, but not limited to: RT and immunotherapy sequencing, RT dose and fractionation, and RTs specific interactions with different immuno-modulatory agents and individual tumor subtypes. It is our hope that the research community continues to vigorously pursue these and other vital questions surrounding the induction of the abscopal effect. The solution to transforming RT from a purely local or palliative therapy to a treatment important for long-term metastatic control, we believe, may lie in the answer to these questions.

AUTHOR CONTRIBUTIONS

ZB, JW, SG, and SZ: wrote the manuscript; TN: created the figures; SZ: created the tables; WM, MK, and SK: edited the manuscript and provided expertise.

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Merkel Cell Polyoma Viral Load and Intratumoral CD8+ Lymphocyte Infiltration Predict Overall Survival in Patients With Merkel Cell Carcinoma

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Introduction: Merkel cell carcinoma (MCC) is linked to the presence of clonally integrated Merkel cell polyomavirus (MCPyV) in up to 80% of the cases. The aim of the study was to determine the prognostic value of baseline MCPyV viral load and lymphocytic infiltration.

Methods: MCPyV DNA prevalence, integration status and viral load were determined by specific quantitative real-time PCR in surgical specimens obtained from 49 patients with MCC treated with ($n = 22$, 45%) or without postoperative radiotherapy (RT). CD8+ tumor infiltrating lymphocytes (TILs) and programmed death ligand 1 (PD-L1) status were assessed using immunohistochemistry. MCPyV characteristics and immune marker expression were correlated with clinicopathological factors and overall survival (OS).

Results: Median age at diagnosis was 74 (range, 42–100); 51% of the patients were female. One-, three, and five-year OS rates were 83.8, 58.6, and 47.1%, respectively. A positive MCPyV status was associated with female gender ($p = 0.042$). Tumor localization (head/arms vs. trunk) positively correlated with PD-L1 status ($p = 0.011$) and combined CD8/PD-L1 expression ($p = 0.038$). Overall CD8+ infiltration was inversely associated with N-stage ($p = 0.048$). Stromal TILs correlated significantly with both PD-L1 expression ($p = 0.010$) and N-stage ($p = 0.037$). A high viral load (>median) was significantly associated with worse OS ($p = 0.029$) and high intratumoral CD8+ infiltration with improved OS for the entire cohort ($p = 0.045$).

Conclusion: These data provide important insight on the role of MCPy DNA viral load and TILs in the context of PD-L1 in patients with Merkel cell carcinoma. Future clinical studies should aim to explore the effect of PD-1/PD-L1 immune-checkpoint inhibitors in combination with existing radiotherapy approaches.

Keywords: merkel cell carcinoma, polyomavirus (MCPyV), CD8+ tumor infiltrating lymphocytes, PD-L1, radioimmunotherpay

INTRODUCTION

Merkel cell carcinoma (MCC) is a rare neuroendocrine, cutaneous malignancy with an incidence rate of 0.13 per 1,00,000 residents in Europe between 1995 and 2002 (1). Therapy consists of surgery only (if N0), surgery followed in most cases by adjuvant radiotherapy (RT) or, more recently, by novel approaches, including immune-checkpoint inhibitors (ICI) in metastatic disease (2–4). The 5-year MCC-specific mortality rate is up to 46% (5, 6). MCC tumorigenesis is linked to the presence of clonally integrated Merkel cell polyomavirus (MCPyV) in up to 80% of the cases, or mutagenesis from ultraviolet light (UV) exposure for MCPyV-negative tumors, as well as advanced age and immunosuppression (3, 7). MCPyV integrates into the host cells genome and persistent expression of MCPyV T antigens is required for MCC tumor cell survival (8). Immunosuppression due to, e.g., organ transplantation or chronic lymphatic leukemia significantly increases the risk for MCC, thus indicating a pivotal role of the host immune system in tumorigenesis (7).

Although it has been reported that patients with high intratumoral CD8+ and CD4+ lymphocyte infiltration show better clinical outcome, including complete spontaneous tumor regression (9–11), the majority of MCC tumors progress despite the presence of T-cells priming MCPyV capsid proteins and oncoproteins. MCC seems to be capable of escaping immune response via down-regulation of major histocompatibility complex class I (MHC-I), Toll-like Receptor 9 (TLR9), and prevention of NF-kB translocation into the nucleus (8, 12). Upregulation of programmed death ligand 1 (PD-L1) expression in response to interferon-γ, released by CD8+ TILs as an adaptive immune-resistance mechanism, can suppress local effector T-cell function. ICI against the PD-1/PD-L1 axis have shown promising results in the treatment of metastatic MCC, and recently resulted in the approval of Avelumab (anti-PD-L1) by the Food and Drug Administration (FDA) (4).

In this study we aimed to correlate MCPyV quantitative viral load, CD8+ tumor infiltrating lymphocytes (TILs), and PD-L1 expression with clinicopathological characteristics and overall survival (OS) in patients with MCC.

PATIENTS AND METHODS

Patients and Treatment

We retrospectively analyzed 49 patients treated for histologically-proven MCC between June 2000 and September 2017 at the Departments of Dermatology and/or Radiotherapy of the University of Frankfurt, Germany. All patients underwent physical examination and complete tumor excision. In case of >cT1 or cN1 cM0, a sentinel lymph node biopsy (SLNB) was performed, followed, in case of positive SLNB, by a regional lymph node dissection and in most cases by adjuvant RT. Depending on tumor site and volume, RT was administered using 3D-conformal or intensity-modulated radiotherapy (IMRT, since 2010) utilizing photon or electron beams and energies ≥6 MV. RT-doses ranged between 20.0 and 70.0 Gray (Gy, median: 60.0 Gy). All patients provided informed consent for sample and clinical data collection. All procedures performed in this study followed approval of our institutional ethics committee (No. 4/09 UCT-03-2017) and were in accordance with the standards of the 1964 Helsinki declaration and its later amendments.

Immunohistochemistry

Formalin fixed paraffin embedded (FFPE) tumor samples derived from the Dr. Senckenberg Institute of Pathology, and the Department of Dermatology, University of Frankfurt, were subjected to an automatic staining procedure with standardized DAKO EnVision™ FLEX Peroxidase Blocking reagent (K8000, DAKO, Hamburg, Germany) on a DAKO Autostainer Link 48 (DAKO). Antigen retrieval was performed by treatment of the sections using an Epitope Retrieval Solution (Trilog, Cell Marque, Rocklin, CA) for 20 min. Slides were stained with the primary antibodies for either CD8 (1:100, clone C8/144B; Dako M7103) or PD-L1 (1:50, clone E1L3N(R); Cell Signaling Technology) for 120 min at room temperature. Next, dextran polymer conjugated horseradish peroxidase and 3,3′-diamino-benzidine (DAB) chromogen were used for visualization and hematoxylin solution (Gill 3, Sigma Aldrich, Munich, Germany) for counterstaining. Blinded samples were evaluated by two investigators (J.V. and P.B.) without knowledge of the clinicopathologic and clinical data as described before (13, 14). In cases of discrepancy, a final decision was made after additional examination of the specimens. The expression of CD8+ TILs was scored semi-quantitatively via measurement of cell density. Scoring was as follows: for the intra-epithelial, invasive front and stromal compartments: (i) no, or sporadic cells; (ii) moderate numbers of cells; (iii) abundant occurrence of cells; and (iv) highly abundant occurrence of cells. The total score was calculated by adding the separate scores from all three compartments (range, 3–12). The median score was used as cut-off to classify patients into two groups: low (<median) or high (≥median) CD8+ infiltration. PD-L1 tumor expression as evaluated for each sample in different representative fields and expression in >1% of the tumor cells were considered positive as reported before (15).

MCPyV Detection and DNA Load Determination

Determination of MCPyV DNA load and MCPyV integration status were performed on five 10 µm FFPE sections using a LightCycler 480 Real Time PCR System (Roche, Mannheim, Germany) as described previously (16, 17). Briefly, viral DNA load was determined using MCPyV-specific LT3-primers and a locked nucleic acid probe binding to the N-terminal part of the large T-antigen gene (18). MCPyV DNA load was expressed as MCPyV DNA copies per betaglobin-gene copy (17). The integration status of the MCPyV DNA into the cellular host genome was assessed with a real-time PCR-based MCPyV T-antigen gene C-terminus deletion assay as described before (16). For statistical analysis, a non-detectable viral DNA load was defined as 0 and the median was calculated for the entire cohort ($n = 48$).

Statistical Analysis

The association of MCPyV, CD8+ infiltration and PD-L1 expression with clinicopathological characteristics was assessed

using Pearson's Chi-squared test for categorical variables and Mann-Whitney U test for continuous variables. The clinical outcome measure was overall survival (OS) as defined from the time-point of histologically confirmed diagnosis of MCC to death from any cause. Differences in OS between groups were plotted using the Kaplan–Meier method and assessed using the Log-rank test (Mantel-Cox; SPSS 25). A $p < 0.05$ was considered as significant.

RESULTS

Patients and Tumor Characteristics

From a total of 49 patients, 25 (51.0%) were female. Median age at diagnosis was 74 (range, 42–100) years. The head was the main tumor site (45.5%), followed by arms (34.0%), and body trunk (20.5%). A total of 54.5% of the patients had positive lymph nodes, and 44.9% received adjuvant RT. Concerning the MCPyV-DNA status, 1 MCC was not evaluable due to low cellularity (betaglobin-gene copy number < 10), 38 of the remaining 48 biopsies were MCPyV-DNA positive (79.2%), and 10 MCC (20.8%) were MCPyV-negative. The median viral DNA load for the entire cohort ($n = 48$) was 0.745 (interquartile range 0.007–4.448; mean 7.072; range 0.000–157.007). Integrated, C-terminally deleted MCPyV-DNA was found in 22.9% of all patients (11/48), episomal or full-length integrated MCPyV-DNA in 33.3% of all patients (16/48), and in 22.9% of the entire cohort (11/48) the integration status could not be evaluated or was negative (20,8%, 10/48). Patient characteristics are given in **Table 1**.

TABLE 1 | Patients characteristics.

Clinical characteristics	*n* (%)
Total (*n* = 49)	
GENDER	
Male	24 (49.0)
Female	25 (51.0)
Age, median (range)	74 (42–100)
TUMOR LOCALIZATION	
Head	20 (45.5)
Arm	15 (34.0)
Body trunk	9 (20.5)
Missing values	5
cN-CATEGORY	
cN0	15 (45.5)
cN+	18 (54.5)
Missing values	16
CD8 SCORE‡	
<median	25 (51.0)
≥median	24 (49.0)
PD-L1*	
≤1%	21 (42.9)
>1%	28 (57.1)
MCPyV DNA STATUS	
Positive	38 (79.2)
Negative	10 (20.8)
Not assessable	1
VIRAL INTEGRATION STATUS	
Integrated,C-terminally deleted	11 (22.9%)
Episomal or full-length integrated	16 (33.3%)
Integration status not assessable	11 (22.9%)
MCPyV-negative	10 (20.8%)
Missing values	1
RADIOTHERAPY	
Yes	22 (44.9)
No	27 (55.1)

*MCPyV, Merkel Cell Polyomavirus. *% PD-L1+ tumor cells. ‡CD8+ tumor infiltration.*

Clinicopathological Characteristics and Their Association With MCPyV Status, CD8 Infiltration, and PD-L1 Expression

For CD8+ TILs, the median score was used as cut-off to dichotomize between low and high infiltration, whereas PD-L1+ expression in >1% of the tumor cells was considered positive (**Figure 1**). Tumor localization (head/arms vs. trunk) positively correlated with PD-L1 status ($p = 0.011$, **Table 2**) and combined CD8/PD-L1 expression ($p = 0.038$, **Supplementary Table 2**). Overall CD8+ infiltration was inversely associated with N-stage ($p = 0.048$, **Table 2**). A high stromal CD8+ infiltration was associated with PD-L1 positivity ($p = 0.010$) and N-stage ($p = 0.037$, **Table 3**). Further, a positive MCPyV status and high viral DNA load were associated with female gender ($p = 0.042$ and 0.021, respectively) (**Table 2** and **Supplementary Table 1**).

Overall Survival and Correlation With MCPyV DNA Load, CD8, and PD-L1

One-, three-, and five-year OS rates were 83.8, 58.6, and 47.1%, respectively (**Figure 2**). Cumulative ($p = 0.078$) and stromal ($p = 0.279$) expression of CD8+ TILs were not associated with OS, whereas elevated levels of intratumoral CD8+ cells correlated significantly with superior OS for the entire cohort ($p = 0.045$, **Figure 2**). High levels of DNA viral load (>median) were significantly related to a worse OS ($p = 0.029$, **Figure 3**). The association remained significant after exclusion of cases that lack detectable viral DNA ($p = 0.034$ for $n = 38$, **Supplementary Figure 1**). PD-L1-positivity did not correlate with OS ($p = 0.966$).

DISCUSSION

MCC is an aggressive disease with various options of the malignant cells to avoid immune response. Accumulating evidence indicates a direct association of higher "immunogenicity" and response to RT in MCC (19), and other virus-associated malignancies, including HPV-16/18 induced oropharyngeal and anal carcinoma (13, 20, 21). A recent investigation in 805 patients with MCC indicated a significantly impaired efficacy of RT in terms of local tumor control and

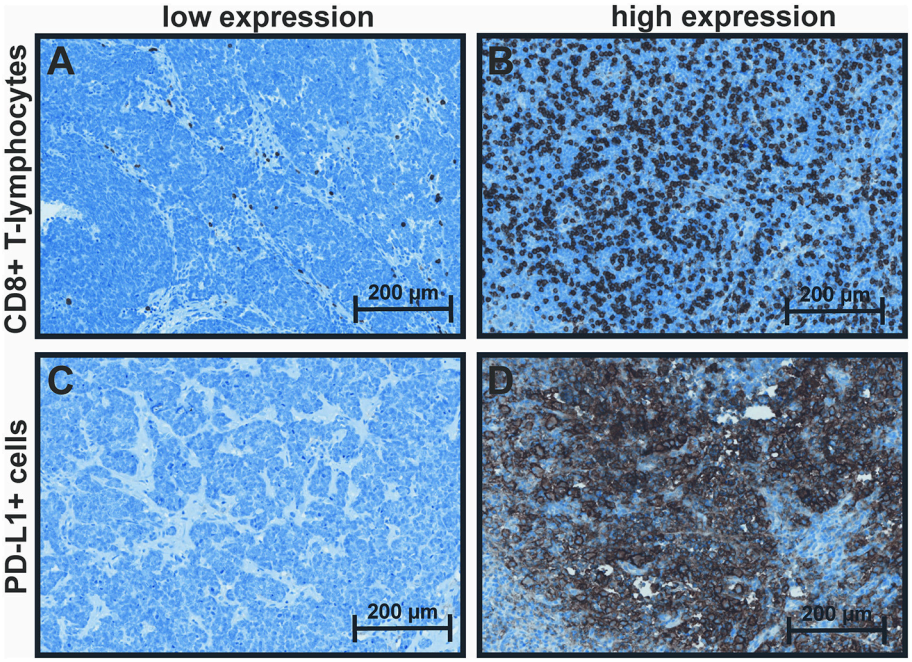

FIGURE 1 | Immunohistochemical staining of CD8 and PD-L1. **(A)** CD8: low: <median score of 5, **(B)** high: ≥median score of 5 (range 3–12); **(C)** PD-L1: ≤1% positive tumor cells (low) and **(D)** >1% positive tumor cells (high).

recurrence-free survival for patients with immunosuppression (22). Further understanding of tumor driving mechanisms may lead to new strategies facing this rare tumor entity.

In the present study, we quantitatively evaluated the prevalence, viral load, and genomic integration into the host DNA of MCPyV in a cohort of MCC-patients and correlated these parameters with OS, PD-L1 status, and CD8+ lymphocyte infiltration. To the best of our knowledge, this is the first study investigating the relationship between MCPyV viral load and survival (9, 23). Vandeven et al. could demonstrate that MCC of unknown primary (MCUP) was associated with higher levels of MCPyV-antibodies and higher mutational load, as surrogate parameters for immunogenicity, and correlated with improved survival when compared to patients with identified primary tumors (24). Additionally, other authors have reported a positive correlation of a high antibody titer with MCPyV status and OS for classical MCC (18, 25, 26). These data provide a strong rationale for a virus-triggered effective immune-activation as a pivotal mechanism underlying tumor elimination.

Intriguingly, a high viral load correlated with worse OS in our cohort while tumors with a lower load or lack of viral DNA displayed increased OS. MCPyV negative tumors are mainly considered to be induced by ultraviolet radiation and present a high mutational burden in general and more specifically high incidence of p53 (75%) and Rb mutations (67%) (27, 28). Emerging evidence shows a clear association of mutational load and prognosis for almost any malignancy, a phenomenon associated with the increased immunogenicity of such tumors (29). The percentage of non-virally induced tumors in our cohort is in accordance with the literature (28, 30). These extensively—mutated cases could have an even better outcome compared to MCPyV-driven tumors, such biasing the survival-analysis. Moreover, a less favorable outcome for MCPyV negative tumors has been reported before (25, 31). However, the significance for the correlation of high viral load and OS still remained after exclusion of cases without any detectable viral DNA. A possible reason for the impaired survival of patients with high viral-load is a missing or ineffective immune response due to immunosuppression or various cancer- and microenvironment-associated mechanisms, including alteration of regulatory T cell function and activation of the PD-1/PD-L1 axis (32, 33). Notably, similar findings have been reported for Epstein-Barr-Virus (EBV) associated nasopharyngeal cancer, where a high EBV-DNA load in the plasma correlated with an impaired outcome (34, 35).

Until the advent of ICI, chemotherapy was standard of care in the treatment of advanced MCC. First-line platinum-based chemotherapy combined with Etoposide showed overall response rates (ORR) of 31–55% with shorter progression-free survival than those recently reported for anti-PD-1/PD-L1 ICI (3). In a recent phase 2 trial the anti-PD-L1 antibody Avelumab was applied to 88 patients with stage IV MCC that had progressed after chemotherapy. Objective response was reached in 32% of the patients indicating superiority of novel immune-modulating therapies (2). These findings resulted in the first approval of a checkpoint inhibitor in MCC (4).

TABLE 2 | Clinicopathological characteristics and their association with MCPyV status and immune microenvironment.

Clinico-pathological characteristics	MCPyV status, *n* (%)		$p^{\dagger}$	CD8‡, *n* (%)		$p^{\dagger}$	PD-L1*, *n* (%)		$p^{\dagger}$
	Negative#	Positive		<Median	≥Median		≤1%	>1%	
N-STAGE (*n* = 33)									
Negative	7 (46.7)	8 (53.3)		4 (26.7)	11 (73.3)		7 (46.7)	8 (53.3)	
Positive	7 (38.9)	11 (61.1)	0.653	11 (61.1)	7 (38.9)	**0.048**	7 (38.9)	11 (61.1)	0.653
TUMOR LOCALIZATION (*n* = 45)									
Head or arms	16 (45.7)	19 (54.3)		20 (57.1)	15 (42.9)		16 (45.7)	19 (54.3)	
Other	3 (33.3)	6 (66.7)	0.504	3 (22.2)	7 (77.8)	0.062	0 (0)	9 (100.0)	**0.011**
PD-L1* (*n* = 49)									
≤1%	9 (45.0)	11 (55.0)		14 (66.7)	7 (33.3)				
>1%	12 (42.9)	16 (57.1)	0.883	11 (39.3)	17 (60.7)	0.058			
CD8‡(*n* = 49)									
<median	11 (45.8)	13 (54.2)					14 (56.0)	11 (44.0)	
≥median	10 (41.7)	14 (58.3)	0.771				7 (29.2)	17 (70.8)	0.058
MCPyV STATUS (*n* = 48)									
Negative#				7 (70.0)	3 (30.0)		4 (40.0)	6 (60.0)	
Positive				17 (44.7)	21 (55.3)	0.155	16 (42.1)	22 (57.9)	0.503
GENDER (*n* = 49)									
Male	14 (58.3)	10 (41.7)		12 (50.0)	12 (50.0)		9 (37.5)	15 (62.5)	
Female	7 (29.2)	17 (70.8)	**0.042**	13 (52.0)	12 (48.0)	0.889	12 (48.0)	13 (52.0)	0.458

*MCPyV, Merkel Cell Polyomavirus; PD-L1,Programmed cell death ligand 1. *% PD-L1+ tumor cells. ‡CD8+ tumor infiltration, overall score. #defined as negative or not assessable*
†p-values according to Pearson's Chi-squared test and calculated after exclusion of missing values. Significant results have been marked with bold.

TABLE 3 | PD-L1 and N-stage and their association with stromal CD8 infiltration.

Clinico-pathological characteristics	Stromal CD8+ infiltration, *n* (%)		$p^{\dagger}$
	<Median	≥Median	
PD-L1* (*n* = 49)			
≤1%	12 (57.1)	9 (42.9)	
>1%	6 (21.4)	22 (78.6)	**0.010**
N-STAGE (*n* = 33)			
Negative	3 (20.0)	12 (80.0)	
Positive	10 (55.6)	8 (44.4)	**0.037**

*PD-L1,Programmed cell death ligand 1. *% PD-L1+ tumor cells. †p-values according to Pearson's Chi-squared test and calculated after exclusion of missing values. Significant results have been marked with bold.*

Other studies investigating anti-PD-1 antibodies Nivolumab (+/- prior chemotherapy, recruiting) and Pembrolizumab (no prior chemotherapy) reported ORR of 68 and 56%, respectively (36, 37). In our cohort, PD-L1 status, however, was not associated with altered outcome, suggesting that this marker may be predictive for response to targeted therapy but not prognostic.

Regarding infiltration with CD8-positive cytotoxic lymphocytes, we identified a significant correlation between intratumoral CD8+ infiltration and OS, and a significant inverse correlation with nodal-stage (a widely accepted negative prognosticator for MCC). Notably, N+ disease in our cohort occurred in 54.5% of the cases while literature reports on 37% (7), a fact attributed to selection bias, as many of the patients included here were referred to the department of radiotherapy. In a larger study by Paulson et al., both clinical stage and CD8-infiltration were of prognostic relevance (10). More recent analyses of larger numbers of samples seem to confirm these assumptions (9, 38, 39) and the same was true when the specificity of T cells for MCPyV was taken to account (40). Interestingly, we did not observe any significant correlation between total tumor CD8-infiltration, PD-L1 expression, and viral load, indicating that mechanisms other than viral infection (e.g., ultraviolet radiation-induced mutations) may contribute to immune response. On the contrary, stromal infiltration with CD8+ TILs significantly correlated with both PD-L1 and

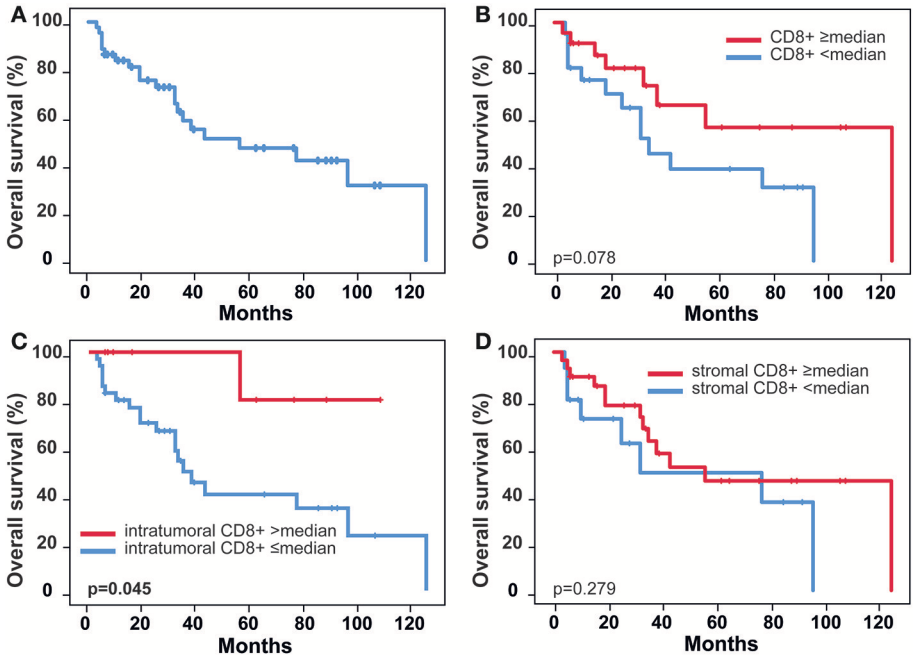

FIGURE 2 | Overall survival stratified by CD8 immune infiltration. **(A)** Overall survival, **(B)** Overall survival stratified by CD8 median score, **(C)** Overall survival stratified by CD8 intratumoral median score, **(D)** Overall survival stratified by CD8 stromal median score; *p*-values according to log-rank test (Mantel Cox).

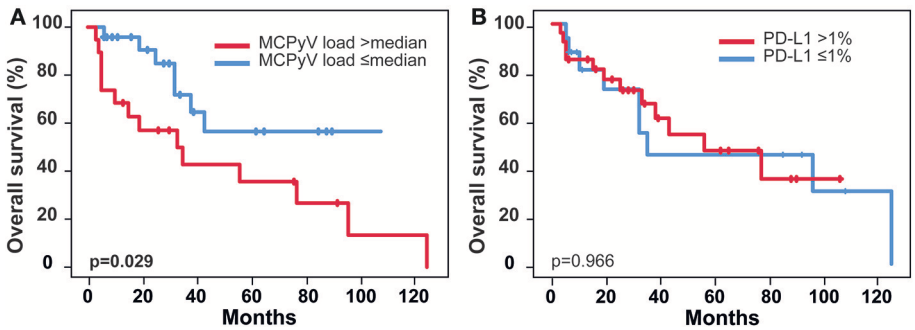

FIGURE 3 | Patients outcome and correlation with MCPyV DNA load and PD-L1. **(A)** Overall survival stratified by MCPyV DNA median load ($n = 48$), **(B)** Overall survival stratified by PD-L1 status; MCPyV, Merkel Cell Polyomavirus; *p*-values according to log-rank test (Mantel Cox).

MCPyV. This argues for a locally restricted, viral antigene-driven immune response that failed to control the tumor in a PD-L1 dependent manner, that could be potentially reversed by ICI (41).

With respect to the correlation of MCPyV status/viral DNA load with clinical and epidemiological parameters, the most important finding in the present cohort was a significant correlation with female gender although the limited number of patients in our study does not allow definite conclusions yet. In line with that, the higher prevalence of MCPyV in female patients has been reported before, but a possible association with tumor site remains controversial (18, 42, 43). There is no molecular explanation readily available for the increased prevalence in women. A putative reason, however, may be the observation that tumors in females were diagnosed more frequently in older patients (median age females 77.0 years vs. median age males 70.5). In line with that, Álvarez-Argüelles et al. recently speculated that there may an immunosuppressive component due to age contributing to the sex effect in MCPyV detection demonstrated in their analyses and in our study (44). Unfortunately we could not prove an association of age and viral load in our data. Another possible explanation could be a higher UV-exposure as casual factor in the male population. Yet there exist no data to undermine this speculation, although a viral etiology has been associated with female sex by many authors (18, 42, 43). Interestingly, male sex, and advanced age were associated with worse prognosis in the literature (7).

We acknowledge that the retrospective evaluation and the small number of patients is a limitation of our study. A potential calculation bias cannot be excluded. However, this is the first

study quantitatively assessing and correlating the MCPyV viral load to clinical parameters that warrant validation in larger, independent cohorts with long-term follow-up.

CONCLUSION

These data provide important insight on the crucial role of MCPyV DNA load and TILs, in the context of PD-L1, in patients with MCC. We consider our findings on a correlation of PDL-1 with tumor localization and CD8+ Tils and a prognostic relevance of intratumoral CD8+ T cell infiltration to be in favor of a future checkpoint immunotherapy in MCC. Moreover, there is growing pre-clinical and clinical evidence on an additional improvement of the effects of checkpoint-inhibition by synergistic effects of radiation therapy (45, 46). Consequently, future clinical studies should aim to explore the effect of PD-1/PD-L1 immune-checkpoint inhibitors in combination with existing radiotherapy approaches.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the local ethics committee (Frankfurt University No. 4/09 UCT-03-2017) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Frankfurt university ethics committee.

AUTHOR CONTRIBUTIONS

JG, RW, FR, and PB conceived the idea. JG, RW, CR, and MM provided patient data and material. RW, MM, UW, and SS contributed to the sample-preparations. RW, UW, and SS carried out the laboratory analyses. JG, RW, FR, and PB performed microscopy. JG, DM, and PB performed the statistics. JG, DM, FR, and PB analyzed and interpreted the data. FR, UW, EF, and CR were involved in the planning and supervising. JG, DM, FR, and EF drafted the manuscript and designed the figures. JG, EF, CR, FR, and PB wrote the manuscript, with contributions from the other authors. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00020/full#supplementary-material

Supplementary Figure 1 | Patients outcome and correlation with MCPyV DNA load. Overall survival stratified by MCPyV DNA median load restricted to patients with any detectable viral DNA independent from integration status ($n = 38$); p-value according to log-rank test (Mantel Cox).

Supplementary Table 1 | Association of MCPyV and gender.

Supplementary Table 2 | Clinicopathological characteristics and their association with combined CD8/PD-L1 expression.

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Functionalized Superparamagnetic Iron Oxide Nanoparticles (SPIONs) as Platform for the Targeted Multimodal Tumor Therapy

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Standard cancer treatments involve surgery, radiotherapy, chemotherapy, and immunotherapy. In clinical practice, the respective drugs are applied orally or intravenously leading to their systemic circulation in the whole organism. For chemotherapeutics or immune modulatory agents, severe side effects such as immune depression or autoimmunity can occur. At the same time the intratumoral drug doses are often too low for effective cancer therapy. Since monotherapies frequently cannot cure cancer, due to their synergistic effects multimodal therapy concepts are applied to enhance treatment efficacy. The targeted delivery of drugs to the tumor by employment of functionalized nanoparticles might be a promising solution to overcome these challenges. For multimodal therapy concepts and individualized patient care nanoparticle platforms can be functionalized with compounds from various therapeutic classes (e.g. radiosensitizers, phototoxic drugs, chemotherapeutics, immune modulators). Superparamagnetic iron oxide nanoparticles (SPIONs) as drug transporters can add further functionalities, such as guidance or heating by external magnetic fields (Magnetic Drug Targeting or Magnetic Hyperthermia), and imaging-controlled therapy (Magnetic Resonance Imaging).

Keywords: nanoparticles, nanomedicine, targeted therapy, immunotherapy, chemotherapy, irradiation, immunogenic cell death

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EVOLVEMENT OF TUMORS AND THEIR TREATMENTS

Mutation and clonal selection are driving forces in carcinogenesis (1). Accumulation of mutations in proto-oncogenes and tumor suppressor genes lead to uncontrolled proliferation of cells. Some of these mutations are recognized by the immune system as "non-self" (tumor associated antigens) and are eliminated, a process known as "immunosurveillance" (2, 3). Cells expressing only low amounts of tumor associated antigens cannot be detected and removed. Thus, the immune system exerts a selective force on the tumor, altering cell composition and promoting survival of the least immunogenic cells ("immunoediting") (4). Tumors evade the immune system by various mechanisms such as downregulation of MHC I expression, development of resistance to cytotoxic T lymphocytes, active suppression of activated T cells, or release of immune suppressive molecules (5). In the clinic, tumors are treated by surgery, radiotherapy, chemotherapy,

photodynamic therapy, and others. All of these procedures can induce the release of immune stimulatory intracellular molecules increasing the immunogenicity of the tumor. Immunotherapies shall further intensify the strength of immune responses. Problematically, monotherapies often cannot remove the tumor completely due to the occurrence of resistant tumor cell populations. Chemotherapy can lead to multiple drug resistance in long term use (6). In radiotherapy the lack of oxygen in hypoxic tumor tissues results in reduced production of reactive oxygen species (ROS) and thus decreased DNA damage (7). Immunotherapy is often effective only in a subgroup of patients. Thus, combinations of therapy concepts exhibiting synergistic effects might overcome limitations of monotherapies, referred to as multimodal tumor therapy. To bring therapeutics to the tumor area, nanoparticles have come into focus. Serving as transporters, various therapeutic cargos can be integrated in one nanoparticle system to combine different functionalities. Here we discuss the use of nanoparticles as multimodal drug transporters with special emphasis on superparamagnetic iron oxide nanoparticles (SPIONs). Based on their magnetic core they can be magnetically guided to the desired place, visualized in magnetic resonance imaging (MRI) and serve as heat transporters in magnetic hyperthermia.

CHALLENGES OF SYSTEMIC TUMOR THERAPIES

After intravenous or oral application of fluid chemo- and immunotherapeutics, the drug circulates in the whole organism and only a fraction reaches the tumor, whereas the majority disappears in the healthy tissues or is ejected. Thus, high doses must be applied for sufficient therapeutic concentrations in the tumor (8). Also, poor solubility can be an obstacle to reach effective therapeutic doses.

Chemotherapeutics are injected in a cyclic schedule to kill the rapidly proliferating tumor cells. Problematically, not only the tumor is affected but also healthy tissues (9) with quickly dividing cells such as cells of the blood, the immune system, hair, or mucosa. Since some cytostatic agents are carcinogens themselves they sometimes induce acute myeloid leukemia after therapy (9). Additionally, the risk of chemotherapy-associated anemia (10) and neutropenia (11) is high. Thus, immune function must be monitored regularly. In case of severe limitations, it may be necessary to reduce or stop the therapy (12). If the number of leukocytes in blood is too low, infections may occur and therefore patients often die due to therapy-related side effects and not the tumor itself (13).

Unlike chemotherapy, immunotherapy does not destroy cancer cells directly. The goal of immunotherapy is to manipulate the immune system to kill cancer without impeding normal tissues. Since checkpoint inhibitors act by blocking the inhibition of T cells, additionally to the wanted reactions such as tumor infiltration and killing of cancer cells, activated T cells can also attack healthy cells, resembling autoimmune reactions (14). While chemotherapy is associated with immunosuppression and infections, some of the recent approaches in immunotherapy can be accompanied by massive inflammatory responses and autoimmune-type like pathologies, which can affect all the organs of the body (14, 15). For Ipilimumab therapy in metastatic melanoma for instance, immune-mediated side effects as dermatitis, hepatitis, enterocolitis, hypophysitis, and uveitis, which can be life threatening, have been described (16). For management of inflammatory side effects systemic steroids or corticosteroids should be considered (16).

Immunotherapies are effective only in a subgroup of cancers and a minority of patients (17, 18). Reasons for this are tumor heterogeneity, previous treatments, variability in tumor type and stage and immunosuppressive phenotype of the cancer (19). Tumors with many mutations seem to have better response rates to immune checkpoint blockade with PD-1, probably due to higher tumor immunogenicity (20). Since immunotherapies are not applied as first line treatments, they are rather given to patients with compromised immune systems due to advanced disease or previous chemotherapy cycles, hindering the development of effective immune reactions (21).

Moreover, immunotherapies are very expensive depending on dosing and scheduling, putting economic pressure on patient and healthcare system (22). In 2016 the one-year per-patient costs for treatment of metastatic melanoma with PD-1 inhibitor Pembrozulimab was $145,010, achieving a progressing-free survival of 6.3 month (23, 24). Combination therapies can even double or triple the costs. These extremely expensive therapies might be denied by health insurances or lead to restrictions for patients who cannot afford additional payments for the drugs (24). Also, only few of the treatments reach complete tumor remission after one treatment cycle, so that multiple rounds of treatments are necessary.

TARGETED THERAPIES USING NANOPARTICLES

Systemic toxicities can hinder the efficacy of potent antitumor drugs. However, side effects caused by the unspecific distribution and low doses in the target area are not only problems in the treatment of tumors but also of various other diseases. To bring therapeutics directly to the target area and to reduce systemic concentrations nanocarriers have come into focus.

Passive Delivery of Nanoparticles

Distribution, pharmacokinetics and retention of medical nanoparticles strongly depend on the route of application and the physicochemical nanoparticle characteristics. For daily medication, oral application is comfortable for the patients. However, orally applied nanoparticles are rather quickly excreted from the body than being absorbed through the intestine into the blood. A possibility to increase nanoparticle absorption from the gastrointestinal tract is the conjugation of nanoparticles with bile acids, employing bile acid transporter-mediated cellular uptake and chylomicron transport pathways (25). With intravenous application nanoparticles tend to be restricted to the vascular system and to organs with a fenestrated endothelium, such as liver and spleen since the pore size of normal intact endothelium

is about 5 nm. Tumors and inflamed areas are accessible as well, since they exhibit fenestrated endothelium and vascular leakiness. Depending on their size, injected nanoparticles undergo renal clearance including glomerular filtration, tubular secretion, and finally elimination through urinary excretion. For globular proteins the filtration-size threshold is <5 nm, and this seems to be comparable for nanoparticles (26, 27). Larger particles are cleared from blood circulation via phagocytic cells of the reticuloendothelial system (RES). Macrophages in the liver (Kupffer cells), the spleen and the circulating blood rapidly take up opsonized nanoparticles and intracellularly degrade them (28, 29). Importantly, systemic inflammation affects nanoparticle distribution by alteration of systemic circulatory properties, modulation of the immune system and increase of vessel permeability (30). Modification of the nanoparticle surface by polyethylene glycol (PEG) reduces non-specific protein adsorption and opsonization and minimizes clearance by the RES, thus resulting in longer blood circulation times and improved pharmacokinetic properties (31). Intraarterial injection in proximity to the tumor site can limit the nanoparticle removal by the RES (32).

When tumors exceed a distinct size transport of oxygen and nutrients by diffusion is insufficient and access to the blood circulation is necessary (33). Contrary to healthy blood vessels, tumor capillaries have large gaps between endothelial cells, a wide irregular lumen and lack of smooth muscle cells, enabling the selective extravasation. The poor lymphatic drainage permits retention of macromolecular drugs or nanoparticles in the tumor microenvironment, referred to as enhanced permeation and retention (EPR) effect (34, 35). So far, several clinically approved chemotherapeutics such as doxorubicin, daunorubicin, or vincristine encapsulated into liposomes have been approved as nanomedicines by the Federal Drug Administration (FDA). Beside these first generation clinically approved nanomedicines, other non-targeted nanosystems are under investigations in clinical studies (phase I/II/III) (36).

Active Delivery of Nanoparticles

Despite preferential accumulation in tumor tissues due to the EPR effect, the fraction of nanoparticles finally entering the tumor is still limited. The majority of the applied nanoparticles is removed from blood in a few hours and only some percent remain in the systemic circulation (37). Finally, only ∼2% of the total intravenously administered dose is deposited in the tumor after 4 h of circulation (38). To increase the intratumoral dose, several studies revealed receptor-based active targeting of nanoparticles to be a promising delivery strategy (39). Targeting ligands such as monoclonal antibodies and antibody fragments, aptamers, peptides and small molecules are under extensive investigation for use in diagnostics, therapy and post-therapeutic follow-up (40). For example, Trastuzumab functionalized nanoparticles targeting Her2 positive tumor cells showed favorable results in experiments with breast cancer cells as diagnostic agents and drug delivery vehicles (41, 42). SPIONs with folic acid as targeting molecule enhanced the uptake by folate receptor exposing tumor cells (43).

Beside use of targeting moieties, nanoparticles can be transported by physical forces to the desired place. For instance, SPIONs can be applied as drug transporters in Magnetic Drug Targeting (MDT). To prevent clearance by RES, SPIONs are applied intraarterially in the tumor supplying vascular system and are enriched in the tumor region using an external magnetic field. Previously, studies with tumor bearing rabbits (squamous cell carcinoma) revealed that the amount of the chemotherapeutic agent mitoxantrone in the tumor region can be increased from 1% after intravenous application to 50–60% with MDT. Complete tumor remissions or slower tumor growth with increased survival times were shown in the majority of the treated animals (44). Also, immune cells from peripheral blood were spared from the toxic effects of the chemotherapy, due to specific accumulation in the tumor (45).

A major challenge remains the treatment of tumors in the brain, due to often being surrounded by important functional structures, which can be injured by interventions such as surgery, intratumoral injections or radiation. In this case, magnetic forces can be used to trap SPIONs at the site of interest. To bypass the first-pass organ clearance of the magnetic nanoparticles, intraarterial administration via carotid artery enhances nanoparticle exposure of the tumor vasculature. Together with an MRI guided subject alignment within the magnetic field and surface modification of the drug with biological membrane permeable polyethyleneimine it is possible to deliver ß-galactosidase selectively to the brain tumor in a rat glioma model, while limiting the exposure of healthy brain areas (32). In this approach, magnetic field topography is essential to prevent magnetic aggregation in the vasculature (32, 46, 47). To prevent nanoparticle aggregation and occlusion of vessels in magnetic fields we found that a proper surface coating and colloidal stabilization of SPIONs is a prerequisite (48).

IMMUNOGENIC CELL DEATH INDUCTION BY TUMOR THERAPIES

Therapeutic strategy of conventional treatments relies on the rationale that rapidly proliferating tumor cells are more sensitive to toxic chemicals or radiation than healthy tissues. In the past, it has been believed that these treatments simply act by killing the tumor cells or inhibiting their proliferation. However, it became apparent that distinct cell death pathways activated during cell stress turn the cells "visible" for the immune system, a process referred to as immunogenic cell death (ICD). Agents inducing ICD in cancer therapy are for example chemotherapeutics from the class of the anthracyclines and their derivatives (e.g., doxorubicin, mitoxantrone), photosensibilisators for photodynamic therapy (PDT) or radiotherapy (49). In contrast to apoptosis, the physiological form of cell death, eliciting inflammatory silent or even anti-inflammatory clearance, ICD induces inflammatory immune reactions. Hallmark of ICD is the release of damage associated molecular patterns (DAMPs) from the dying cells in a timely resolved fashion (50). The early cell surface exposition of calreticulin, the active release of heat shock proteins (HSPs)

and ATP as well as the *post mortem* leakage of HMGB1 have been described to act as endogenous adjuvants, recruiting and activating immune cells. Professional antigen presenting cells take up tumor derived antigens, process them, migrate to the tumor draining lymph nodes and cross present them to T cells. Subsequently, antigen specific T cells differentiate to effector T cells, proliferate, and are attracted to the tumor region by chemokines (51). There, effector T cells kill the tumor cells via cytotoxic granules or Fas-induced apoptosis and thereby create a new wave of released tumor antigens which boost the immune response (52). By inducing ICD radiation, photodynamic therapy (PDT) and/or chemotherapy may activate immune responses and immunize a patient against cancer by turning the tumor into an *in situ* vaccine (53). Radiation and chemotherapy both can induce DNA damage resulting in cell cycle arrest and/or cell death. Furthermore, cellular mutations with the development of neoantigens are provoked, resulting in higher immunogenicity (**Figure 1A**).

NANOPARTICLE-BASED THERAPIES

Due to induction of ICD by several routine treatment regimens, the combination of those therapies with immunotherapeutic agents can induce or increase anti-tumor responses from the immune system. A multitude of various nanoparticle systems has been developed for medical application and multimodal tumor therapy, which are discussed elsewhere (54). SPIONs can be tailored in size, morphology and functionalization, enabling their use in a wide range of applications (55). SPIONs can be loaded as drug transporters with various cargos (chemotherapeutics, photosensibilisators, immune modulators), serve as contrast agents in MRI, provide heating capacity in alternating magnetic fields, and enable magnetic targeting (**Figure 2**). Due to these additional possibilities, a special focus will be set on SPIONs here.

SPIONs as Drug Transporters

Prerequisite for use of nanoparticles in biomedicine is their biocompatibility. Due to their inorganic nature, SPIONs on their own are not sufficiently biocompatible. One strategy to circumvent this compatibility issue is to coat the SPIONs with biocompatible polymers (56). For SPIONs comprehensive studies have been performed with partially contradictory results dependent on size, coating, applied concentration and exposure time of the nanoparticles (57). Reported toxicities in experimental studies include reduced mitochondrial activity, cellular stress mediated generation of ROS, inflammation and chromosome condensation (58). In our hands, coating of nanoparticles with biocompatible substances such as crosslinked dextran or formation of an artificial protein corona of serum albumin not only increased colloidal stability of the particles but also their biocompatibility (59–64). Some formulations of magnetite-based nanoparticles have already been approved for

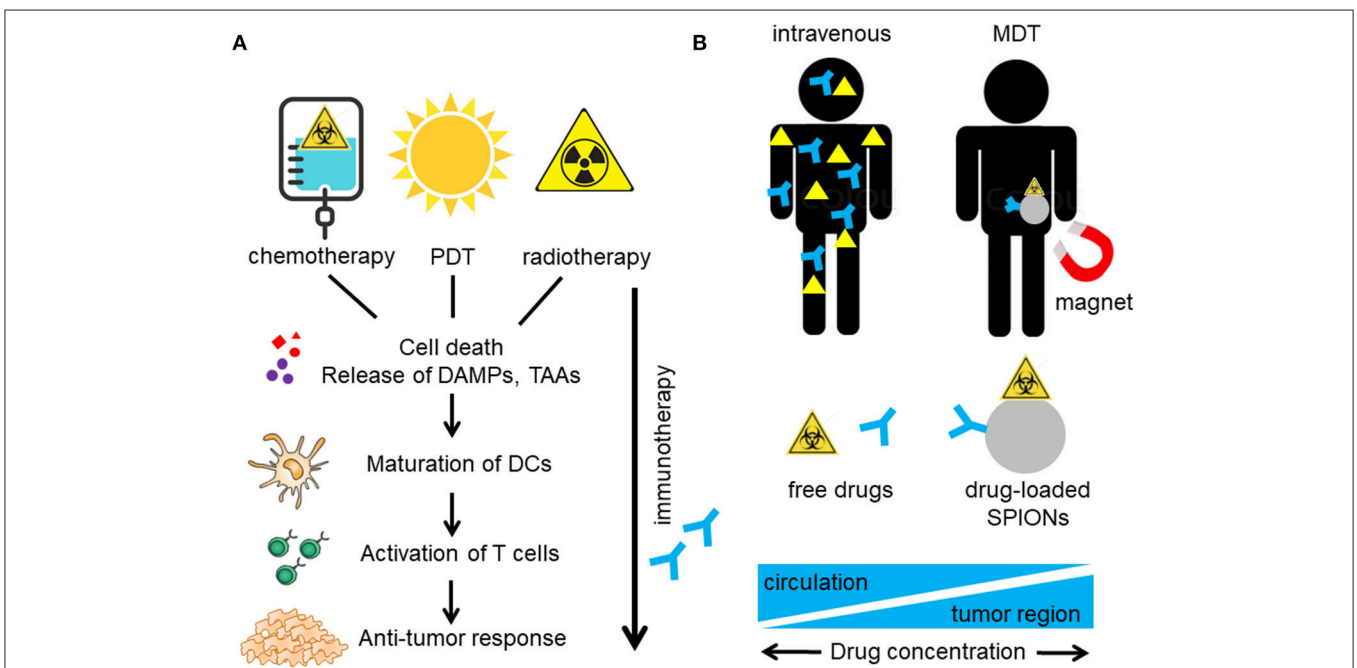

FIGURE 1 | Induction of anti-tumor immune reactions by multimodal therapy. **(A)** Chemotherapy, radiotherapy, and photodynamic therapy (PDT) induce immunogenic cell death (ICD) in the tumor with release of damage associated molecular patterns (DAMPs) and tumor associated antigens (TAA). TAA are taken up by antigen presenting cells (APC), such as dendritic cells (DCs) and are processed and presented to T cells, which are activated to proliferate. Accompanying immunotherapy (e.g., with anti-PD-1) blocks PD-1 (on T cells) and PD-L1 (on tumor cells and APCs) interaction, resulting in immune activation and increase of anti-tumor immune responses. **(B)** Integrating several treatment functionalities on one nanoparticle and active targeting to the tumor region e.g. by magnetic drug targeting (MDT) might increase the therapeutic doses in the tumor and reduce systemic distribution with accompanying side effects such as immune deprivation.

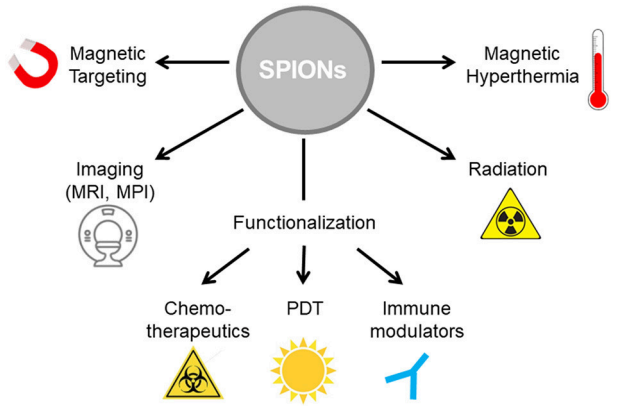

FIGURE 2 | SPIONs as nanoparticle platform for multimodal tumor therapy. SPIONs can be functionalized with various cargos such as cytotoxic agents for chemotherapy, photosensibilisators for photodynamic therapy and/or immune modulators for immunotherapy. To increase treatment efficacy, magnetic hyperthermia can be induced in alternating magnetic fields. Radiation induces release of ROS on the particle surface. Imaging controlled therapy is enabled by magnetic resonance imaging (MRI).

use in humans as iron deficiency therapeutics and as MRI contrast agents by the FDA (e.g., Feraheme®, Feridex I.V.® and Gastromark®) (65). Once the SPIONs are administered intravenously, they enter liver and spleen (66). SPIONs are taken up into the lysosmes of cells, where the iron oxide is broken into iron ions presumably due to hydrolysing enzymes effective at low pH and ultimately get incorporated into hemoglobin (57, 67).

Combination of Nanocarriers (SPIONs) With Chemotherapy

Challenges in routine chemotherapy are systemic toxicities. Despite several chemotherapeutics have shown the ability to induce ICD, systemic applications are accompanied by severe side effects, in particular destruction of the immune system (11). That's why some of the current chemotherapeutics are also used as immunosuppressive agents (e.g., cyclophaosphamide, methotrexate) for the treatment of severe autoimmune diseases. By loading chemotherapeutic drugs onto nanoparticles this challenge can be addressed. With targeting of nanoparticles to the tumor region, the systemic concentration is reduced while effective intratumoral doses are increased. Several chemotherapeutic agents such as doxorubicin, daunorubicin, or vincristine encapsulated into (PEGylated) liposomes have been approved as e.g., Doxil®/Caelyx®, DaunoXome®, or Marqibo, respectively. Paclitaxel bound to lyophilized human albumin as carrier protein is registered as Abraxane® for breast cancer treatment (68). Presensitization of tumor cells with antisense miRNA (against miRNAs expressed during cancer) or siRNA (against a developmental transcription factor reactivated in cancers) prior to chemotherapy can reduce the effective doses of chemotherapeutics needed or can overcome chemoresistance (69, 70).

To induce anti-tumor immune reactions, inducers of ICD such as oxaliplatin or doxorubicin have been loaded into nanocarriers (71–74). Exemplarily, after intravenous injection of oxaliplatin or doxorubicin-loaded amphiphilic diblock copolymer nanoparticles, the nanoparticle-encapsulated ICD inducer led to significantly enhanced ICD and consequently improved anti-tumor effects in pancreatic cancer xenograft compared to the free form (71). Active targeting of nanoformulations using magnetic forces have been explored to maximize drug accumulation of ICD inducers as well. We and others loaded chemotherapeutic drugs such as mitoxantrone or doxorubicin onto SPIONs and showed improved targeting and anti-tumor efficacy in the presence of magnetic fields *in vivo* (44, 75, 76). When we treated rabbits suffering from induced squamous cell carcinomas with SPIONs functionalized with mitoxantrone and targeted the particles to the tumor by an external magnet, the tumors were continuously shrinking until complete tumor disappearance after several weeks, indicating rather an immunological process than immediate tumor lysis by mitoxantrone (44). We proved that mitoxantrone functionalized SPIONs can induce ICD with concomitant release of DAMPs such as HSPs, ATP, HMGB1, and foster maturation of DCs (77).

Improving chemotherapy (probably by synergistically inducing ICD), pH sensitive magnetically guidable iron oxide nanocarriers loaded with doxorubicin and a photosensibilisator showed beneficial effects in U87 tumor bearing nude mice, thus overcoming chemoresistance (78).

Combination of Nanocarriers (SPIONs) With Immunotherapy

Anti-cancer immunotherapies shall increase the strength of immune responses against the tumor by either stimulating activities of the immune system or block signals produced by cancer cells to suppress immune responses. In the evolving field of immunotherapy, therapeutic antibodies against tumor antigens (e.g., Herceptin targeting HER-2/neu on breast cancer) or antibodies inhibiting the proliferation of tumor-supplying vessels, stimulatory cytokines (e.g., interferon α and β), and immune checkpoint inhibition (e.g., PD-1 inhibitors) have shown clinical activity in many different types of cancer.

Several pathways influence the intensity of an immune reaction to prevent autoimmune reactions. Inhibitory pathways induce downregulation of T cell activation or effector functions (79). T cells with receptors recognizing non-self structures on tumor cells are the key players to trigger anti-tumor immune responses. Binding of the T cell receptor accompanied by a co-stimulatory signal leads to T cell activation. The tight control of this process is essential to inhibit excessive activation leading to autoimmune reactions, whereby the proteins CTLA-4 and PD-1 on T cells play major roles as brakes of T cell activation. Blocking CTLA-4 and/or PD-1 by antibodies can restore immune activation, referred to as immune checkpoint therapy (80, 81). Examples for antibodies that target PD-1 are Pembrolizumab or Nivolumab, applied in several types of cancer including tumors of the skin, kidney, bladder, head and neck, lung, and Hodgkin lymphoma (82).

Challenges of current immunotherapies are systemic autoimmune reactions, low response rates, tremendous costs, and application to patients with compromised immune systems. Loading immunotherapeutics onto nanoparticulate transporters can increase their therapeutic potential (83). Thus, currently nanoparticles are being investigated as transporters for antigens, adjuvants, or siRNA to activate the immune system (5). To target nanoparticles to PD-L1 expressing cancer cells, PD-1 antibody was not only used on nanoparticles as targeting ligand but also for disturbing the interaction between PD-L1 on tumor cells and immune cells (84).

Tumor accumulation of nanoparticulate immunotherapeutics can further be increased by targeting T cells in the circulation since leukocytes are the first cells intravenously applied nanoparticles get in contact with. Additionally, lymphocytes can deeply penetrate into the tumor tissue. Thus, nanoparticles targeting PD-1 expressed on T cells and inhibition of TGF-β signaling have been shown to increase survival of tumor bearing mice. With this approach dosing can be significantly reduced, thus limiting potential toxicity (85). In this context first pilot experiments have been performed to load T cells *ex vivo* with SPIONs as transporters for (immune modulatory) drugs to subsequently inject and guide them to the tumor area using an external magnetic field (86).

Combination of Nanocarriers (SPIONs) With Hyperthermia and Radiotherapy

Mild hyperthermia can elicit cell death by denaturation of proteins and/or damage of DNA and other mechanisms, resulting in apoptosis (87). Inefficient blood flow and supply with oxygen through the quickly generated blood vessels in tumors results in an acidotic and nutrient-deprived milieu making cancer cells more thermo sensitive to acute increases in temperature than healthy cells (88). Major problem with conventional methods to induce hyperthermia is the generation of homogenous therapeutic temperatures deep in the tumor. Here, SPIONs can act as controllable heat source: in alternating magnetic fields, the magnetic polarity rapidly flips. However, there is some hysteretic loss involved in the flipping, revealing as heat. Thus, a tumor can be heated in alternating magnetic fields if preloaded with SPIONs. Although there are some reports on use of magnetic hyperthermia alone to treat and/or cure cancer in animal models, magnetic hyperthermia is often used in combination. Radiotherapy and hyperthermia have complementary effects: Poorly perfused tumor cores are sensitive to hyperthermia but resistant to ionizing radiation which depends on the formation of toxic oxygen radicals in well perfused areas. Also, in the S phase of the cell cycle tumor cells exhibit radioresistancy, but are highly sensitive to heat. Thus, hyperthermia can act as radiosensitizer to radioresistant cancer cells (89).

Radiosensitizers, such as histone deacetylase inhibitors, which inhibit DNA double strand repair can enhance the response of tumor cells to radiation through the prolongation of γ-H2AX foci as shown with polymer nanoparticles (90). Also, binding of radionuclids to SPIONs, particularly β emitters, induced DNA damage due to free radicals, resulting in apoptosis of target cells (91). Also, SPIONs have shown their potential as X ray-enhancer for low-dose irradiation therapy. After radiation the amount of toxic ROS in tumor cells with engulfed nanoparticles has substantially increased (92, 93).

SUMMARY

For efficient cancer treatment including long-term immune reactions, the immunogenicity of the tumor must be increased and the tolerance of the immune system against tumor associated antigens abrogated. Importantly, at the same time, immune compatibility has to be preserved. With nanoparticles as platform technology immunotherapeutics and/or chemotherapeutic drugs can be targeted towards the tumor. Compared to systemic application, the intratumoral drug concentration can be increased and healthy tissues spared from the drug related side effects by nanoparticle-mediated transportation (**Figure 1B**). Concurrent radiation and/or hyperthermia of the tumor induces cell death and increases immunogenicity of the tumor cells. Employing SPIONs as drug transporters enables multimodal therapy concepts since compounds of various therapeutic classes (e.g., chemotherapeutics, immune modulators, phototoxic compounds) can be bound and adapted to the individual profile of the patient. Using SPIONs as nanoparticle platform additionally enables monitoring of tumor targeting in MRI (Theranostics) (62, 63).

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Low-Dose Total Body Irradiation Can Enhance Systemic Immune Related Response Induced by Hypo-Fractionated Radiation

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A systemic immune related response (SIME) of radiotherapy has been occasionally observed on metastatic tumors, but the clinical outcomes remain poor. Novel treatment approaches are therefore needed to improve SIME ratio. We used a combination of hypo-fractionated radiation therapy (H-RT) with low-dose total body irradiation (L-TBI) in a syngeneic mouse model of breast and colon carcinoma. The combination therapy of H-RT and L-TBI potentially enhanced SIME by infiltration of $CD8^+$ T cell and altering the immunosuppressive microenvironment in non-irradiated subcutaneous tumor lesions. The frequency of IFN-γ, as a tumor-specific $CD8^+$ T cells producing, significantly inhibited the secondary tumor growth of breast and colon. Our findings suggest that L-TBI could serve as a potential therapeutic agent for metastatic breast and colon cancer and, together with H-RT, their therapeutic potential is enhanced significantly.

Keywords: systemic immune related response, hypo-fractionated radiation therapy, low-dose total body irradiation, immune enhancement, immunosuppressive microenvironment

INTRODUCTION

Radiotherapy (RT) is one of the main approaches used in cancer treatment, along with the induction of DNA damage that leads to tumor cell apoptosis. It also activates the anti-tumor immune response by exposing the tumor antigens to the host immune factors (1–3). Activation of the host immune system then leads to remissions even at sites distant from the loco-regional irradiated tissues, a phenomenon known as SIME. However, SIME induced by RT alone is rarely described, with only few published case reports. In a recent review, Reynders et al. retrieved only 23 case reports from 1973 to 2013 on the perceived SIME after RT alone (4). A common strategy of improving the SIME is to combine ionizing RT with immunotherapy (IT), which has been reported to increase the percentage of patients with abscopal tumor regression to 20% (5–7). Notably, most immunotherapeutic strategies, when used alone, failed to establish long-lasting tumor rejection in clinical trials on large patient groups (8, 9). This is most likely due to high heterogeneity of different tumor types and poor immunogenicity and evolving capability to escape immune recognition (10, 11). RT combined with IT (RT-IT) effectively changed the phenomenon (12–14). However, the repertoire is sheer endless, ranging from different RT-IT strategies including many different

radiation treatments, numerous IT approaches, and choosing the right patient population and a reasonable stage of the disease. So far, no conclusive explanation could be given regarding the best strategy providing the best platform for combination approaches. Another major obstacle to precisely evaluating the effects of RT and IT combination on tumor progression is posed by the still limited available imaging modalities especially in the clinical setting (10). In addition, most patients cannot bear the costs of IT, indicating the urgent need for better strategies.

Low-dose irradiation approach, defined as ≤0.2Gy at low linear energy transfer (LET) or ≤0.05Gy at high LET, is known to induce both innate and adaptive anti-tumor immune responses (15, 16). It can activate T-cells and natural killer (NK) cells and increase T-cell proliferation, while reducing the infiltration of the immunosuppressive regulatory T-cell (Treg) in tumor tissues (17, 18). Interestingly, low-dose irradiation has been shown to inhibit or retard the development of both primary and metastatic tumors (19, 20). Since developing tumors create microenvironments that not only support neoplastic growth and metastasis but also significantly reduce the potency of both innate and adaptive anti-cancer immunity (21), the potential SIME of the combination of low-dose irradiation with RT is worth investigating.

Accumulating evidence demonstrate that the dose, mode of delivery and RT schedule are important determinants in the anti-tumor immune response, with the most vital question of "to fractionate or not to fractionate?" Due to genetic and epigenetic changes in the neoplastic cells, they may become "invisible" to immune effectors through the loss or aberrant expression of the MHC class I receptors or other molecules (22, 23). Local irradiation of tumors during standard RT can stimulate anti-cancer immunity and partially reverse the immunosuppression triggered by cancer cells. However, these effects are often induced by moderate (0.2–2.0Gy) or high (>2Gy) doses of ionizing radiation, which also harm healthy tissues, impede normal immune functions, and increase the risk of secondary neoplasms (15). Recently, Vanpouille-Box et al. revealed that single fraction doses above 12–18Gy on different cancer cells induced DNA exonuclease Trex1, which inhibits the immunogenicity of the cells by degrading their DNA that then is accumulating in the cytosol. In the Hypo-fractionated RT (H-RT), the total dose is split into large doses and administered over a short period of time (8Gy × 3), resulting in a significant increase in cytosolic dsDNA and down regulation of Trex1, which enhances the immunogenicity of colorectal and breast cancer cell lines (24, 25). Although these studies highlight the immunological effect of H-RT, as a monotherapy it rarely induces effective anti-tumor immunity that can result in systemic tumor rejection. According to the effect of low dose total body irradiation (L-TBI) in antitumor immunity, we therefore hypothesized that the combination of H-RT with our low dose total body irradiation (L-TBI) protocol might enhance the systemic anti-tumor effect and elicit the SIME as well.

Hence, in this work, we established tumors in a murine model using mouse mammary carcinoma 4T1 and colon carcinoma CT26 cells. Our results showed that tumor growth was not inhibited by L-TBI alone. Local tumor growth inhibition by H-RT did not translate into increased survival due to lung metastases and progression of the proliferation of the secondary tumor. Notably, we demonstrated for the first time that the combination of L-TBI and localized H-RT to the primary tumor activated $CD8^+$ T-cell dependent anti-tumor immunity, inhibited spontaneous lung metastases and retarded secondary tumor growth, all of them significantly increasing the survival of the treated mice. These results suggested that the combination of H-RT and L-TBI might be a promising therapeutic approach for managing metastasis in cancer patients.

Abbreviations: H-RT, hypo-fractionated radiation therapy; L-TBI, low-dose total body irradiation; LET, linear energy transfer; NK, natural killer; MDSCs, myeloid-derived suppressor cells; TAMs, tumor-associated macrophages; Treg, regulatory T-cell; TME, tumor microenvironment; G-MDSCs, granulocytic-myeloid-derived suppressor cells; M- MDSCs, monocytic-myeloid-derived suppressor cells; ROI, irregular region of interest; SUV, standard uptake value.

MATERIALS AND METHODS

Mice

BALB/C mice (female, aged 6–8 weeks, weighing 20–25 g) were obtained from Chongqing Tengxin biotechnology Co. Ltd. (Chongqing, China). Mice were housed in standard laboratory cages under at 20–22°C, 50–60% relative humidity and 12 h light/12 h dark cycles (starting at 07:00 and 19:00, respectively), with free access to food and water. All animal experiments were approved by the Institutional Animal Care and Treatment Committee of Southwest Medical University (Luzhou, China), and all mice were treated humanely.

Cells and Reagents

BALB/C mouse-derived mammary carcinoma 4T1 and colon carcinoma CT26 cell lines were obtained from the State Key Laboratory of Biotherapy of Sichuan University (Chengdu, China) and Army Medical University laboratory (Chongqing, China), respectively. Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Cellmax, Australia) and 1% penicillin–streptomycin (Sigma-Aldrich, St Louis, MO, USA). Cell cultures were incubated at 37°C with 5% CO_2 in a humidified incubator. Cells were found free of mycoplasma contamination with the help of a detection kit.

Irradiation

All the mice were not anesthetized, positioned on a dedicated transparent radiotherapy box over the linac couch. Mice were fixed in our radiotherapy box and showed the whole right leg by a small hole, making the right leg in the tensile state and left leg natural state. All right leg and primary tumor were placed in the radiation field (**Supplementary Figure 1**). Our radiotherapy box has been tested by ionization chamber before radiotherapy. We tested the dose rate of the radiation field center and the middle plane. Also, we stacked in the vicinity of the tumor with thermoluminescence piece to verify dose. Radiation (L-TBI or H-RT) was delivered at a source-to-surface distance of

100 cm with a 6 MV linear accelerator (Varian Clinac 600C, USA). In this study, L-TBI was defined as a irradiation to the whole body at 0.1Gy with a dose rate of 24 cGy/min. Also local H-RT (primary tumor) was applied at 8 Gy × 3 with a dose rate of 400 cGy/min.

Tumor Challenge and Treatment

4T1 mammary carcinoma cells (1.5×10^5) and CT26 colon carcinoma cells (2.5×10^4) were subcutaneously injected in the right flank of each BALB/C mouse on day 0 separately. Also the same amount of cells were injected in the contralateral flank on day 3. The tumor arising from day 0 inoculum was designated as "primary" tumor and was irradiated, while the "secondary" tumor from the second inoculum was not irradiated (**Figure 1A**). On day 14, when the primary tumor reached an average size of 60–80 mm^3, mice were randomly divided into four groups according to the RT administered: (a) control group: non-irradiated; (b) L-TBI: low-dose total body irradiation at 0.1 Gy on day 14; (c) H-RT: 3 doses of localized radiations at 8 Gy each dose on the primary tumor on day 17, 18, and 19; (d) H-RT+L-TBI: L-TBI on day 14 followed by H-RT on day 17–19. Tumor size was monitored every 2 days, and tumor growth or regression was recorded. The perpendicular diameter of each tumor was measured using Vernier calipers, and tumor volume was calculated using the following formula: length×width^2 × 0.52, by two researcher independently (26, 27). On day 24, some of mice were anesthetized and sacrificed by cervical dislocation. The requisite organs were harvested and processed for further analysis. The remaining mice were used to observe survival and make survival curves. Meanwhile, we measured the tumor volume of these mice until death. When the tumor volume exceeded 4 cm^3, all mice were sacrificed.

Measurement of Lung Surface Nodules

After sacrificing the mice on day 24 post-inoculation, their lungs were resected and fixed in 10% neutral-buffered formalin for 24 h. The pulmonary metastatic nodules were counted and their diameters were measured under a dissecting microscope. The nodules were classified into 4 levels according to their diameter as follows: I. <0.5 mm, II. 0.5–1 mm, III. 1–2 mm, and IV. >2 mm. Then, the lung surface transfer nodule was calculated using the formula: I × 1 + II × 2 + III × 3 + IV × 4 (28). As regard histopathological examination, the fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E), according to standard protocols. Microscopically analysis of all the slides was performed by a light microscopy (Olympus Cor, Tokyo, Japan) linked to computerized image system (Image-Pro Plus V6.0, Silver Spring, MD).

Micro 18F-FDG PET/CT Imaging

The early effects of different treatments were evaluated using micro PET/CT scans and all images were analyzed by using an Inveon micro PET/CT animal scanner (Siemens, Germany). Mice were fasted for 12 h and then anesthetized by intraperitoneal injection with 1% pentobarbital (5 ml/kg). Mice were then placed in the center of the scanner, intravenously injected with 200–300 μCi FDG, and then scanned. PET/CT images were exported one h after injection of 18F-FDG trace. The parameters used for PET/CT scanning were as follows: 80 kV, 500 μA, slice thickness of 1.5 mm, and 10 min per bed position.

The image plane with the largest tumor appearance on the PET/CT fusion image was selected for analysis, and the irregular region of interest (ROI) covering the entire tumor was manually drawn. ROIs were also drawn on the paraspinal muscles. The tracer uptake value in both the tumor and muscle tissue was determined in the attenuation-corrected transaxial tomographic slices by calculating the standard uptake value (SUV), and was measured by means of ROI. The 18F-FDG maximum SUV of each lesion was obtained from the selected ROI and then compared to the SUVs of the contralateral paraspinal muscles to calculate the tumor/muscle (T/M) ratio.

Flow Cytometry Analysis

The breast cancer tumors were resected, and then homogenized in 0.2% collagenase type IV, 0.01% hyaluronidase, and 0.002% DNase I (all enzymes from Solarbio science, Beijing, China) in DMEM medium at 37°C for 40 min. Also, spleen tissue was resected, grinded and filtered into a single cell suspension, according to standard protocols. The blood cell lysate kits were used for removing red blood cells (BD Biosciences, CA, USA). The single cell suspension thus obtained was stained with the fixable viability stain 780, and then the harvested cells were labeled with the following antibodies: CD45-PerCP, CD11b-APC, Gr1-FITC, Siglec-F-PE, Ly6G-PE-Cy7, Ly6c-FITC, CD11c-PE, F4/80-APC/Cy7, CD206-FITC, CD3-PerCP-Cy5.5, CD4-FITC, CD8-PE-Cy7, CD86-FITC, and INF-γ-APC antibodies according to the manufacturer's protocol (BD Bioscience, CA, USA). For INF-γ staining, cells were stimulated *in vitro* with a cell stimulation cocktail (plus protein transport inhibitors) (BD Bioscience) for 6 h. After surface labeled with CD3-PerCP-Cy5.5 and CD8-PE-Cy7 antibodies, cells were then processed using a fixation and permeabilization kit (BD Bioscience) and stained with antibodies from BD to IFN-γ. In order to identify the frequencies of $CD8^+$ cell, mouse anti-CD8/Lyt2.1 monoclonal antibody (clone HB129/116-13.1) and corresponding isotype control (clone C1.18.4) were purchased from BioXcell (West Lebanon, NH, USA). The 4T1-bearing mice were intraperitoneally treated with 400 μg of anti-CD8/Lyt2.1 monoclonal antibody and isotype control as described in **Supplementary Figure 4A**. The stained samples were analyzed using a Beckman Coulter Gallios flow cytometry (Beckman Coulter, Miami, FL, USA). All flow cytometry data were analyzed with FlowJo software (version 10.0). Isotype-matched control antibodies were all purchased from BD (BD bioscience, CA, USA) and used at the same concentration as test antibodies. Fluorescence minus one (FMO) controls was used for determining the percentage of positive cells.

Immunohistochemistry

Tumor tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin, and 4 μm thick sections were cut and

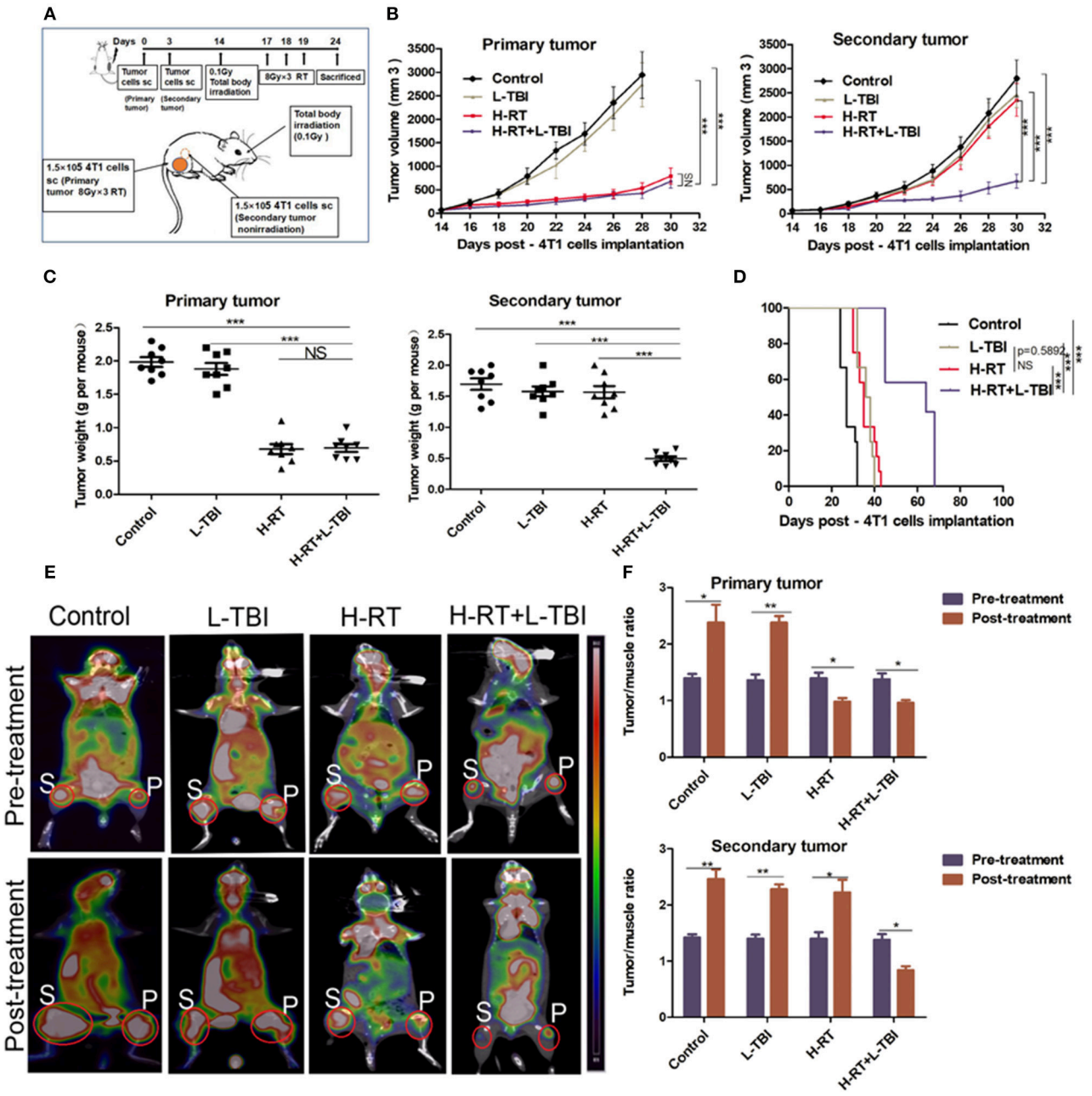

FIGURE 1 | H-RT on 4T1-derived subcutaneous tumor combined with L-TBI. **(A)** Experimental groups were treated as represented in the timeline. Immunocompetent mice were injected s.c. with syngeneic 4T1 cells (1×10^5) into the right (primary tumor) and left (secondary tumor) flank, respectively. H-RT was administered locally to the primary tumor from day 17 to 19, and L-TBI was administered on day 14. Primary and secondary tumor volumes were measured. On day 24, mice were sacrificed and tumors weighed. **(B)** Tumor growth of primary tumors (right panel) and secondary tumor (left panel) in mice treated with control (black line), H-RT (yellow line), L-TBI (red line), and combination of the H-RT and L-TBI (blue line). Data are the mean ± SE of 12 mice/group. **(C)** Primary tumor weight (right panel) and secondary tumor weight (left panel) on day 24 ($n = 8$ mice/group). **(D)** Overall survival of the tumor bearing mice of different treatment groups ($n = 12$ mice/group). **(E)** Representative pre- and post-treatment 18F-FDG PET images of tumor-bearing mice in control, and treatments groups (L-TBI, H-RT, H-RT+L-TBI; $n = 5$ mice/group). **(F)** Tumor/muscle ratio of primary (right panel) and secondary (left panel) tumor in the pre-treatment (on day 13) and post-treatment (on day 24) period ($n = 5$ mice/group). The experiment has been repeated in similar result (*$P < 0.05$, **$P < 0.01$, *** $P < 0.001$ and NS = not significant).

used for immunohistochemistry (IHC). The sections were labeled with the following antibodies: gamma-H2AX, TUNEL, CD3, and CD86, according to the manufacturer's instructions (Bioworld Technology, Nanjing, China). Images were taken using an optical microscope (Olympus, Tokyo, Japan). For each tumor section, the total number of cells and those positive for gamma-H2AX,

CD3, and CD86 were counted in five randomly selected fields (original magnification ×200), and the percentage of positively stained cells was calculated. Similarly, TUNEL-positive brown nuclei were also counted, and the percentage of apoptotic cells per field was calculated.

ELISA Measurements

Levels of INF-γ were measured by standard ELISA method by specific-antibody ELISA kits according to the manufacturer's instructions (Cheng Lin biotechnology, Beijing, China). In details, 0.5 mL of the blood samples were collected from the retro-orbitally sinus on day 24 post inoculation. Blood samples were left undisturbed at room temperature (20–25°C) for 20 min, and then were centrifuged at 2,000 g for 20 min. The serum was aspirated under sterile conditions and was stored at −80°C till further analysis.

Statistical Analysis

All statistical analysis were performed using SPSS 17.0 software (Chicago, Illinois, USA). Comparisons between two groups were made using Student's *t*-test, as well as one-way or two-way analysis of variance (ANOVA) was used for more than two groups. Survival curves were plotted based on the Kaplan-Meier method. Data are presented as mean ± standard error (SE). For all tests, two-sided $p < 0.05$ and high statistical significance at < 0.01 and < 0.001 were considered statistically significant. All charts were designed by Prism 5.0 (GraphPad, La Jolla, CA, USA).

RESULTS

L-TBI (0.1 Gy) Combined With H-RT (8 Gy × 3) Suppressed the Primary Tumor, and Effectively Inhibited the Secondary Tumor

BALB/C-derived mammary carcinoma 4T1 cells were used to establish a tumor model in order to test whether local H-RT can trigger systemic antitumor effects outside the radiation field when combined with L-TBI. According to a reported research that the dose of 0.1 Gy total body irradiation can enhance immune effect (29), mice were subjected to total body irradiation at 0.1Gy. We induced subcutaneous tumors in the mice at two separate sites: the primary tumor was irradiated by H-RT to determine the direct therapeutic effect of H-RT±L-TBI, while the secondary tumor was not irradiated and served to measure the potential indirect, systemic effect of H-RT± L-TBI (**Figure 1A**).

L-TBI alone did not delay the growth of either the primary or secondary tumors, as the tumor volume did not significantly change compared with the non-irradiated control group ($P > 0.05$). In line with the previous reports, H-RT indeed led to a significant growth delay of the irradiated primary tumors ($P < 0.001$ from day 18) but did not have a SIME on secondary tumors. Of note, we found that the combination of L-TBI and H-RT significantly delayed the growth of both the primary and secondary tumors ($P < 0.001$ from day 22; **Figure 1B**). Consistently, the weight of the harvested abscopal tumors was also significantly reduced in the combination therapy group compared to the others (with complete regression in 2 mice; $P < 0.001$), while reduction in primary tumor weight was similar in the H-RT and H-RT+L-TBI groups (**Figure 1C**). Taken together, local H-RT combined with L-TBI showed the highest tumor inhibitory effect and SIME was also elicited. The anti-tumor efficacy of H-RT+L-TBI translated to the best overall survival. H-RT+L-TBI treated mice showed a median survival time of 64 days compared to the 35 days in H-RT, 37 days in L-TBI, and 27 days in the control group ($P < 0.001$; **Figure 1D**).

Micro 18F-FDG PET/CT imaging (representative images in **Figure 1E**) showed significant differences between the pre-treatment and post-treatment T/M values within all four groups (**Figure 1F**). The primary tumor of the H-RT+L-TBI and H-RT group showed a significant decrease in the T/M values following treatment. In contrast, the post-treatment T/M values of secondary tumors showed a significant decrease only in the H-RT+L-TBI group, further indicating a better systemic anti-tumor response (SIME) of H-RT and L-TBI combination.

Impact of the Duration and Sequence of Combination Therapy on SIME

To determine whether the post L-TBI interval could impact the therapeutic effect of the combination therapy, we started the local H-RT at 48, 72, 96, and 120 h after L-TBI (scheme shown in **Figure 2A**). Compared to the non-irradiated control, the primary tumor volume of the other groups showed a significant decrease regardless of the post L-TBI interval, while the maximum growth delay of the secondary tumor was achieved by the administration of H-RT at 48 and 72 h after L-TBI before 30 days (**Figures 2B,C**). In addition, local H-RT 72 h after L-TBI therapy led to the best overall survival (**Figure 2D**).

To test the therapeutic impact of the sequence of the combination therapy, we administered local H-RT 3 days before L-TBI (b-L-TBI), 3 days after L-TBI (a-L-TBI), or simultaneously with L-TBI (s-L-TBI) (**Supplementary Figure 2A**). a-L-TBI achieved the best therapeutic effect represented by a significant tumor growth delay and improved survival of the treated mice (**Supplementary Figures 2B–D**).

Effect of Combination Therapy on Apoptosis

RT is known to induce apoptosis of cancer cells. To determine whether the direct and abscopal anti-tumor effect of the combined therapy was also related to apoptosis, tumor tissue sections were stained with TUNEL. Compared to the sporadic apoptotic cells seen in the primary tumor in the non-irradiated control and the L-TBI treated group, a significantly higher number of apoptotic cells was observed in the H-RT and H-RT+L-TBI group (**Figure 3A**). However, the primary tumor of the H-RT group showed a higher apoptosis rate than the tumor of the H-RT+L-TBI group ($P < 0.05$; **Figure 3B**). In contrast, little apoptosis was observed in the secondary tumor in all groups ($P > 0.05$; **Figure 3B**). Taken together, the percentage of apoptotic cells in the primary tumor was dramatically higher in the H-RT group compared to the others, while apoptosis was not the main underlying mechanism of the anti-tumor immune response.

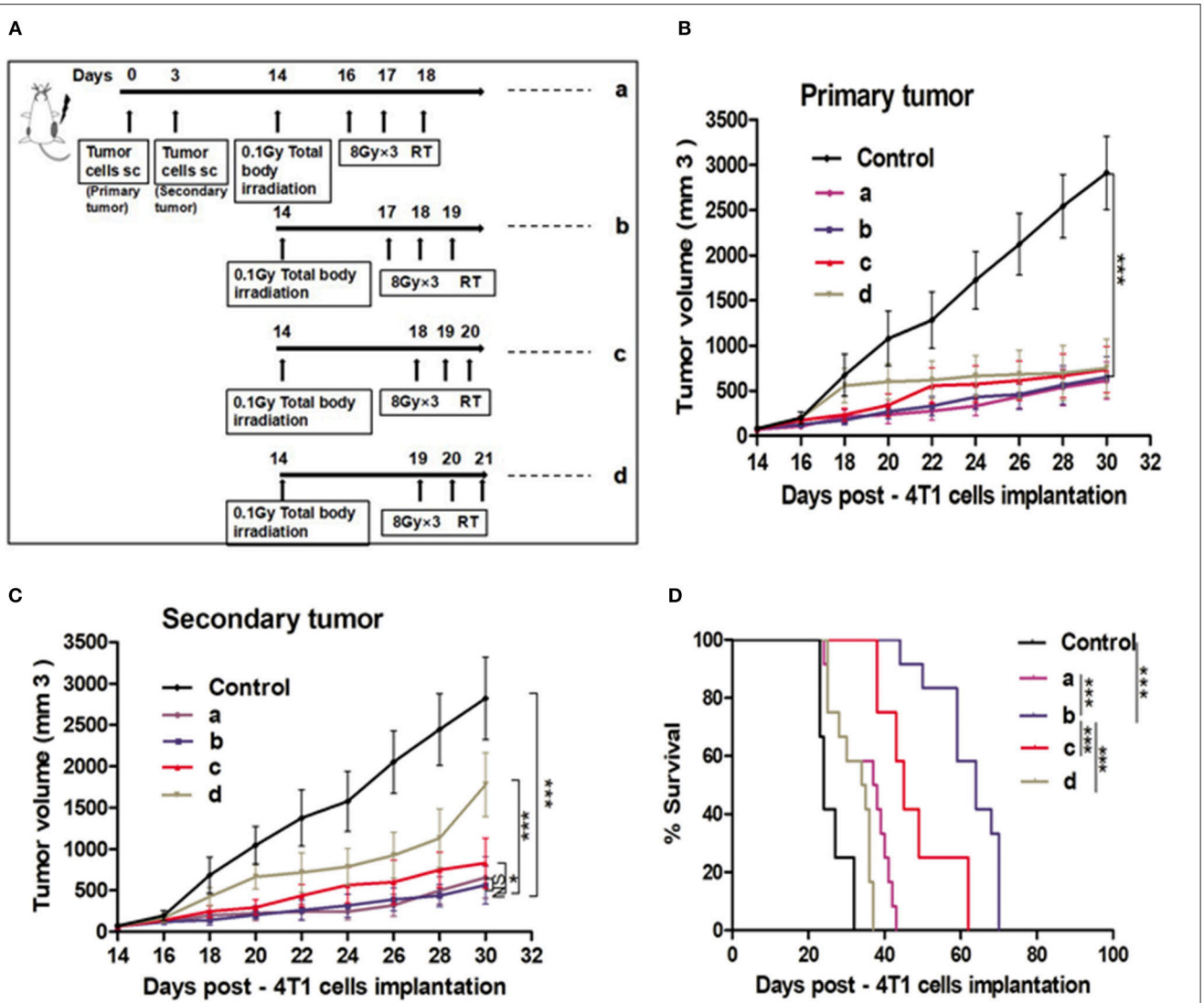

FIGURE 2 | Effect of different time intervals of the H-RT and L-TBI combination therapy in 4T1 tumor-bearing mice. **(A)** Treatment timeline of the 4T1 mammary carcinoma BALB/C mouse model. Tumor growth of primary tumors **(B)** and secondary tumors **(C)** in different experimental groups. **(D)** Overall survival curves of the treatment groups. Immunocompetent mice were injected s.c. with syngeneic 4T1 cells (1×10^5) into the right (primary tumor) and left (secondary tumor), respectively. The 12 mice/group irradiated with H-RT (8 Gy x 3) at 48 h (a), 72 h (b), 96 h (c), and 120 h (d) after L-TBI. Primary and secondary tumor volumes were measured. Data are expressed as mean ± SE (*$P < 0.05$, ***$P < 0.001$, and NS = not significant).

To determine whether DNA damage mediated the primary and secondary tumor growth inhibition, gamma-H2AX staining was performed on the tumor tissue (**Figure 3C**). A significantly higher number of gamma-H2AX positive cells were seen in the primary tumor tissue of the H-RT and H-RT+L-TBI group compared to the L-TBI and control group, while H-RT induced significantly more gamma-H2AX foci compared to H-RT+L-TBI ($P < 0.05$; **Figure 3D**). However, in the secondary tumor, very low level of gamma-H2AX staining was observed in all groups ($P > 0.05$; **Figure 3D**). In conclusion, H-RT resulted in more DNA damage compared to H-RT+L-TBI. Therefore, L-TBI reduced DNA damage caused by H-RT.

Increased Secondary Tumor Infiltration of $CD8^+$ T-Cells After H-RT+L-TBI Is Probably Dependent on IFN-γ

Since irradiation triggers an immune response, we also assessed the infiltration of $CD3^+$ and $CD86^+$ lymphocytes in the primary and secondary tumor tissue (**Figures 4A,C**). The primary tumor of the H-RT and H-RT+L-TBI group showed a higher percentage of $CD3^+$ cells compared to the L-TBI and control group, and no significant difference was observed between H-RT+L-TBI and H-RT group (**Figure 4B**). Furthermore, the percentage of $CD86^+$ cells in the primary tumor was the highest in the H-RT+L-TBI group (**Figure 4D**). A significantly increased number of $CD3^+$ and $CD86^+$ positive cells were seen in

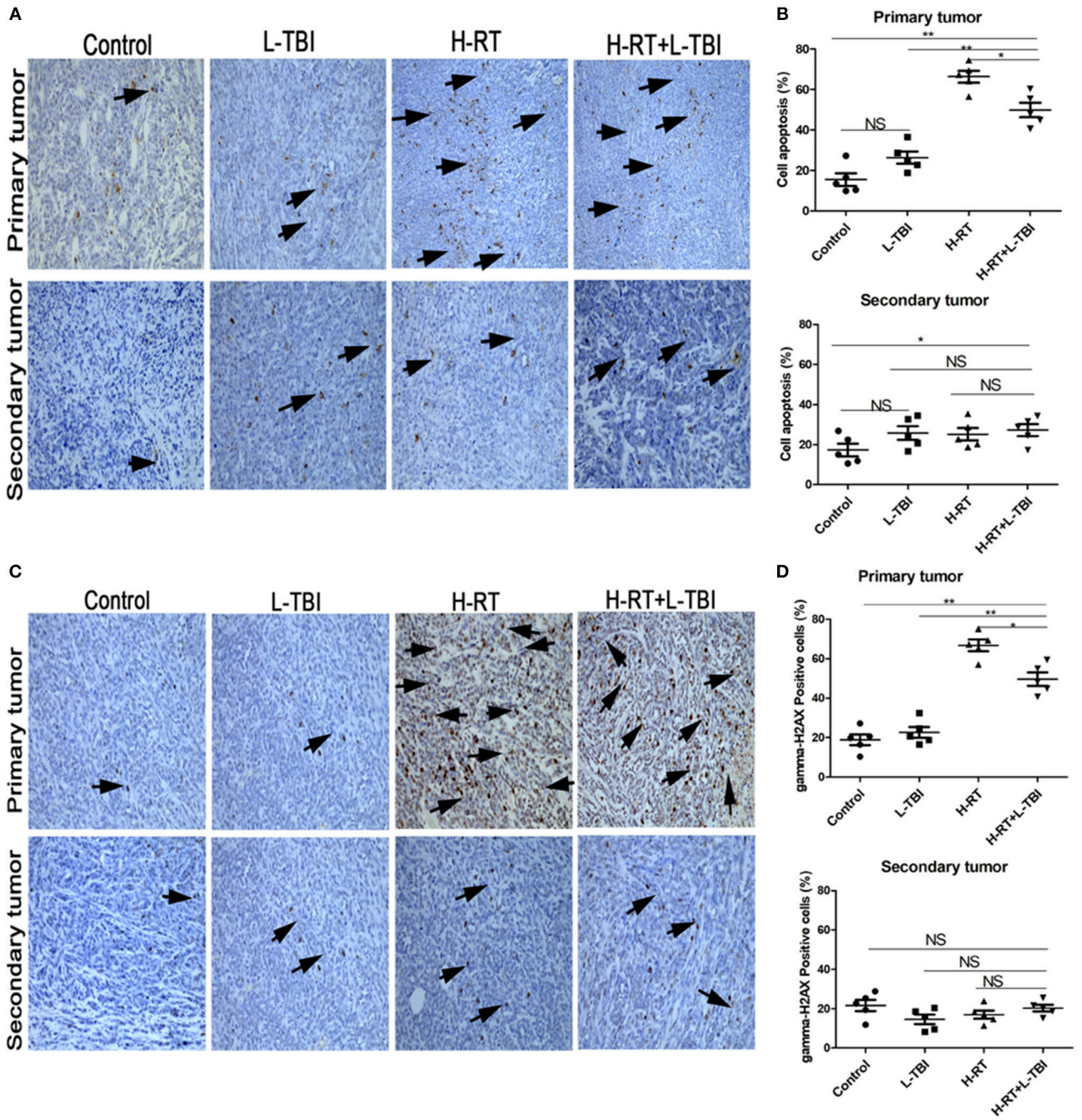

FIGURE 3 | Effect of combination H-RT and L-TBI therapy on apoptosis in 4T1 tumor-bearing tissues. **(A)** Comparison of representative TUNEL IHC-stained in different treatment groups. **(B)** Percentage of TUNEL positive cells in the primary and secondary tumor. **(C)** Representative gamma-H2AX IHC staining image in different treatment groups. **(D)** Percentage of gamma-H2AX positive cells in the primary and secondary tumor. The arrows point to the TUNEL and gamma-H2AX positive cells in the tumor tissue (original magnification ×200). Data are expressed as mean ± SE of 5 mice/group. (*$P < 0.05$, ** $P < 0.01$, and NS = not significant).

the secondary tumor of the H-RT+L-TBI group compared to the other groups (**Figures 4B,D**). Due to activated tumor-associated $CD11c^+$DCs, which higher expression of CD86, we further evaluated the expression of tumor-associated $CD86^+$DCs ($CD45^+CD11b^+CD11c^+CD86^+$) within the tumor tissue by flow cytometry. In the secondary tumor, the number of $CD86^+$DC cells was significantly increased after combination therapy (**Supplementary Figures 3A,B**).

Combination therapy increased activated $CD8^+$ T cells in the secondary tumor. A dramatic increase of infiltrating $CD8^+$

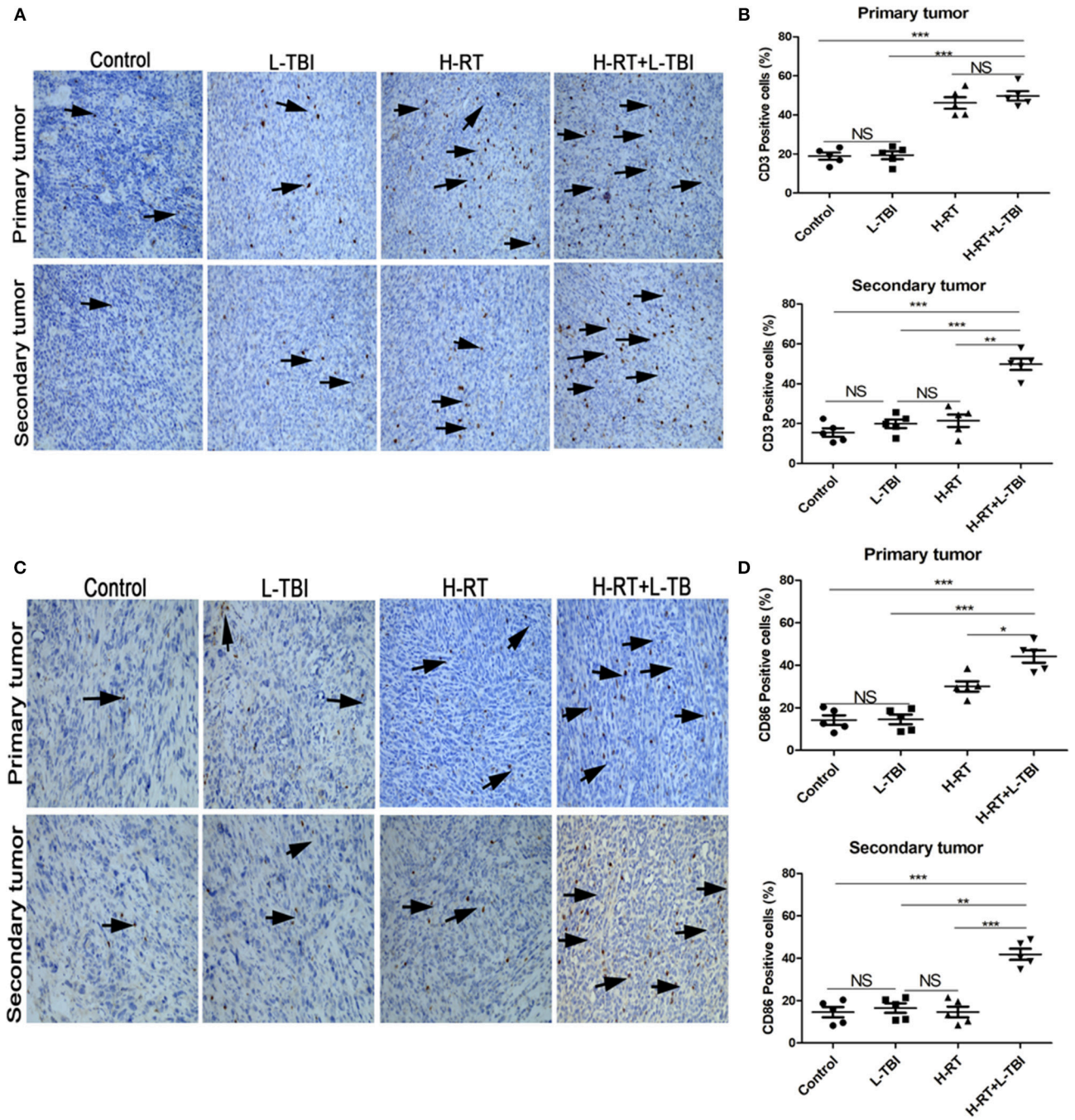

FIGURE 4 | Comparison of $CD3^+$ and $CD86^+$ lymphocytes in different treatment groups. **(A)** Representative images of CD3 IHC in tumor tissues of different treatment groups. **(B)** Percentage of CD3 positive cells in the primary and secondary tumor. **(C)** Representative IHC images of CD86 infiltration in the tumor tissue of different treatment groups. **(D)** Percentage of CD86 positive cells in the primary and secondary tumor. The arrows point the CD3 and Cd86 positive cells in tumor tissues from mice that received different treatments (original magnification ×200). Data are expressed as mean ± SE of 5 mice/group. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and NS = not significant).

T-cells in the secondary tumor of the H-RT+L-TBI group (**Figures 5A,B**), suggesting that cell-mediated immunity was responsible for the SIME of the combined RT. Since tumor-infiltrating $CD8^+$ T-cells induce anti-tumor immune response via cytokines such as IFN-γ (30–32), we assessed the levels of IFN-γ in the mouse serum by ELISA. H-RT+L-TBI led to a significant increase in IFN-γ levels (**Figure 5C**). In order to identify the frequencies of $CD8^+$ IFN-γ, we performed

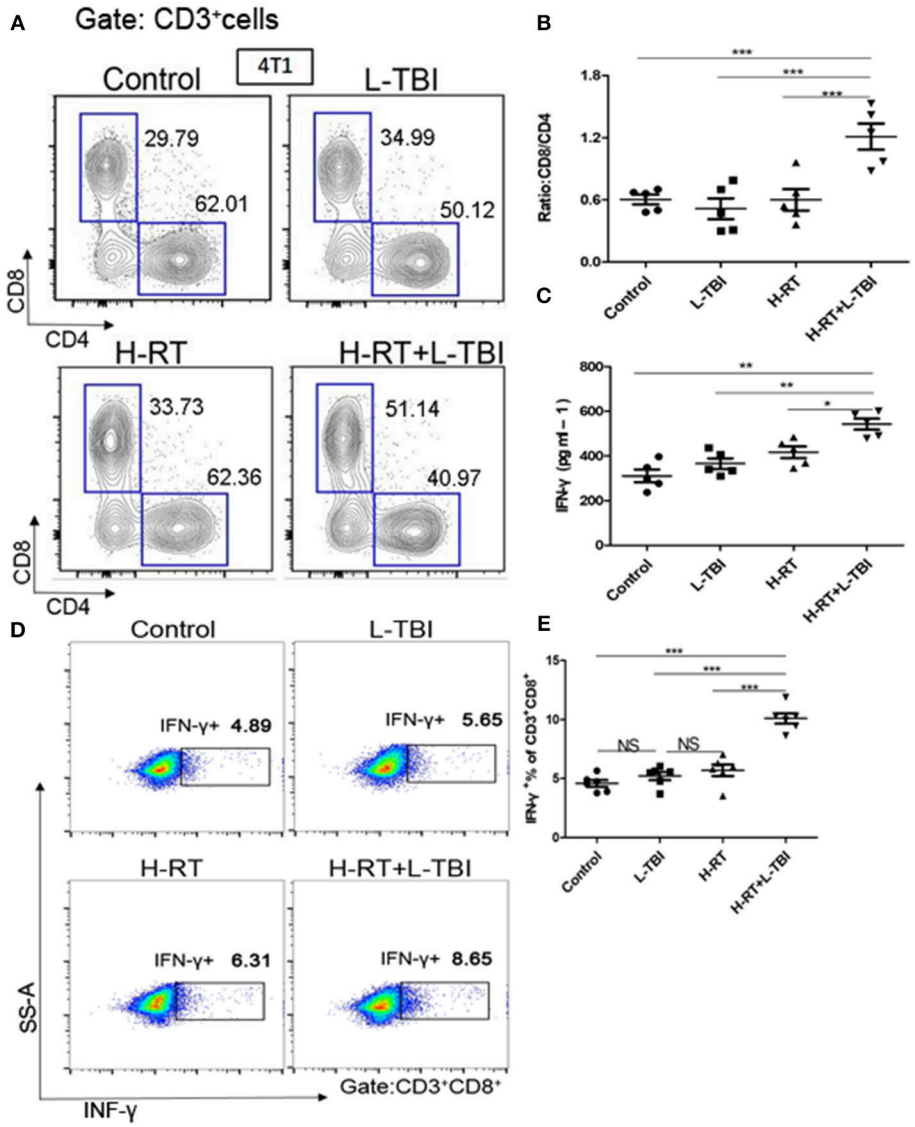

FIGURE 5 | Expression of $CD8^+/CD4^+$ T cells in mice treated with H-RT and L-TBI radiotherapy. **(A)** The frequencies of $CD8^+$ and $CD4^+$ cells induced in the secondary tumor of each group ($n = 5$ mice/group). **(B)** Ratio of $CD8^+/CD4^+$ cells in the secondary tumor of each group ($n = 5$ mice/group). **(C)** ELISA results of the INF-γ levels (pg/ml) in various groups ($n = 5$ mice/group). **(D)** Representative dot plots of $CD3^+CD8^+INF\text{-}\gamma^+$ cells in the secondary tumor tissue of control, L-TBI, H-RT, and H-RT+L-TBI group ($n = 6$ mice/group). **(E)** Comparison plot of $CD3^+CD8^+INF\text{-}\gamma^+$ cells in the secondary tumor tissue of different various groups ($n = 6$ mice/group). The cells were gated on living lymphocytes and then on $CD8^+$ and $CD4^+$ cells and the percentages of $CD3^+CD8^+INF\text{-}\gamma^+$ T-cells were determined by flow cytometry analysis. Data are representative charts or the percentages of individual subjects. The lines indicate median values for each group. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, NS = not significant).

intracellular $CD3^+CD8^+IFN\text{-}\gamma^+$ staining (**Figure 5D**). The combination therapy increased the number of $IFN\text{-}\gamma^+CD8^+$T cells in the secondary tumor (**Figure 5E**), confirming the induction of tumor-specific immune response. Collectively, these results demonstrate that the combined treatment with H-RT and L-TBI induced tumor-specific T cell responses that, when sufficiently strong, could result in complete remission of abscopal tumors.

$CD8^+$T cells were indispensable for SIME with combination therapy. To confirm that tumor-specific $CD8^+$T cells induced by combination therapy contributed to growth suppression of distant metastatic tumors, $CD8^+$ cells were depleted by anti-CD8/Lyt2.1 monoclonal antibody (**Supplementary Figure 4A**). The tumor volume was statistically not significant in either the primary or the secondary tumors between control and H-RT+L-TBI after the percentage of $CD8^+$ T cells decreased (**Supplementary Figures 4B,C**). We confirmed depletion of $CD8^+$ cells using flow cytometry (**Supplementary Figure 4D**). The result showed that the decrease of $CD8^+$ cells ended the suppressive

effects of the combination therapy in both primary and secondary tumors.

H-RT+L-TBI Altered the Immunosuppressive Microenvironment of Secondary Tumors

To further explore the underlying mechanism of the anti-tumor effect of the combined RT, we investigated the secondary tumor microenvironment in the different groups. Large solid tumors can evade anti-tumor immunity partly by inducing an immunosuppressive/tolerogenic microenvironment that includes regulatory cells such as myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory $CD4^+$ T-cells (Tregs) (33–38). Therefore, we analyzed these populations in the tumor tissues by flow cytometry (**Supplementary Figures 5**, **6**). The percentage of the granulocyte (G)-MDSCs was the lowest and that of the monocytic (M)-MDSCs was the highest within the total cell population in the H-RT+L-TBI group, ($P < 0.001$; **Figures 6A,B**). In addition, the tumor of the L-TBI, H-RT and control group showed an increase in the number of G-MDSCs post treatment, while the proportion of M1 cells in the total cell population was similar in all groups (**Figure 6C**), and the proportion of M2 cells was the lowest in the H-RT+L-TBI group (**Figure 6D**). In contrast, treatment with L-TBI or H-RT alone led to an increase in the percentage of M2 cells. Taken together, the combination treatment reversed the immunosuppressive tumor microenvironment (TME) in the distant tumor by reducing the percentage of G-MDSCs and M2 cells. Since eosinophil infiltration is associated with tumor inhibition, we also examined the percentage of Eosinophils (Siglec-F^+Gr1^{lo}) within the tumor tissue (**Supplementary Figure 7**). Both L-TBI and H-RT treatment led to an increase of eosinophil population. Notably, such an expansion was further increased by L-TBI+H-RT combination therapy (**Figure 6E**). Taken together, the combination treatment reversed the immunosuppressive tumor microenvironment (TME) in the distant tumor by reducing the immunosuppressive G-MDSCs and M2 macrophages and increased the percentage of anti-tumor eosinophil population.

H-RT+L-TBI Inhibited 4T1 Lung Metastasis

The murine 4T1 tumor closely resembles human breast cancer both in terms of immunogenicity and metastasis. Since 4T1 cells primarily metastasize to the lungs, we examined the lungs for metastatic nodules and tumor cell infiltration. In addition to considerably less metastatic infiltration (**Figures 7A,B**), H-RT+L-TBI mice had significantly fewer and smaller lung metastatic nodules ($P < 0.001$; **Figure 7C**). Three of the 5 H-RT+L-TBI mice had no visible nodules larger than 2 mm. Thus, the combination therapy significantly inhibited lung metastases, which was most likely the reason for improved survival.

Combination therapy induces SIME. To further confirm the effect of the combination therapy on the systemic immune system, we observed the number of IFN-γ^+CD8^+T cells, G-MDSC, M-MDSC, M1, M2 and Eosinophils in the spleen from different groups (**Supplementary Figure 8**). The combination treatment reduced the percentage of G-MDSCs and M2 cells and increased the percentage of anti-tumor eosinophil and IFN-γ^+CD8^+ T cell population in the spleen (**Figure 7D**). Taken together, the combination treatment induced systemic immune related responses.

4T1 Breast Tumor Responded to Accelerated L-TBI in a Manner Similar to CT26 Tumor

To determine whether the efficacy of H-RT+L-TBI was dependent on the tumor type and/or genetic background of the mice, we established another tumor model in BALB/C mice using the murine CT26 colon carcinoma cells, and subjected them to the same RT protocols (**Figure 8A**). As observed in the 4T1 model, L-TBI did not have any effect on the growth of primary or secondary CT26 tumor, H-RT caused a significant growth delay only in the primary tumor ($P < 0.001$), while the combined treatment significantly inhibited the growth of both primary and secondary tumor (**Figures 8B,C**). Therefore, H-RT+L-TBI triggered a SIME in the CT26 model as well. In addition, a 80-day follow-up showed a significant survival benefit in mice treated with H-RT+L-TBI as compared to H-RT alone ($P < 0.001$; **Figure 8D**). However, we could not observe a survival benefit using L-TBI alone.

DISCUSSION

To the best of our knowledge, this is the first report to demonstrate that H-RT (8 Gy×3) combined with L-TBI (0.1 Gy) enhanced the systemic or abscopal anti-tumor effect of RT, in addition to the other local effects of irradiation. The enhanced therapeutic efficacy was manifested by increased primary tumor regression and decreased metastasis, resulting in improved survival. These findings indicate that this novel combination approach could potentially control metastasis in advanced cancer patients.

We also observed an L-TBI-induced adaptive immune response by sequential H-RT treatment in this mouse model. L-TBI administration before H-RT not only protected the immune system of the mice, but also resulted in a maximum inhibition of primary tumor growth compared to the other groups. Interestingly, when the immune function was impaired by b-L-TBI, the primary tumor could still be inhibited to some extent. The therapeutic effect of simultaneous administration of L-TBI and H-RT was similar to that of b-L-TBI. TUNEL and gamma-H2AX staining showed that H-RT alone and L-TBI+H-RT could both inhibit primary tumor growth by inducing apoptosis and DNA damage, while the combination treatment induced less apoptosis and DNA damage than H-RT alone. Therefore, we speculated that another reason might induce this phenomenon, such as the immune effect. The combination treatment resulted in $CD8^+$ T-cells, IFN-γ^+CD8^+ T cells and DCs infiltration in the non-irradiated tumors as well, resulting in a marked attenuation of tumor growth. However, in L-TBI alone and H-RT alone group, these two treatments did not delay the growth of the non-irradiated tumor. It was the improved immune response that played a key role in L-TBI+H-RT induced abscopal tumor inhibition. This indicated that L-TBI

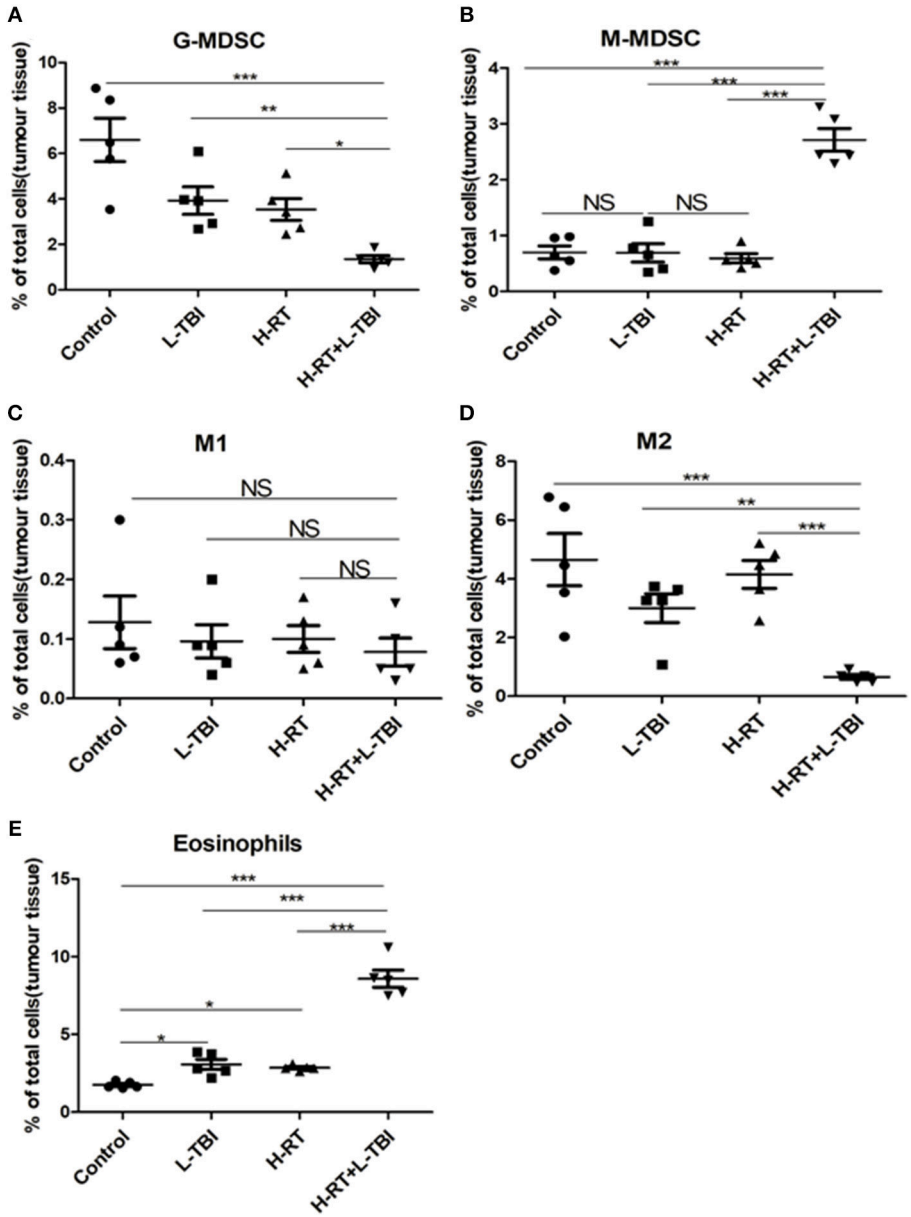

FIGURE 6 | Immunosuppressive microenvironment effects of H-RT and L-TBI combination therapy on 4T1 tumor-bearing mice. The percentage of the G-MDSCs (defined as $CD45^+CD11c^-CD11b^+Ly6G^+Ly6c^{low}$) **(A)**, M-MDSCs (defined as $CD45^+CD11c^-CD11b^+Ly6G^-Ly6c^{hi}$) **(B)**, M1 (defined as $CD45^+CD11b^+F4/80^+CD206^-$) **(C)**, M2 (defined as $CD45^+CD11b^+$ $F4/80^+CD206^+$) **(D)**, and Eosinophils (defined as Siglec-F^+Gr1^{low}) **(E)** were analyzed by flow cytometry analysis. Data are representative charts or the percentages of individual subjects. Data are expressed as mean ± SE of 5 mice/group. The statistical significance of differences was determined by ANOVA. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and NS = not significant).

was the key determinant of SIME via induction of the adaptive immune response. These findings emphasize the importance of the immune response in tumor RT, and might help to promote the application of low dose RT as a novel approach in treating metastasis.

RT alone rarely induces SIME because the tumor microenvironment not only support neoplastic growth and metastasis, but also inhibits host anti-cancer immunity through various strategies (39, 40). Growth of the 4T1 and CT26 tumor is accompanied with increased MDSCs, TAMs and Treg cell population, which have immunosuppressive functions (41–43). MDSCs, especially the G-MDSCs, enable tumor immune escape by inhibiting the activation of T-cells, DCs and NK cells (44). MDSCs also promote tumor metastasis and progression (45, 46). The tumor associated macrophages (TAMs) are classified into the classic/pro-inflammatory M1 and the anti-inflammatory M2 macrophages. M1 are cytotoxic cells that identify tumor antigen through antigen presentation, and kill the tumor cells. M2 inhibit T-cell and NK cell activation and proliferation, and inhibit the anti-tumor immune response by producing anti-inflammatory

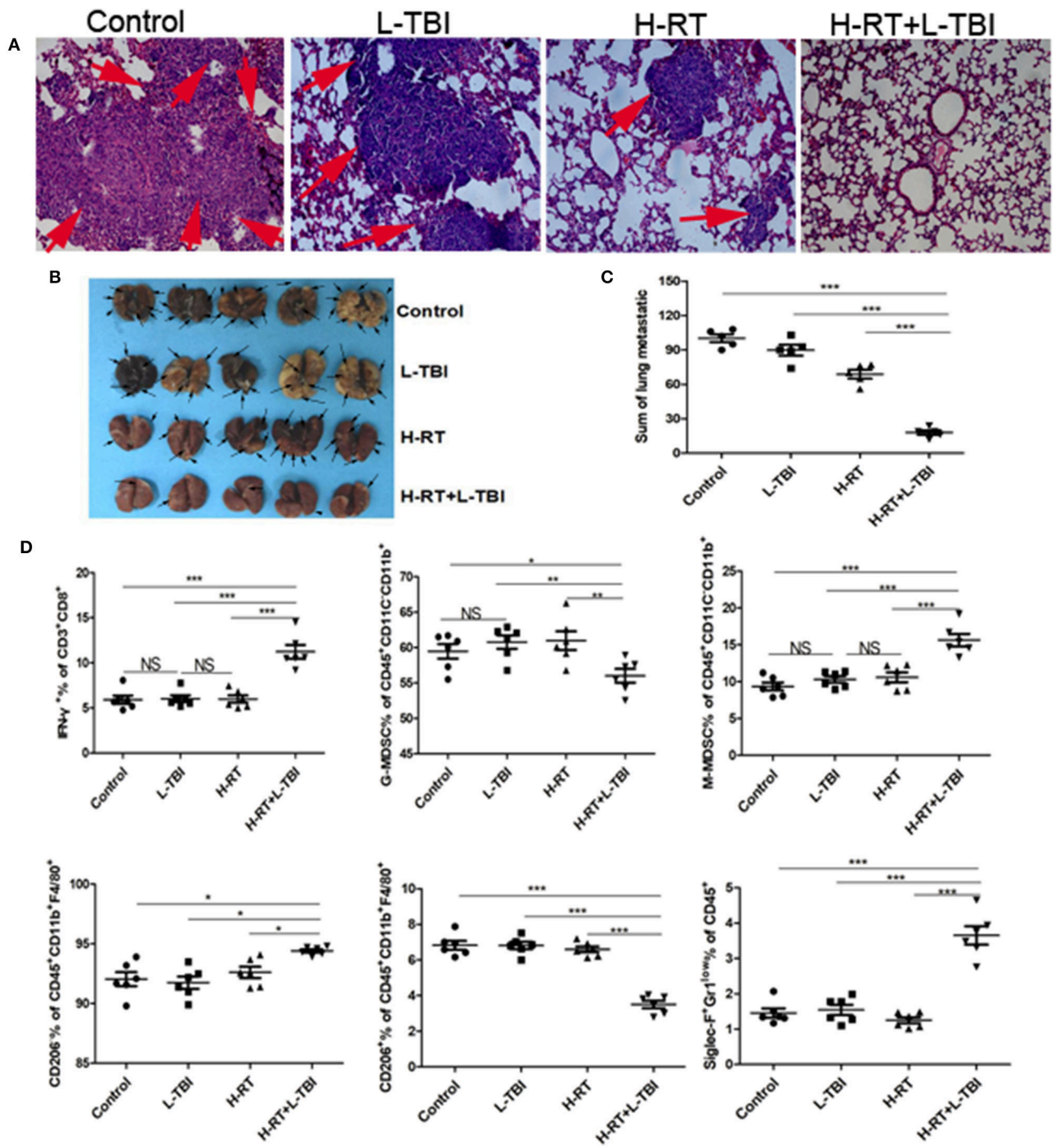

FIGURE 7 | Anti-metastatic effect of the H-RT and L-TBI combination therapy. **(A)** Comparison of representative H&E-stained in lung tissue sections at 24 days after 4T1 cells implantation (original magnification ×100). The arrows point to the metastatic infiltration. **(B)** Representative macroscopic images of the lungs in different groups. The arrows point to the metastatic nodules in the lung ($n = 5$ mice/group). **(C)** Comparison of the lung metastatic nodules between control, L-TBI, H-RT, and H-RT+L-TBI group ($n = 5$ mice/group). **(D)** Frequency of IFN-γ^+ $CD8^+$ T cells ($CD3^+CD8^+$IFN-γ^+), G-MDSC ($CD45^+CD11c^-CD11b^+Ly6G^+Ly6c^{low}$), M-MDSC ($CD45^+CD11c^-CD11b^+Ly6G^-Ly6c^{hi}$), M1 ($CD45^+CD11b^+F4/80^+CD206^-$), M2 ($CD45^+CD11b^+F4/80^+CD206^+$), and Eosinophils ($CD45^+$Siglec-$F^+Gr1^{low}$) in mice spleens ($n = 6$ mice/group). Data are representative charts or the percentages of individual subjects. Data are expressed as mean ± SE. In general, combined therapy of H-RT+L-TBI significantly reduced the number and diameter of lung metastatic nodules ($P < 0.001$). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, NS = not significant).

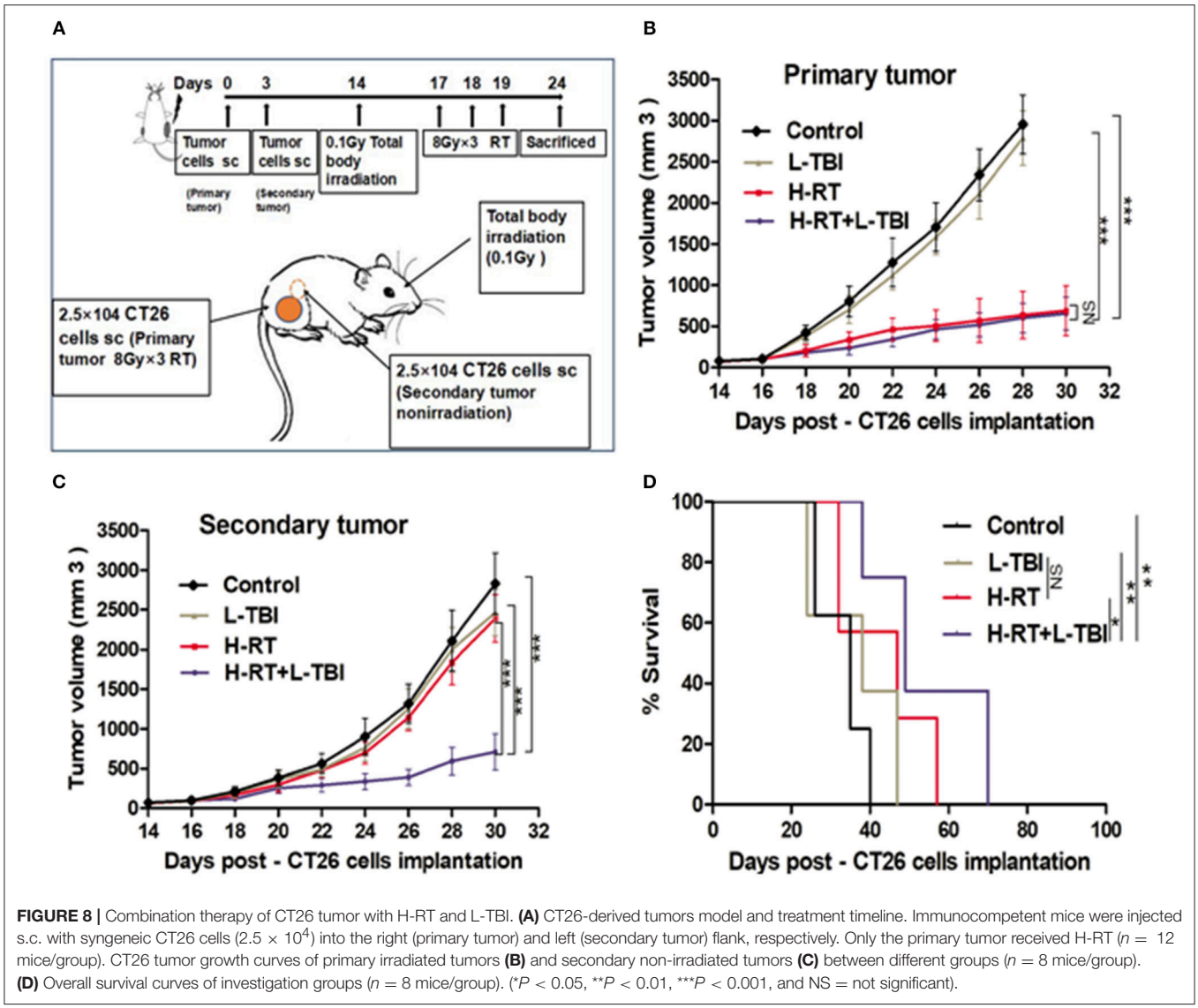

FIGURE 8 | Combination therapy of CT26 tumor with H-RT and L-TBI. **(A)** CT26-derived tumors model and treatment timeline. Immunocompetent mice were injected s.c. with syngeneic CT26 cells (2.5×10^4) into the right (primary tumor) and left (secondary tumor) flank, respectively. Only the primary tumor received H-RT ($n = 12$ mice/group). CT26 tumor growth curves of primary irradiated tumors **(B)** and secondary non-irradiated tumors **(C)** between different groups ($n = 8$ mice/group). **(D)** Overall survival curves of investigation groups ($n = 8$ mice/group). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and NS = not significant).

factors such as IL-10, TGF-β and prostaglandin E2 (43, 44, 47). The balance between immunosuppression and activation ultimately results in a successful tumor elimination. Due to the secondary tumor apparent regression in H-RT+L-TBI in our study, changes in tumor microenvironment in various groups were evaluated by flow cytometry. Previous studies showed that L-TBI alone can inhibit tumor growth and reduce metastasis in experimental mouse models, mainly by reversing the tumor-associated immune suppression (20, 48). In contrast, L-TBI alone had no effect on tumor growth in our study, and did not significantly reduce MDSCs. However, in H-RT+L-TBI group G-MDSCs and M2 were significantly decreased compare to other groups, as shown in **Figure 6**. This could be due to the absence of H-RT induced immunogenic tumor cell death. It is reported that if the total dose is split into large doses and administered over a short period of time (8 Gy×3), they can enhance the immunogenicity (24). As a result, mutual promotion of L-TBI and H-RT activates system anti-tumor immune response.

Demaria et al. showed that abscopal tumor regression was totally dependent on the presence of T cells (49), while Dewan et al. further associated this effect with cytotoxic $CD8^+$ T cells (50). Subsequently, several studies showed that T-cells play a crucial role in abscopal tumor regression (51–53). In our research, we also found that H-RT+L-TBI led to the recruitment and activation of T-cells and DCs in the abscopal tumors. This is consistent with the observation that secretory signals of tumor cells might be central for the recruitment of myeloid cells (54, 55). Similarly, DCs also migrate *in vitro* toward irradiated tumor cells, as seen by the increased expression of the activation marker CD86. In our study, the combination treatment resulted in $CD8^+$ T-cells and DCs infiltration in the non-irradiated tumor as well, resulting in a markedly attenuation of tumor growth. Furthermore, the anti-tumor $CD8^+$ T cells can kill MDSCs via

production of TNF-α, IFN-γ, or the expression of apoptotic FasL, and thereby reduce MDSC tumor infiltration (30–32). In our study, its combination with L-TBI increased the number of total $CD8^+$ and IFN-γ^+ $CD8^+$ T cells at both secondary tumor and spleen (**Figures 5D**, **7D**). The decrease of $CD8^+$cells ended the suppressive effect of the combination therapy at both primary and secondary sites (**Supplementary Figures 4B,C**). Therefore, the remission of both tumors depended on IFN-γ^+ $CD8^+$ T cells. These findings suggested that $CD8^+$ T cells induced by combination therapy were capable of suppressing metastatic and recurrent tumor growth by increasing activated DCs, the level of IFN-γ and the loss of tumor MDSCs. Eosinophil count is increased in a variety of tumors and blood malignancies. The infiltration of eosinophils in the tumor tissue has been associated with improved 5-year survival rate in cancer patients (56). Consistent with this, Eosinophils were significantly increased in the L-TBI+H-RT group, indicating the anti-tumor role of innate immune cells.

Taken together, the combination of H-RT and L-TBI significantly delayed both primary and secondary tumor growth. This approach is more convenient, simpler, and cost-effective compared to RT and IT. Therefore, it is worth studying its underlying mechanisms in greater detail and further testing it in clinical settings. Future optimization of dosing and administration schedule is expected to further increase its efficacy. Our findings highlight the importance of the adaptive immune response in tumor RT and might help to promote the application of low dose RT as a novel approach in treating metastases. In summary, the success of the combination radiation therapy over several weeks in the induction of abscopal remission suggests that $CD8^+$T cell infiltration might be the critical factor in controlling the secondary tumor via altering the tumor microenvironment. In addition, the pre-clinical data presented here on the chronology of immune cell infiltration into tumors should help optimize clinical radio-IT protocols.

AUTHOR CONTRIBUTIONS

JL, JW, JZ, SL, and SF designed the study and wrote the manuscript. JL, JZ, and MW performed and analyzed the experiments. JL assisted in the establishment of the mouse models and data analysis. CH, JY, DL, PW, YuC, and YoC performed the experiments. YoC, SF, JW, and PC provided critical suggestions and discussions throughout the entire study. JW provided the initial idea of the study.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Rationale for Combining Radiotherapy and Immune Checkpoint Inhibition for Patients With Hypoxic Tumors

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In order to compensate for the increased oxygen consumption in growing tumors, tumors need angiogenesis and vasculogenesis to increase the supply. Insufficiency in this process or in the microcirculation leads to hypoxic tumor areas with a significantly reduced pO2, which in turn leads to alterations in the biology of cancer cells as well as in the tumor microenvironment. Cancer cells develop more aggressive phenotypes, stem cell features and are more prone to metastasis formation and migration. In addition, intratumoral hypoxia confers therapy resistance, specifically radioresistance. Reactive oxygen species are crucial in fixing DNA breaks after ionizing radiation. Thus, hypoxic tumor cells show a two- to threefold increase in radioresistance. The microenvironment is enriched with chemokines (e.g., SDF-1) and growth factors (e.g., TGFβ) additionally reducing radiosensitivity. During recent years hypoxia has also been identified as a major factor for immune suppression in the tumor microenvironment. Hypoxic tumors show increased numbers of myeloid derived suppressor cells (MDSCs) as well as regulatory T cells (T_{reg}s) and decreased infiltration and activation of cytotoxic T cells. The combination of radiotherapy with immune checkpoint inhibition is on the rise in the treatment of metastatic cancer patients, but is also tested in multiple curative treatment settings. There is a strong rationale for synergistic effects, such as increased T cell infiltration in irradiated tumors and mitigation of radiation-induced immunosuppressive mechanisms such as PD-L1 upregulation by immune checkpoint inhibition. Given the worse prognosis of patients with hypoxic tumors due to local therapy resistance but also increased rate of distant metastases and the strong immune suppression induced by hypoxia, we hypothesize that the subgroup of patients with hypoxic tumors might be of special interest for combining immune checkpoint inhibition with radiotherapy.

Keywords: immunotherapy, radiotherapy, hypoxia, T cells, cancer, T_{reg}s, immune checkpoint inhibition

INTRODUCTION

Solid tumors are prone to encounter chronic or intermittent hypoxic microenvironment. Hypoxia results from an imbalance of O_2 consumption by the tumor and O_2 delivery by perfused tumor vessels. The latter is limited since tumor vasculogenesis and angiogenesis usually lags behind expansion of tumor mass. In addition, tumor vessels often show aberrant architecture, may have dilated or blind-ending lumina, and lack normal vessel walls (1). As a consequence, increasing intratumoral pressure may compress the vessel lumen accentuating malperfusion of the tumor. Concomitant to insufficient O_2 and nutrient supply, this malperfusion restricts delivery of systemically administered drugs such as chemotherapeutics or immunomodulating antibodies limiting the efficacy of these therapies in hypoxic tumor areas (2). Beyond that, hypoxia attenuates DNA damages conferred by ionizing radiation.

Oxygen tensions vary considerable in areas of diffusion-limited chronic hypoxia or perfusion-limited cycles of intermittent hypoxia and reperfusion, hence, triggering a plethora of different cellular adaptation processes (3). Oxygen-sensing processes comprise stabilization of hypoxia-inducible factor (HIF), nutrient depletion-induced down-regulation of the mTOR (mammalian target of rapamycin) pathway (4), impairment of oxidative folding of proteins in the endoplasmic reticulum and unfolded protein response (5), DNA replication stress (6), or oxygen-dependent remodeling of chromatin (7–9). Adaptations to hypoxia include metabolic reprogramming that maintains structural integrity (10), as well as energy (4), redox (11, 12), pH (13), and lipid (14) homeostasis of the hypoxic tumor cell. These complex adaptations, however, induce tumor heterogeneity and may be accompanied by adoption of more malignant phenotypes (15).

Therefore, intratumoral hypoxia has major implications in cancer biology and treatment resistance. Based on the knowledge of an increased radioresistance of hypoxic cancer cells and impaired prognosis for patients with hypoxic tumors, imaging modalities for hypoxia and treatment strategies to overcome the disadvantages of hypoxia have been developed in radiation oncology. With the rise of immunotherapy in cancer over the recent years and the establishment of immune checkpoint inhibition as a standard treatment for several cancer entities, well-known concepts in cancer and radiobiology have been evaluated for their effects on immune responses to cancer. For hypoxia, pronounced immunosuppressive properties have been described by several groups. This article aims at giving an overview and converging the knowledge about tumor hypoxia in the context of radiotherapy and immunotherapy of cancer patients, hypothesizing that patients with hypoxic cancers might benefit most from combination treatments in curative treatment settings.

HYPOXIA-ASSOCIATED MALIGNANT PROGRESSION OF TUMOR CELLS

Master regulators of metabolic reprogramming under hypoxia are the O_2-sensitive hypoxia-inducible transcription factors (HIFs), the cellular nutrient sensing mTOR and the energy-sensing AMP kinase, as well as the unfolded protein response. They induce downregulation of anabolic metabolism, up-regulation of nutrient import and glycolysis, a switch from oxidative phosphorylation to lactic acid fermentation, up-regulation of acid extrusion pathways such as monocarboxylate transport, adaptation of glutamine metabolisms to maintain fuelling of the citrate pool, alteration of lipid metabolism, attenuation of mitochondrial reactive oxygen species (ROS) formation and/or up-regulation of oxidative defense [for recent reviews (4, 16, 17)].

Metabolic reprogramming may be paralleled by a HIF-regulated phenotypic switch leading to cellular plasticity of tumor and stroma cells which drives tumor heterogeneity. In particular, a hypoxic microenvironment may stimulate in a subset of tumor cells neuroendocrine differentiation, epithelial-mesenchymal transition (EMT) (or neural/glial-mesenchymal transition in brain tumors) or induction of cancer stem (-like)/tumor initiating cells (CSCs) (11). Signaling cascades that induce CSC phenotypes in distinct hypoxic niches are probably triggered by ROS that are formed during the metabolic adaptation to hypoxia (**Figure 1**). Notably, EMT and CSC induction seems to be highly interrelated and involve HIF signaling [for review see (18, 19)]. Importantly, EMT and upregulation of CSC properties are accompanied by a change from a "grow" to a "go" phenotype. As a consequence, hypoxic tumors are at higher risk of tissue infiltration and metastasis (18, 19).

Moreover, hypoxia and in particular ROS formation during reoxygenation have been shown to favor genetic instability and to increase mutagenesis in tumors by induction of DNA damage and/or deregulation of DNA damage response and apoptotic pathways fostering malignant progression of tumor cells (10, 11). Notably, genetic instability has been associated with response to immune checkpoint inhibition on the one hand and decreased tumor immunogenicity by formation of immune-evasive subclones on the other hand (20, 21). Beyond malignant progression and immune evasion, hypoxia confers resistance to chemo- (2) and radiation therapy as described in the next paragraphs.

RADIORESISTANCE OF HYPOXIC TUMOR CELLS

About half of all cancer patients undergo radiation therapy often applied in fractionated regimens. Conceptually, a radiation dose of 1 Gy with high energy photons causes about 20 DNA double strand breaks (DSBs) per nucleus on average in normoxic tissue (22). Nuclear DNA DSBs have been proposed to be most hazardous for the cell since when left unrepaired they inevitably provoke chromosome aberrations in mitosis. Tumors are thought to become eradicated if the quantity of radiation induced DSBs exceeds the capacity of DNA DSB repair by non-homologous end joining in G1 phase of cell cycles and additional homologous recombination in S and G2 phase (23). Hypoxia has turned out to be a negative predictive factor for the response to radiation therapy (24) due to lowering the efficacy

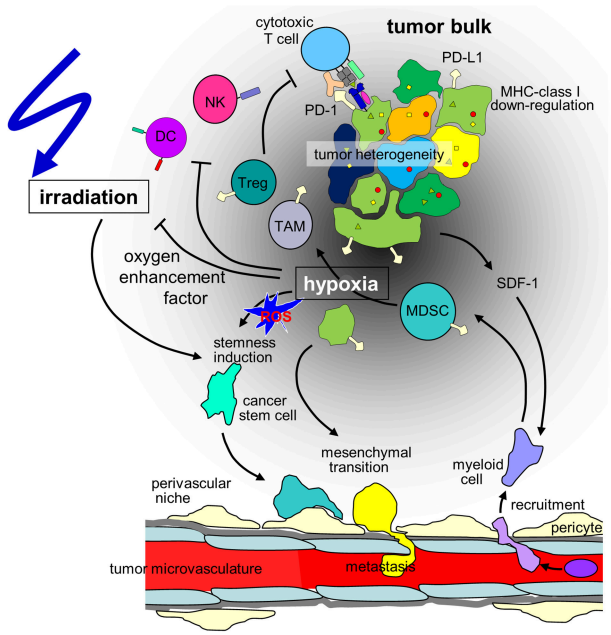

FIGURE 1 | Hypothesis of the influence of hypoxia on cancer cells and the immune microenvironment in the context of radiotherapy of solid tumors. Hypoxia may stimulate in a subset of tumor cells mesenchymal transition and metastasis or induction of cancer stem(-like) cells. The radioresistant phenotype of the latter together with the decline in radiation-induced DNA damage with decrease in oxygen tension (oxygen enhancement factor) contribute to the radioresistance of hypoxic tumors. Moreover, hypoxia/radiation-induced migration may lower locoregional tumor control by radiotherapy. In addition, tumor hypoxia recruits immunosuppressive cell types such as regulatory T cells (T_{reg}s) and myeloid derived suppressor cells (MDSCs) that mature to M2-polarized tumor associated macrophages (TAMs) via stromal cell-derived factor-1 (SDF-1) chemokine signaling. Dendritic cell (DC) function is modulated to T_H2 polarized immune responses which suppress anti-tumor immunity. Finally, hypoxia may induce downregulation of MHC class-I molecules and Natural Killer (NK) cell-activating ligands and upregulation of programmed death-ligand-1 (PD-L1) on tumor cells. (ROS: reactive oxygen species).

of ionizing radiation by a factor of 2–3. Mechanistically, this so-called oxygen enhancement ratio (OER) most probably reflects three processes in irradiated cells: O_2 fixation of DNA damages, O_2-dependent formation of ROS by the mitochondria, as well as hypoxia-induced acquisition of a radioresistant phenotype.

O_2 Fixation of DNA Damages

Radiation therapy damages cells by ionization of molecules. Among those, H_2O with the far highest concentration (more than 50 M) of all molecules in a cell absorbs the largest fraction of the radiation energy. Energy transfer to H_2O leads to formation of hydrogen ($^{\bullet}H$) and hydroxyl radicals ($^{\bullet}OH$) in a process referred to as radiolysis of H_2O. Formation of $^{\bullet}H$ radicals has been proposed to confer reductive stress to the irradiated cells (25) while the high reactivity and low lifetime of $^{\bullet}OH$ radicals may remove hydrogen atoms from neighboring macromolecules resulting in formation of macromolecule radicals. With a lower stochastic probability formation of macromolecule radicals also occurs upon direct absorption of radiation energy by the macromolecules. Now, the O_2 tension comes into the play. Under normoxia, at high O_2 partial pressure in the cell, the radical atom within the macromolecule has been suggested to become oxidized which may be associated with the cleavage of molecular bonds of the macromolecule. Under hypoxia, however, at low cellular O_2 tension and reductive cellular redox state (which comprises a high ratio between reduced and oxidized glutathione and a high capacity of oxidative defense), macromolecule radicals have been proposed to become "repaired" chemically (**Figure 1**).

Thus, a high O_2 tension may evoke DNA strand breaks whenever radiation-induced radical formation occurs within the phosphate deoxyribose backbone of the DNA. If radical formation concurs in close vicinity in both anti-parallel DNA strands, high oxygen pressure promotes formation of DNA DSBs. This so-called oxygen fixation hypothesis which was developed in the late 1950's, however, explains only insufficiently the oxygen enhancement ratio in radiation therapy. It neither considers hypoxia-mediated effects on DNA repair (26) nor radiation-induced secondary cell damages by mitochondrial ROS formation. The latter are also highly O_2-dependent as discussed in the following paragraphs.

Mitochondrial ROS Formation

Early microbeam technologies which allow irradiation of cellular substructures provided strong evidence for a much higher efficacy of ionizing radiation when the nucleus was targeted as compared to selective irradiation of the cytoplasm (27). Therefore, as central dogma of radiation therapy, the genotoxic effects of radiation has been attributed for many years to an interaction between ionizing radiation and the nucleus as primary mechanism (25). Notwithstanding, more recent work, however, suggests that nuclear DNA damage does not exclusively require irradiation of the nucleus and even can be observed in unirradiated bystander cells [for review see (28)]. Notably, inhibiting ROS formation reportedly prevents nuclear DNA damage of the beam-targeted and the bystander cells (29) indicating ROS mediated spreading of the absorbed radiation energy. Furthermore, experiments comparing cells with mitochondrial DNA-proficient (ρ^+) and -deficient (ρ^0) mitochondria strongly suggest the involvement of mitochondrial electron transport chain in genotoxic damage mediated by radiation (29–33). Most importantly, the fraction of mitochondrial ROS formation-dependent DNA damage has been proposed to increase with O_2 tension (34).

Mechanistically, ionizing radiation reportedly increase intracellular free Ca^{2+} concentration in several tumor entities such as lymphoma (35), leukemia (36, 37), or glioblastoma (38). Intracellular Ca^{2+} buffering experiments demonstrated that Ca^{2+}, in turn, stimulates in the presence of O_2 mitochondrial ROS formation (30) probably in concert with the transient energy crises observed in irradiated cells (39, 40). Both, low ATP/ADP ratios and high Ca^{2+} concentrations disinhibit mitochondrial electron transport chain, leading to hyperpolarization of the inner mitochondrial membrane potential $\Delta\Psi_m$ which is directly linked to superoxide anion ($^{\bullet}O_2^-$) formation by

slippage of single electrons to O_2 [for review see (41)]. Ca^{2+}-mediated $^{\bullet}O_2^-$ formation by the electron transport chain, in turn, provokes mitochondrial membrane permeability transition and eventually dissipation of $\Delta\Psi_m$ and mitochondrial disintegration (42). Of note, radiation-stimulated permeability transition of few affected mitochondria and consequent local release of mitochondrial Ca^{2+} has been proposed to stimulate Ca^{2+}-overflow, ROS formation, and Ca^{2+} re-release of adjacent mitochondria, thereby propagating radiation-induced mitochondrial ROS formation through the mitochondrial network in a spatial-temporal manner (30).

As a matter of fact, inhibitors of mitochondrial permeability transition blocked radiation-induced mitochondrial ROS formation (30) and in some but not all cell lines O_2-dependent radiosensitivity (43). Combined, these observations strongly suggest that O_2 tension-dependent mitochondrial ROS formation and adjunct DNA damage contribute significantly to the OER phenomenon. Beyond stimulation of mitochondrial ROS formation, radiation has been reported to up-regulate activity of uncoupling proteins (UCPs) in the inner mitochondrial membrane (34). UCPs shortcircuit $\Delta\Psi_m$ thereby directly counteracting radiation-stimulated mitochondrial ROS formation [for review see (41)]. As described in the next paragraph, adaptation to hypoxia may also involve up-regulation of mitochondrial uncoupling.

Radioresistant Phenotypes Induced by Hypoxia

Adaptation of cells to hypoxia has been described for highly oxidative phosphorylation-dependent normal proximal tubule cells. By repeatedly subjecting these cells to hypoxia and re-oxygenation cycles over weeks strong up-regulation of oxidative defense and mitochondrial uncoupling was induced. Besides diminishing reoxygenation-induced $\Delta\Psi_m$ hyperpolarization, $^{\bullet}O_2^-$ formation, and consecutive cell damage, mitochondrial uncoupling confers cross-resistance to ionizing radiation (44). Importantly, tumors such as proximal tubule-derived renal clear cell carcinoma show high upregulation of mitochondrial uncoupling proteins (44) pointing to hypoxia-induced mitochondrial uncoupling as one potential mechanism of induced resistance *in vivo*. Similarly, cyclic hypoxia and reoxygenation reportedly upregulates *in vitro* the mitochondrial citrate carrier SLC25A1 in cancer cell lines that contributes to an increased radioresistance-conferring oxidative defense (11). Beyond that, further metabolic pathways up-regulated in hypoxic cells such as glutamine-dependent glutathione formation (12) or glycolysis-associated pyruvate accumulation [for review see (4)] result in increased capacity of radical scavenging that may confer radioresistance.

Moreover, the above mentioned hypoxia-triggered induction/selection of CSCs reportedly associates with an increased intrinsic radioresistance (**Figure 1**). CSCs have been supposed to express higher oxidative defense, pre-activated and highly efficient DNA repair and anti-apoptotic pathways rendering them less vulnerable to ionizing radiation [for review see (18)]. Beyond that, CSCs may overexpress certain Ca^{2+} and electrosignaling pathways that improve stress response upon irradiation (45, 46) as demonstrated for the mesenchymal subpopulation of glioblastoma stem cells (47).

Finally, at least in theory, the above mentioned hypoxia-induced migratory phenotype of tumor cells might limit efficacy of radiotherapy in fractionated regimens. One might speculate that highly migratory cells evade from the target volume covered by the radiation beam. In glioblastoma, stabilization of HIF-1α stimulates auto/paracrine SDF-1 (CXCL12)/CXCR4-mediated chemotaxis the programming of which strongly depends on electrosignaling as one key regulator of chemotaxis (48). Likewise, ionizing radiation stimulates the same pathways also by activating the HIF-1α/SDF-1/CXCR4 axis (48). It is, therefore, tempting to speculate that hypoxia and radiation cooperate in stimulating hypermigration during fractionated radiotherapy. Evidence, however, that hypermigration indeed has any relevance for local tumor control by radiation therapy in the clinical setting is missing. Nevertheless, tumor hypoxia is a severe obstacle of radiation therapy. The next section deals with concepts of visualization and effective treatment of hypoxic tumors for radiation therapy.

TREATMENT MODIFICATIONS TARGETING HYPOXIA IN RADIATION ONCOLOGY

Cellular effects on radiation-response under hypoxia *in vitro* (49, 50) cannot be directly transferred to xenografts *in vivo* and tumors in patients. The OER (determined to be 2–3 *in vitro* (51), as described above) seems to be lower *in vivo*. This is on the one hand due to the fact that parts of the tumor volume are sufficiently oxygenated since oxygen tension is decreasing only gradually around perfused blood vessels (52–54). On the other hand, depending on the tumor entity, decrease of the bulk tumor mass during fractionated radiation may lead to tumor reoxygenation (55, 56). Extensive research on the tumor microenvironment (hypoxia, vasculature, necrosis and metabolism) and its impact on radioresistance has been done in *xeno*graft models for head and neck squamous cell carcinoma (HNSCC), glioblastoma, non-small cell lung cancer (NSLCL) and colorectal carcinoma and sarcoma cell lines (51, 57–61). *In vivo* models were also used to show the predictive value of functional tumor imaging with hypoxia sensitive tracers for positron emission tomography (PET) imaging (62–64). Based on hypoxia imaging, different approaches including dose escalation, HIF1α-inhibitors, hypoxia activated prodrugs and hyperbaric oxygen (HBO) or carbogen breathing were studied to overcome treatment resistance with promising results (65–67).

In a clinical setting of HNSCC and cervix cancer, an association between oxygen tension and radioresistance could be shown. For 35 patients with locally advanced HNSCC invasive pO2-measurement with oxygen sensitive electrodes with >15% of pO2 values below 2.5 mm HG, was associated with reduced local control at 2 years (68). In a prognostic validation study as well as in a multicenter study with more than 390 patients, the

results could be confirmed (69). There are matching results of worse prognosis for patients with cervical cancer with decreased pO2 values before radiotherapy (70, 71). With advances in imaging methods, non-invasive measurement of hypoxia, based on positron emission tomography (PET) with different hypoxia specific tracers, e.g., [^{18}F]fluoromisonidazole (FMISO), [^{18}F]fluoroazomycin arabinoside (F-AZA), [^{18}F]fluortanidazole (HX4) and [^{64}Cu]diacetyl-bis(N^4-methylthiosemicarbazone (Cu-ATSM), and magnetic resonance imaging (MRI) were established and could be correlated to outcome in HNSCC, cervical cancer and NSCLC (72–81). Hypoxia imaging is also closely related to other functional imaging modalities such as FDG-PET or functional MRI (82–84). Based on this evidence, there were major efforts to target hypoxia in the curative setting of radiotherapy during the last decades.

In parallel to the findings of hypoxia as a common phenomenon in solid tumors in the fifties, efforts were started to increase tumor oxygenation by HBO treatment under 2 to 4 atmospheres (85). Due to small numbers of patients in these trials and difficulties of irradiation in pressure chambers, the promising results could not advance into clinical use. Inhalation of carbogen with nicotinamide was the topic of a large phase III trial, which showed decreased regional failure (86). Another approach is the use of hypoxia specific agents like nitroimidazoles. In a trial of The Danish Head and Neck Cancer group (DAHANCA 5) the addition of nimorazole to standard treatment showed an increase in locoregional control (LRC) as well as disease-free survival (DFS) for patients with increased osteopontin levels (87) or a specific gene expression profile (88), both linked to hypoxia. Since then nimorazole is standard of care in Denmark during radiotherapy of HNSCC. To evaluate this combined approach, a large European Organization for Research and Treatment of Cancer (EORTC) phase III trial was conducted with results pending (NCT01880359). With the possibilities of modern radiotherapy techniques like intensity modulated radiotherapy (IMRT) and image-guided radiotherapy (IGRT), first trials with dose escalation based on [^{18}F]fluorodeoxyglucose (FDG) or FMISO are conducted with conflicting results for toxicity and local control data pending (89, 90). A large meta-analysis of all studies with hypoxic modification in HNSCC of 32 trials with more than 4,800 patients included, showed a significant survival benefit of the intervention vs. the control group (91). In a phase II trial an increased radiation dose could not overcome the worse prognosis of hypoxic NSCLC (92). In summary, the big hopes of targeting hypoxia could not be translated directly into the clinic (93).

IMMUNOSUPPRESSION IN THE HYPOXIC TUMOR MICROENVIRONMENT

Hypoxia in the tumor microenvironment influences the interaction between cancers and the immune system on all levels. Cancer cells regulate the interaction surface with immune cells, the cytokine microenvironment is altered, and immune cell function is reshaped.

Immune-Relevant Changes in Cancer Cells Under Hypoxia

Cancer cells under hypoxic conditions show a downregulation of MHC class-I molecules (94) (**Figure 1**), which are crucial for the immune recognition and immune mediated lysis of tumor cells (95). Several immune checkpoints are upregulated in hypoxic conditions. HIF-1α mediates the upregulation of HLA-G (96), which has been described as immunosuppressive (97, 98). In pancreatic cancer HLA-G is a negative prognostic marker, and downregulation of ILT-2 (the receptor of HLA-G) in immune cells activates anti-tumor immunity (99). In addition, hypoxia induces upregulation of CTLA-4 and PD-L1 on tumor cells via HIF-1α in several different mouse and human tumor cell lines (**Figure 1**). Enhanced PD-L1 abundance could be linked to a HIF-1α binding site in the PD-L1 promotor (100). In renal cell carcinoma elevated PD-L1 levels were correlated with HIF1α levels linked to impaired function of the Von-Hippel-Lindau (VHL) protein (101). In patient samples, HIF1α genes and expression also correlated with PD-L1 expression. The functional link of PD-L1 expression and HIF1α was established by knock-down experiments (101, 102). In hepatocellular carcinoma patient samples PD-L1 expression also was linked to hypoxia and showed prognostic value (103).

Hypoxia has also been linked to downregulation of DNA damage response proteins such as RAD51 in prostate cancer (104), and RAD51 and BRCA1 in breast cancer (105), respectively. BRCA1 downregulation has been shown to be epigenetically regulated in different cancer cell lines (106). Impaired DNA-double-strand-break repair under hypoxic condition might lead to a higher mutation rates and more malignant phenotypes (104). On the other hand, more mutations might also lead to more neoantigens possibly supporting tumor-immune responses. Intriguingly, mutational burden is one of the most promising predictive factor for treatment with immune-checkpoint-inhibition (107). In concordance, the antigenic landscape of prostate cancer is modified by the applied oxygen tension (108) *in vitro*.

Hypoxic Immune Microenvironment

The immune microenvironment of tumors also undergoes profound changes with the development of intratumoral hypoxia. Hypoxia induced downregulation of ADAM-10 (109) and upregulation of CCL28 (110, 111) and IL-10 (112) all lead to immunosuppression via shedding of MHC class I chain-related molecule A (MICA) and hampering cytolytic action of immune cells, T_{reg} recruitment and enhancing suppressor MDSc, respectively. Hampered anti-tumor immunity in hypoxic tumors is mainly mediated by adenosine receptor signaling (113). Adenosine is formed by hydrolysis of tumor cell-derived ATP in the extracellular space (114). Adenosine receptors are a direct target of HIF1α and have been reported to enable stem (like) cell enrichment in breast cancer (115). Clinical data as well as *in vivo* data in an autochthonous mouse model linked adenosine A2A receptor with carcinogenesis

and immune resistance of HNSCC (116). Tumor reactive $CD8^+$ cells express A2A receptors and show enhanced activity upon downregulation or blockade thereof (117). Oral A2A receptor inhibitors have been developed and tested preclinically (118). *Ex vivo* testing suggests synergistic effects with immune checkpoint blockade (119).

Consequently, several cell subsets required for efficient anti-cancer immune responses have been described to be impaired or inhibited by hypoxia. Mechanisms of the innate immune system, such as NK cell-mediated killing of cancer cells is disturbed due to downregulation of the respective activating ligands on tumor cells (120). Concerning adaptive immunity, several critical steps are hampered under hypoxic conditions. Dendritic cell function is modulated to T_H2 polarized immune responses, consequently, T cells primed under hypoxia preferably are T_H2-polarized and thus suppress anti-tumor immunity (121) (**Figure 1**). At the same time, the development of anti-cancer T_H1 cells is inhibited (122) and $CD8^+$ effector T cells are inhibited in their proliferative activity under hypoxia, possibly via IL-10 (112).

Regulatory T Cells

In addition, major immunosuppressive cell types in the tumor microenvironment are upregulated under hypoxic conditions, such as regulatory T cells (T_{reg}s) and myeloid derived suppressor cells (MDSCs) and tumor associated macrophages (TAMs) (**Figure 1**). T_{reg}s have been described as major players in cancer immunosuppression by inhibiting effector T cells and fostering angiogenesis (123) and have been described to be increased in hypoxic tumors (124). Several mechanisms for this phenomenon have been proposed. In gastric cancer, FoxP3 (as a marker for T_{reg}s) is strongly associated with HIF-1α and TGFβ and acts as negative prognostic factor. *In vitro*, TGFβ blockade diminished the T_{reg} induction under hypoxic conditions (125). This has been linked to hypoxia-induced NANOG expression (126). SDF-1/CXCR4 signaling induced by hypoxia also has been linked to T_{reg} recruitment (127). Another major mechanism described for ovarian as well as for liver cancer is the induction of CCL28. In ovarian cancer CCL28 recruits T_{reg}s and leads to accelerated tumor growth *in vitro* as well as in orthotopic models of intraperitoneal tumors (110). These findings have been confirmed for hepatocellular carcinoma (111). The interplay of these different factors for T_{reg} accumulation has not been clarified yet.

Myeloid-Derived Suppressor Cells (MDSCs) and Tumor Associated Macrophages (TAMs)

Hypoxia leads to the recruitment of MDSCs (128) as well as their accumulation (129) in a hepatocellular carcinoma model as well as in gliomas (130). In the tumor microenvironment MDSCs differentiate to macrophages (131). In hypoxia, macrophages are preferably polarized to the immunosuppressive M2 phenotype (132, 133). M2 macrophages support tumor growth directly (134–136) and simultaneously prevent immune destruction (137, 138). Interestingly, myeloid cells have also been described to be involved in the formation of pre-metastatic niches in secondary organs (139, 140).

RATIONALE FOR COMBINING RADIOTHERAPY AND IMMUNOTHERAPY

Immune Checkpoint Inhibition for Cancer Therapy

Immune checkpoint inhibition (ICI) gained increasing interest as a new paradigm in cancer treatment as several encouraging clinical trials were published (141–143). However, in some other studies, ICI showed less promising results (144, 145). There is still a considerable number of patients who do not response at all, solely achieve a partial response or relapse in spite of notable initial response, yet. Several other immunotherapy approaches are being developed (146) [such as cytokine based therapy (147–149) or vaccines (150, 151)], however, the clinical development is most advanced for CTLA-4 and PD-1/PD-L1 blockade.

As reviewed in Wolchok et al. (152) CTLA-4 has been identified as a negative regulator of T-cell activation binding to the B7 protein on antigen presenting cells. This interaction prevents the binding of CD28 to B7, a necessary costimulatory signal for T cell activation following the recognition of respective antigens by the T-cell-receptor representing a very early step in the immune cascade (153). CTLA-4 deficient mice show massive lymphoproliferation, multi-organ tissue destruction and early letality (154). Blockade of CTLA-4 has been shown to induce T cell activation (155, 156) and anti-tumor immunity in preclinical models (157). These findings translated into clinical benefits and long-term cancer control first in patients with malignant melanoma (158, 159). A recent compilation of finished and ongoing clinical trial shows the application of CTLA-4 blockade in numerous cancer entities, therapeutic settings and combinatorial approaches (160).

In clinical cancer therapy, blockade of the PD-1/PD-L1 axis has become even more prominent as indicated by the numbers of ongoing clinical trials (160). The inhibitory effect of PD-1/PD-L1 interaction is predominant during the inflammatory phase in peripheral tissues (161). Similar to CTLA-4, mice deficient for PD-1 developed severe autoimmune symptoms indicating an inhibitory function of PD-1 on immune activation (162). It was soon linked to immune-evasion of tumors as cancer cells show a high expression of PD-L1 and thus directly inhibit T-cell activation in the tumor microenvironment (163). PD-1 also plays a major role in T-cell exhaustion in chronic inflammatory processes and cancer (164). After initial signs of safety and activity of blocking PD-1 for cancer treatment (165), numerous randomized trials have shown clinical benefit of single-agent or combined treatment using PD-1 or PD-L1 antibodies (166).

Immune Effects of Radiation

Rare abscopal effects (response of distant, non-irradiated lesions) in irradiated patients have been described many years ago

[reviewed in (167)], but the interaction of radiation and tumor specific immune responses was increasingly understood later on (168).

In addition to direct cytotoxic effects of radiotherapy and reoxygenation in solid tumors during fractionated radiation, local irradiation also affects the tumor immune microenvironment. In contrast to the predominant perception of radiotherapy being basically immunosuppressive, several mechanisms have been identified how irradiation might lead to better anti-tumor immune responses as summarized by Demaria and Formenti (169). Radiation influences every step of the "cancer immunity cycle" (170). The cancer cell death induced by irradiation does not only lead to antigen release, but has been characterized as immunogenic cell death characterized by the release of danger signals (171, 172) such as membranous calreticulin exposure and release of HMGB1 and ATP into the extracellular space leading to activation of the innate immune system (173, 174) (**Figure 2**). Radiation induces upregulation of MHC-I complexes on cancer cells (175) and priming and maturation of antigen-presenting cells (176, 177). After traveling to draining lymph nodes, these antigen-presenting cells are able to prime T cells specific for tumor associated antigens (178). The primed and activated effector T cells show increased infiltration into irradiated tumors (179–181). In addition to the effects on T cell based anti-tumor immune responses, irradiation is able to repolarize macrophages to a tumor inhibiting M1-subtype (182) and activate natural killer cells (183) (**Figure 2**).

On the other hand (and explaining the scarce clinical evidence for anti-tumor immune induction by radiotherapy alone) irradiation induces immunosuppressive mechanisms in solid tumors (184). One major mechanism is the upregulation of PD-L1 in irradiated tumors (185–187). Even combined treatment of CTLA-4 blockade with irradiation led to upregulated PD-L1 level and treatment resistance, which could be overcome by adding PD-1/PD-L1 blockade to the regimen in a preclinical model (188). In addition, radiation leads to the accumulation of T_{reg}s (189, 190) as well as the release of immunosuppressive molecules such as TGFβ (191, 192). Curative, normofractionated radiotherapy leads to significant changes in the peripheral immune status of the patients with a decrease of naïve $CD4^+$ lymphocytes and an increase in T_{reg}s (193–195). These findings led to the rationale of

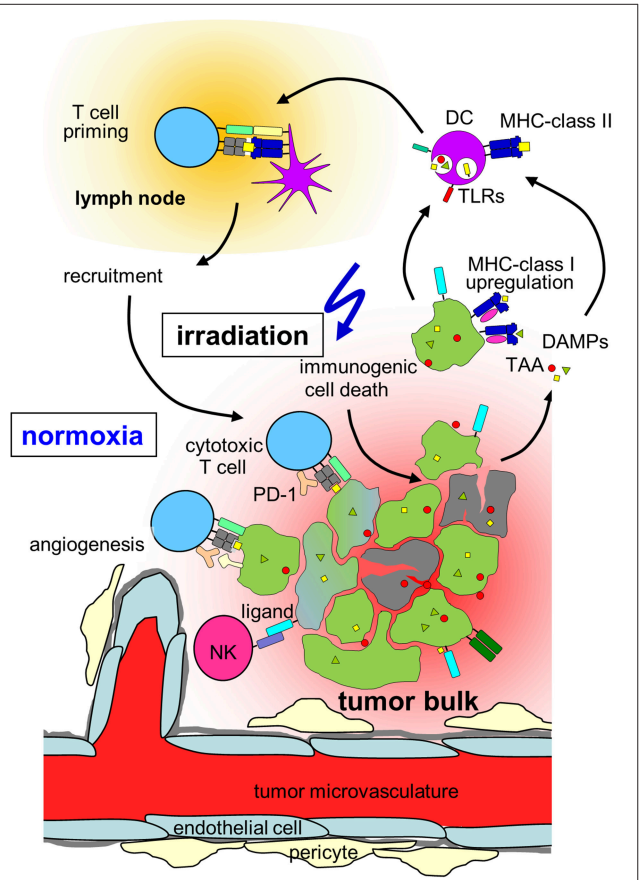

FIGURE 2 | Hypothesis on radiation-induced immunogenic cell death in normoxic tumors. In a normoxic tumor microenvironment, irradiation may lead to effective anti-tumor immune responses by induction of upregulation of MHC class-I on the tumor, immunogenic cell death, release of danger associated molecular patterns (DAMPs) activating toll-like receptors (TLRs) and induction of new tumor associated antigens (TAAs). Maturation of dendritic cells (DCs) and upregulation of MHC-class II is followed by T cell priming in the draining lymph node, cytotoxic T cells and natural killer (NK) cells travel back to the tumor and lead to lysis of tumor cells. Please note, that radiation also induces immunosuppressive processes in normoxic tumors (which are not depicted) such as up-regulation of programmed death-ligand-1 (PD-L1) or T_{reg}s (for details, see chapter Immune effects of radiation).

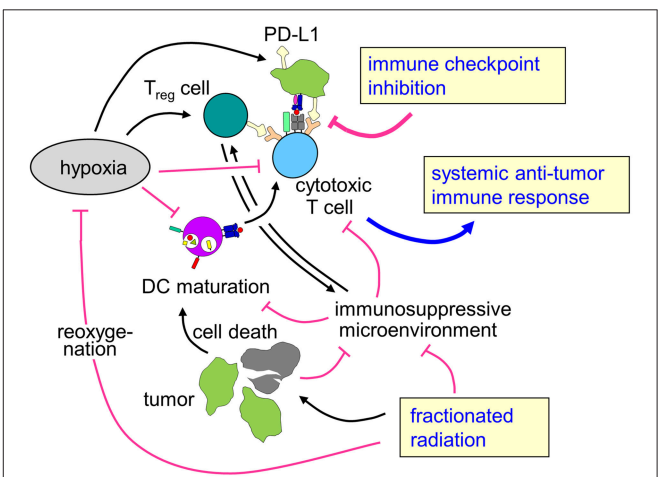

FIGURE 3 | Rationale for combining radiotherapy and immune checkpoint inhibition to overcome therapy resistance of hypoxic tumors. Tumor hypoxia is a key player for the prognosis of cancer patients and resistance to radiotherapy and possibly also for anti-tumor immune response. Fractionated radiotherapy may lead to reoxygenation. The profound immune suppressive microenvironment (see chapter Immunosuppression in the hypoxic tumor microenvironment) predominantly in hypoxic tumors as well as upregulation of immune checkpoint molecules might hint at a rationale to combine fractionated radiotherapy with immune checkpoint inhibition in patients with hypoxic tumors to enhance local control and systemic anti-tumor immune effects.

combining cancer radiotherapy with immune checkpoint inhibition (196).

Combined Radiation and Immune Checkpoint Inhibition

The rationale of combining immunotherapy and radiotherapy has been discussed intensely in several review articles [e.g., (197, 198)]. Initial clinical signs of synergistic and abscopal effects after combination therapy of radiotherapy and immune checkpoint inhibition were reported in a patient with malignant melanoma who had progressed on Ipilimumab but showed a second systemic response after palliative radiotherapy for a paraspinal lesion (199). Initial phase II studies in melanoma showed an abscopal response rate of 18% (200). Immune checkpoint inhibition has been combined with palliative radiotherapy (201) as well as with ablative stereotactic irradiation (202). Furthermore, a recent trial in stage III non-small cell lung cancer encourages efforts of combining both therapeutic strategies in curative settings as well (203). Here, Durvalumab (a monoclonal PD-L1-antibody) consolidation after definitive radiochemotherapy showed significantly prolonged progression-free survival rates and increased overall survival compared to the placebo group with short time between end of radiochemotherapy and start of checkpoint-blockade showing an even larger effect in a subgroup analysis (203, 204).

However, in spite of first efforts (205), the optimal regimen of timing, target organ, dosage and fractionation remains elusive and future trials and translational research need to address these important questions to maximize the potentially beneficial combination effects of radiotherapy and immunotherapy (206). The underlying molecular mechanisms are being investigated intensely and might lead to more promising designs for future clinical trials. PD-1 signaling has been linked to abscopal responses by knock-out and inhibition in *in vivo* models of stereotactic radiotherapy (207). The identification of radiation fractionation schedules leading to abscopal effects in combination with CTLA-4 blockade in an *in vivo* model of breast cancer was linked to the induction of cytosolic double-stranded DNA. With high radiation doses, the induction of the exonuclease TREX-1 degrading the DNA fragments, no abscopal effects were observed (208).

RATIONALE FOR SELECTING PATIENTS WITH HYPOXIC TUMORS FOR COMBINATION TREATMENT

To the best of our knowledge, there are no data on combined radiotherapy and immune checkpoint inhibition focusing on hypoxic tumors. However, as hypoxic tumors are intrinsically more radioresistant than normoxic counterparts and show reduced local control and higher rates of distant metastases, there is a specific clinical need in this subgroup of patients for more effective therapies. As hypoxia also leads to dramatically impaired anti-tumor immune responses, enhancing immune-mediated tumor control mechanisms might be a promising strategy, especially because the combination of immune checkpoint inhibition and radiotherapy has been described to improve local control as well as to induce abscopal effects leading to better systemic tumor control. The here described effects of hypoxia with increased mutational load and upregulation of immune checkpoints such as PD-L1 might even hint at improved responsiveness of hypoxic tumors to immune checkpoint inhibition, further strengthening the hypothesis that patients with hypoxic tumors might be a subgroup of specific interest for combination concepts of radiotherapy with immune checkpoint inhibition (**Figure 3**).

AUTHOR CONTRIBUTIONS

FE and SH designed the concept and wrote the manuscript. KZ wrote the chapter Rationale for combining radiotherapy and immunotherapy. SB wrote the chapter Treatment modifications targeting hypoxia in radiation oncology. DT, DZ, and all authors read and approved the manuscript.

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REVIEW
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Turning "Cold" Into "Hot" Tumors—Opportunities and Challenges for Radio-Immunotherapy Against Primary and Metastatic Brain Cancers

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The development of immunotherapies has revolutionized intervention strategies for a variety of primary cancers. Despite this promising progress, treatment options for primary brain cancer and brain metastasis remain limited and still largely depend on surgical resection, radio- and/or chemotherapy. The paucity in the successful development of immunotherapies for brain cancers can in part be attributed to the traditional view of the brain as an immunologically privileged site. The presence of the blood-brain barrier and the absence of lymphatic drainage were believed to restrict the entry of blood-borne immune and inflammatory cells into the central nervous system (CNS), leading to an exclusion of the brain from systemic immune surveillance. However, recent insight from pre-clinical and clinical studies on the immune landscape of brain cancers challenged this dogma. Recruitment of blood-borne immune cells into the CNS provides unprecedented opportunities for the development of tumor microenvironment (TME)-targeted or immunotherapies against primary and metastatic cancers. Moreover, it is increasingly recognized that in addition to genotoxic effects, ionizing radiation represents a critical modulator of tumor-associated inflammation and synergizes with immunotherapies in adjuvant settings. This review summarizes current knowledge on the cellular and molecular identity of tumor-associated immune cells in primary and metastatic brain cancers and discusses underlying mechanisms by which ionizing radiation modulates the immune response. Detailed mechanistic insight into the effects of radiation on the unique immune landscape of brain cancers is essential for the development of multimodality intervention strategies in which immune-modulatory effects of radiotherapy are exploited to sensitize brain cancers to immunotherapies by converting immunologically "cold" into "hot" environments.

Keywords: GBM, brain metastasis, tumor immunology, immune therapy, immune checkpoint blockade, ionizing radiation, radiotherapy

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INTRODUCTION

Primary and metastatic brain tumors represent a challenging clinical issue. Glioblastoma (GBM) with an incidence of 2–3 per 100,000 population, is the most common primary brain tumor making up 54% of all gliomas and 16% of all primary brain tumors (1). Brain metastases (BrM), that most frequently arise from melanoma, breast- or lung cancers, are the most common intracranial tumor in adults and exceed the number of primary brain tumors by ∼5-fold (2, 3). With the advent of improved control of systemic disease and increased life expectancy of cancer patients, the number of patients with brain metastases is rising (4). The development of cerebral tumors is associated with deteriorated quality of life due to headaches, epileptic seizures, and gradual cognitive impairment (5). Surgical resection, chemo- and radiotherapy (RT) remain the standard of care treatment for patients with brain tumors. Despite recent advances in the development of novel therapies against extracranial tumors, only very little progress has been made in the treatment of cerebral cancers. The majority of clinical trials with immunotherapies for GBM or BrM showed only moderate responses and did not significantly improve progression free survival (PFS) and overall survival (OS) (6). The lack of progress in the development of novel therapies for brain tumors can at least in part be attributed to the unique physiology of the central nervous system (CNS) and in consequence the highly complex brain tumor microenvironment (7). Brain tumors establish an immune suppressive tumor microenvironment that is characterized by high myeloid cell content together with relatively low tumor infiltrating lymphocyte (TIL) numbers and signs of T cell exhaustion (7). While immunotherapy alone fails to provide significant survival benefits for brain cancer patients, there is accumulating evidence, that adjuvant radiotherapy increases tumor immunogenicity and sensitizes brain tumors toward immunotherapy (8, 9).

The primary goal of radiotherapy is the induction of DNA damage in rapidly dividing tumor cells to induce different forms of cell death such as apoptosis or mitotic catastrophe (10, 11). In contrast to malignantly transformed tumor cells with impaired DNA repair mechanism, non-transformed stromal cells experience less damage given their post-mitotic state and intact DNA repair machinery (10). Although a link between irradiation and the immune system was proposed already 100 years ago (12), anti-tumor effects of radiotherapy were attributed to genotoxic effects on tumor cells, while effects on bystander cells were largely neglected for decades. Radiation dose and fractionation was therefore chosen to induce maximal damage in tumor cells and to spare bystander cells. However, traditional dose regimens might blunt important immune reactions directed against tumors. The discovery of immunogenic cell death (ICD) and abscopal effects provide formal proofs for immunological effects of radiation (13, 14). Abscopal effects describe the phenomenon that radiotherapy exerts anti-tumor effects in lesions outside the radiation field by triggering systemic anti-tumor effects (15). Therefore, exploiting the immune modulatory functions of radiotherapy represents an attractive tool to convert immunologically "cold" environments into "hot" environments to increase response rates of immunotherapy. This review will discuss preclinical and clinical evidence that support the applicability of radiotherapy as a sensitizer of immunologically inert tumors, such as GBM and BrM toward immunotherapy with a focus on immune checkpoint blockade (ICB). The field of radio-immunology is just at the beginning to understand the complex cellular and molecular effects of ionizing radiation (IR) on tumor cells and tumor-associated stromal cells that lead to more pronounced and long-lasting immune responses. In addition to clinical observations, it is important to employ preclinical models for systematic evaluation of different treatment regimens in terms of scheduling and dosage to maximize the synergy of radio-immunotherapy. Insight into cellular and molecular effects of radio-immunotherapy is critical to provide a strong scientific rationale for the development of multimodality intervention strategies.

Detailed understanding of the immune landscape of the central nervous system (CNS) at steady state and under pathological conditions is critical to appreciate immunological effects of radiotherapy in brain tumors. This review will therefore first summarize current knowledge on immune surveillance in the CNS and discuss how the development of primary and secondary brain tumors modulates the cellular composition of the TME and alters effector functions of tumor-associated immune cells. Based on this knowledge, different immunological aspects of brain tumors will be discussed to provide insight into the molecular basis of immunotherapy and radiotherapy in combination settings with a particular focus on differences in immune modulation depending on dose and fractionation of IR.

IMMUNE SURVEILLANCE IN THE CENTRAL NERVOUS SYSTEM

The CNS has traditionally been regarded as an immune privileged site that is excluded from systemic immune surveillance (16). Several observations constituted the concept of the immune privileged status of the CNS. First it was noted that the CNS fails to elicit an immune response against immunogenic material that was implanted into the brain parenchyma when avoiding the ventricles and meninges (17, 18). Moreover, the presence of the blood-brain-barrier (BBB) or blood-cerebrospinal fluid barrier (BCB) as well as the absence of lymphatic vessels as a route to the lymph node for antigen presenting cells (APC) further underpinned the concept of the CNS immune privilege (16). However, more detailed insights into the anatomical structures of the brain that represent an interface between the CNS and the periphery led to recent revisiting of the immune privilege of the CNS (16, 19). The use of single cell sequencing and single cell cytometric approaches helped to elucidate the complexity of immune populations in the steady state CNS (20). In this regard it is important to discriminate between brain regions that are excluded from systemic immune surveillance such as the parenchyma and areas at the border between the CNS and the periphery, including the meninges and the choroid plexus. The presence of the BBB and BCB restrict the entry of immune cell-types as well as the exchange of

macromolecules into the brain parenchyma under physiological conditions (16, 21). Host defense is therefore performed by microglia, the brain-resident macrophages that constitute the largest population of immune cells in the CNS (22, 23) (**Figures 1A,B**). Parenchymal microglia are long living myeloid cells with self-renewal capacity that arise exclusively from the yolk sac and populate the brain during embryogenesis before the establishment of the BBB (24–27). As the innate immune cell of the brain, microglia exert key functions in immune surveillance, resolution of infection, wound repair, phagocytosis and debris removal (28). Moreover, microglia are involved in maintaining tissue homeostasis by mediating synaptic pruning, myelo- and neurogenesis as well as neuronal apoptosis (29, 30). However, compared to other cells of the macrophage lineage, microglia show lower antigen-presenting capacity (20, 31). While the brain parenchyma is tightly shielded from the systemic immune system, there are routes that peripheral leukocytes can utilize to enter the cerebral spinal fluid (CSF), choroid plexus, meninges, and the perivascular space (32). Border-associated myeloid cells (BAMs) including meningeal macrophages (mMF), choroid plexus macrophages (cMF), and perivascular macrophages (pvMF) populate those specialized locations in the CNS (33) (**Figures 1A,B**). While microglia exclusively originate from yolk sac-derived progenitors, BAM progenitors are of mixed ontological origin deriving from the yolk sac during primitive hematopoiesis and the fetal liver or bone marrow during definitive hematopoiesis (33). Single cell sequencing and mass cytometry (CYTOF) approaches indicate that BAMs show distinct gene expression signatures compared to microglia (20). Importantly, certain subsets of BAMs showed high CD38 and MHCII expression indicating a role in antigen presentation (20). In addition to the tissue-resident macrophages, Ly6Chi and Ly6Clow monocytes as well as dendritic cells constitute the myeloid compartment of the CNS (20, 28). Monocytes and DC are primarily localized in the meninges and choroid plexus (34–36). It was recently demonstrated that myeloid cells, i.e., neutrophils migrate through vascular channels in the skull-dura interface, indicating a direct local interaction between the brain and the skull bone marrow through the meninges as a route for immediate response to brain damage (37).

Moreover, a range of lymphoid cells including B- and T cells as well as innate lymphoid cells (ILC), natural killer (NK), and natural killer T (NKT) cells constitute the lymphoid compartment in the CNS (20). Lymphocytes are absent from the brain parenchyma but can be found within the CSF of the meninges, choroid plexus and the ventricles. Importantly, the recent discovery that the CNS is directly connected to secondary cervical lymph nodes via a standard lymphatic drainage system fundamentally changed the concept of peripheral immune responses within the CNS (38, 39). There are three known routes by which intracranial antigens can traffic to CNS draining lymph nodes (40). The first is via ventricular and subarachnoid CSF that is able to cross the cribriform plate and enter the lymphatics draining into the deep cervical lymph nodes (41). Secondly, CSF is able to enter meningeal lymphatics located in the dura that also drain to the deep cervical lymph nodes (39). The third route results from parenchymal interstitial fluid trafficking through the basement membrane of the wall of capillaries and arteries of the brain (42). The first two routes are accessible to immune cells such as T cells, monocytes and DC as well as soluble antigens, while the third route is limited to soluble antigens (40).

The immune landscape in the CNS under steady state has recently been shown to be more complex than previously noted. Based on recent observations it was proposed to refer to the brain as an immunologically distinct rather than privileged site. Despite the description of the cellular constituents of the immune landscape in the CNS, it will be critical to evaluate to which extent the presence of lymphoid and myeloid cells in border-associated areas affects the immune privileged state of the brain parenchyma. In this regard, it is important to identify pathological stimuli that trigger infiltration of immune cells from border-associated areas as a route for immediate response or lead to recruitment of immune cells from the periphery and induction of a systemic response. There is accumulating evidence that different pathological conditions, including neurodegenerative disorders as well as cerebral cancers, induce fundamental changes in the cellular composition of the immune infiltrate and activation state of key players in neuro-inflammation (43). Importantly, recent studies revealed that in particular cells that are recruited from the periphery are implicated in the generation of an immune suppressive and cancer permissive environment, while brain-resident cells rather maintain host defense functions (31). This review will focus on tumor-associated inflammation in primary and metastatic brain cancers and highlight similarities and unique characteristics of immune responses that are provoked in the CNS during tumor progression. Understanding the complex immune landscape of brain cancers is critical to develop strategies to overcome the generation of an immune-suppressive environment and perturb traits of tumor cells to escape immune surveillance.

BRAIN TUMORS ESTABLISH AN IMMUNE-SUPPRESSIVE ENVIRONMENT

The development of primary and metastatic brain tumors disrupts the BBB leading to pronounced influx of blood-borne myeloid and lymphoid cells that are usually absent from the brain parenchyma. Tumor-associated macrophages (TAM) represent the most abundant stromal cell type in GBM and BrM often constituting up to 30% of the tumor mass (44, 45). Microglia and BMDMs share many phenotypic and functional similarities. The discrimination of both cell types in the context of brain cancers was previously challenging due to their overlapping marker expression and similar morphology in brain tumors. Lineage tracing approaches (46) and the recent discovery of specific markers (31, 47, 48) significantly contributed to our understanding of cell type specific functions of microglia and BMDMs during disease progression. It was long believed that tumor-associated microglia (TAM-MG) and tumor-associated bone marrow-derived macrophages (TAM-BMDM) exert similar functions in brain tumors. However, gene expression analysis of GBM-associated microglia and macrophages revealed that TAM-MG maintain gene signatures

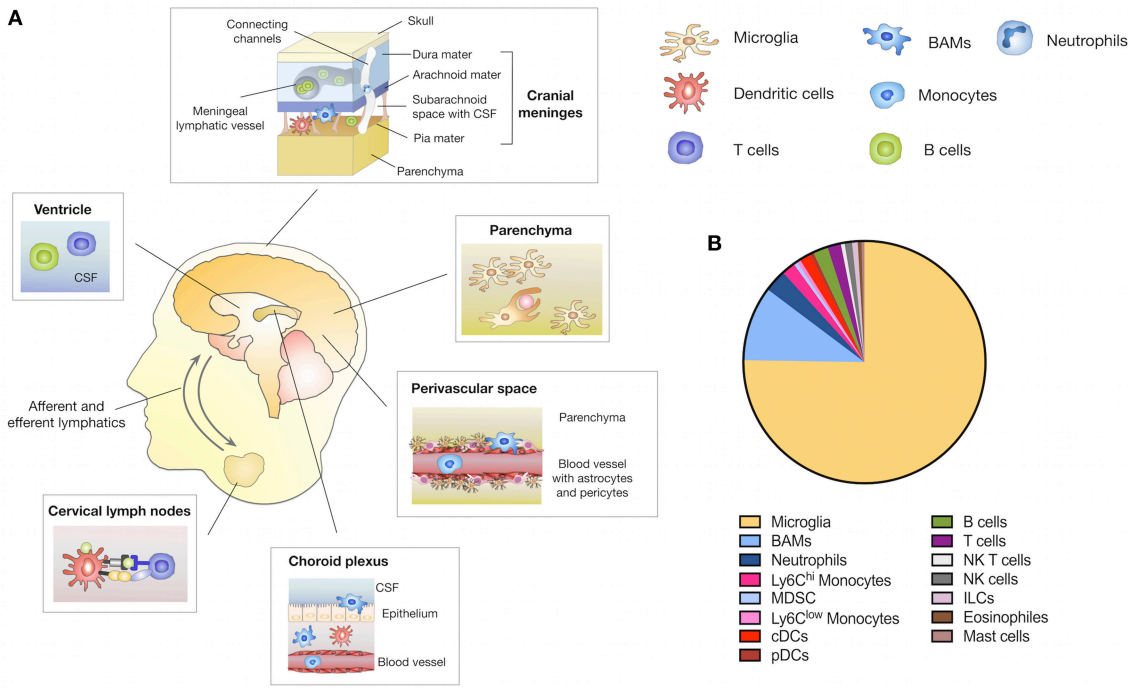

FIGURE 1 | Immune compartments in the CNS. **(A)** The brain parenchyma is tightly shielded from the systemic immune system by the BBB and BCB that restricts the entry of peripheral immune and inflammatory cells. Yolk sac-derived microglia represent in the innate immune cell type within the brain parenchyma and exert key functions in host defense, immune surveillance and tissue homeostasis. In addition to the brain parenchyma, the CNS comprises structural interfaces that connect the CNS with the periphery, such as the choroid plexus, the meninges and the perivascular spaces. Border-associated macrophages (BAMs) are located in each of these niches. Circulating immune cells such as dendritic cells (DC), B- and T-cells are found in the CSF predominately in the meninges, ventricles and choroid plexus. A meningeal lymphatic system allows DC trafficking from the CNS to CNS draining lymph nodes i.e., cervical lymph nodes. Direct channels that connect the skull bone marrow with the dura enable neutrophils to migrate to the CNS as immediate responders to CNS damage. **(B)** Relative amount of distinct CNS immune cell populations located in the parenchyma and border areas of the CNS. Depicted amounts are based on data published in Mrdjen et al. (20).

that are associated with house-keeping functions such as synaptic pruning and host defense and induce pro-inflammatory responses. In contrast, recruited TAM-BMDM showed gene signatures that are associated with wound healing, antigen presentation and immune suppression (31, 49) (**Figure 2**). Hence, functional differences of TAMs based on their ontological origin affect their contribution to disease progression and the ratio of TAM-BMDM to TAM-MG is expected to determine prognosis and therapeutic response especially of intervention strategies that aim to reinstate an effective anti-tumor immune response. In addition to evidence from the mouse models, single cell RNAseq analysis confirmed functional differences between TAM-BMDM and TAM-MG based on their ontological origin in human GBM (50). Interestingly, Müller et al. found that TAM-BMDM signatures correlate with significantly shorter survival in low-grade glioma (LGG) with similar trends in GBM, while there is no correlation between survival and TAM-MG signatures (50). Similar to GBM, it was also reported, that TAM-BMDM infiltrate BrM, although to a lesser extent (31). However, it remains unclear if BrM induce similar gene signatures in TAM-MG and TAM-BMDM as described in GBM. In addition to TAM-BMDM, tumor-infiltrating dendritic cells (DC) represent the most important antigen presenting cell type in brain tumors (51). The ability of DC to collect antigens in peripheral organs and to migrate to draining lymph nodes to activate and prime T cells is fundamental for cytotoxic T cell responses directed against specific antigens (52, 53). Given the immune privileged status of the brain, it remained unclear whether tumor-specific antigens in the CNS are surveyed by the immune system involving trafficking of DCs from CNS tumors to draining lymph nodes as well as trafficking of primed T cells from cervical lymph nodes into CNS tumors. To address the question on T cell trafficking, Prins et al., performed cell-tracking experiments to follow tumor antigen specific T cells after adoptive transfer of *in vitro* activated Pmel T cells (54). Imaging of T cell trafficking in this experimental system that is based on systemic vaccination revealed an early accumulation of T cells in all lymphoid organs including the cervical lymph nodes that drain the CNS and a subsequent accumulation in the bone marrow and brain tumors (54). Moreover, Garzon-Muvdi et al. employed an OVA-expressing GBM model with adaptive transfer of OT-1 T cells to identify the site of antigen presentation of tumor-antigens (55). The authors found proliferating OT1 T cells in cervical lymph nodes indicating that antigen presentation and T cell priming against tumor antigens might take place in the CNS draining lymph nodes (**Figure 2**). T cell expansion in the lymph

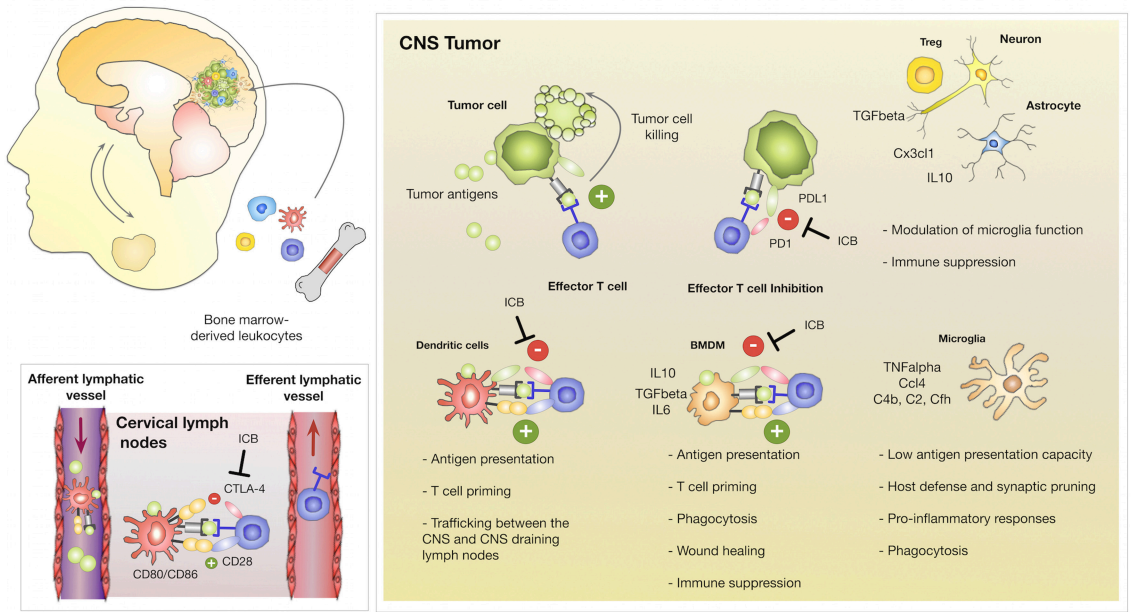

FIGURE 2 | Innate and adoptive immune responses in CNS tumors. Brain tumors induce the recruitment of myeloid and lymphoid cells. Brain-resident and recruited cell types exert different functions within tumor-associated inflammation. Brain resident microglia maintain functions associated to their role as the innate immune cell of the CNS including host defense and synaptic pruning while bone marrow derived macrophages are associated with antigen-presentation, immune suppression and wound healing/tumor promoting functions. TAM-BMDMs express high levels of checkpoint molecules including PD-L1 to inactivate T cells. Dendritic cells traffic between CNS tumors and the cervical lymph nodes to prime T cells against tumor neo-antigens. T cells receive activating signals through interactions of the T cell receptor with antigens presented on MHC molecules and co-stimulation through interactions with CD28 and CD80/CD86. DC express the checkpoint molecule CTLA-4 that binds to CD28 on T cells to prevent activation of auto-reactive T cells. It remains unclear to which extent DCs activate or inhibit cytotoxic T cell functions in CNS tumors. Cytotoxic T cell responses are further inhibited by aberrant expression of checkpoint molecules on tumor cells as well as the secretion of immune-suppressive cytokines by Tregs, astrocytes and neurons. Checkpoint inhibitors (Immune Checkpoint Blockade; ICB) that block CTLA-4, PD-1, or PD-L1 unleash T cell effector functions to induce cytotoxic activity against tumor cells.

nodes and anti-tumor effects were most pronounced in response to DC activation by the Toll-like Receptor (TLR)-3 agonist poly(I:C) in combination with PD-1 mediated immunotherapy (55), indicating that a strong proliferative stimulus is needed for effective T cell expansion. Moreover, it remains to be elucidated whether endogenous tumor antigens can elicit T cell priming or if this only occurs in response to highly immunogenic epitopes such as OVA. While the studies by Prins et al. and Garzon-Muvdi et al. provide evidence that under experimental conditions the proposed route of DC migration, T cell priming and local expansion might take place, it is important to acknowledge that the systems are based on strong experimental stimuli that are not expected in naturally grown CNS tumors. Hence, the formal proof of the route in which DCs migrate to cervical lymph nodes to prime T cells that subsequently traffic to CNS tumors to exert cytotoxic functions is still missing to date. While the question on the natural route for DCs and T cells remains to be addressed in CNS tumors, there is strong evidence that activated, cytotoxic T cells that infiltrate CNS tumors encounter a highly immune-suppressive milieu (56–59). Immune-suppression is particularly well-documented in GBM that are almost completely devoid of T cells (60). Quantitative deficits in the T cell compartment i.e., lymphopenia have been described for GBM patients since the late 1970 (61). It was recently demonstrated that glioblastoma as well as other intracranial tumors induce lymphopenia through sequestration of T cells in the bone marrow leading to a decline in T cell numbers at the tumor site and in lymphoid organs (62). Moreover, T cell apoptosis is induced through interactions of tumor cells via CD70-CD27 signaling (63, 64) or through astrocytes-derived FasL (65) at the tumor site. In addition to quantitative effects on T cells, qualitative deficits of T cells are a common phenomenon in patients with intracranial tumors (66). T cell dysfunction in brain tumors can be induced by a variety of mechanisms (67). High levels of immune-suppressive cytokines such as IL6, IL10, and TGFβ dampen T cell proliferation and effector functions (68). Tumor infiltrating lymphocytes (TILs) show high levels of PD-1, CTLA-4, LAG3, TIM3, TIGIT, and CD39 indicating T cell exhaustion (69–73). Regulatory T cells (Treg) comprise up to 30% of the TILs in GBM that further suppress T cell responses (57, 74). Tumor-associated macrophages and microglia have also been shown to support immune suppression and inhibit the expansion of CD4+ and CD8+ T cell expansion while inducing Treg production (31, 49, 75, 76).

The immune landscape of BrM and its consequences on systemic and CNS immunity are less well-characterized compared to primary brain cancers. The question, if immune responses in BrM are predominately driven by the tissue

environment or if the cellular identity of the tumor of origin (e.g., melanoma, lung, renal or breast cancer) shapes the mode of inflammation, is currently a field of active research. In contrast to GBM, BrM show moderate or even pronounced T cell infiltration. Several studies reported that the extent and pattern of T cell infiltration depends on the primary tumor entity that metastasizes to the brain (77, 78). T cell density is highest in melanoma with a diffuse pattern throughout the metastatic lesion, while renal-, lung- and breast cancer lead to a moderate T cell influx and T cells are dominantly localized within the stromal compartments of the tumor (77). Data from different studies indicate that T cell exhaustion also appears in brain metastasis. For example, expression of PD1 has been found in ∼63% of TILs in melanoma brain metastasis (79, 80). A study by Harter et al. demonstrated that high TIL level, PD1+/CD8+ and PDL1 staining were associated with smaller lesions, however there was no significant association with survival (77). In contrast, a study by Berghoff et al., reported a significant correlation of the density of CD3+, CD8+, and CD45RO+ TILs with favorable median survival (78). As observed in GBM, immune suppressive cell types such as Tregs have also been shown to infiltrate experimental models of metastatic melanoma, breast and colon cancer within the brain and were found in patient brain metastases (57, 81–84).

Taken together, thorough investigation of the tumor microenvironment in GBM and BrM indicate that brain cancers contain the cellular and molecular constituents for therapeutic intervention by checkpoint inhibition. However, clinical data revealed that checkpoint inhibitors as mono-therapy often fail to significantly improve survival rates (85, 86). A possible explanation for the inability of checkpoint inhibitors to reinstate an anti-tumor response might be that the majority of infiltrating T cells are bystander cells that are not directed against specific tumor antigens (87). However, it is also possible that T cells with anti-tumor activity are present within brain tumors, but local immune suppression efficiently blunts their cytotoxic activity even in the presence of checkpoint inhibitors (**Figure 2**). If this is the case, therapeutic strategies that block immune suppression are required to sensitize brain tumors toward immunotherapy. In this context, immunological effects of radiotherapy recently attracted attention and a series of clinical trials have been initiated to test the efficacy of radiotherapy in combination with immunotherapy. The next paragraph will summarize the current status of standard of care and discuss insights from clinical trials with a focus on trials with ICB in GBM and BrM.

CLINICAL MANAGEMENT OF GBM AND BRM

Standard of Care

The current standard of care for newly diagnosed GBM is maximal surgical resection with concurrent radiotherapy and temozolomide (TMZ) chemotherapy followed by 6 months of adjuvant TMZ treatment (88). However, despite multimodality therapeutic intervention, GBM has an almost 100% relapse rate with a median time to recurrence of 7 months (89). The clinical situation for patients with recurrent GBM is extremely dire. Surgery is only considered for ∼25% of the patients and re-irradiation is only possible as a palliative option in rare cases (90). Moreover, response rates to chemotherapy including TMZ rarely exceed 10% and no effects on OS have been reported (91–93).

Previous radiation regimen for brain metastasis patients involved whole brain radiotherapy (WBRT) with fractionated doses of 30 Gy in 10 fractions or 20 Gy in 5 fractions. Different clinical trials report a cerebral response following WBRT in 60% of patients and tumor volume reduction after WBRT has been associated with better neurocognitive function and prolonged survival (94). Median survival following WBRT alone in patients with multiple brain metastasis ranges from 3 to 6 months, with 10–15% of patients still alive at 1 year. However, numerous detrimental effects of WBRT in terms of acute and delayed neurotoxicity such as leuko-encephalopathy and loss of memory function as well as radiation necrosis have been described (95, 96). Given the lack of survival benefit and both short- and long-term toxicities associated with WBRT, recent guidelines from the European Association of Neuro-Oncology (EANO) recommend a deferment and replacement of WBRT by stereotactic radiosurgery (SRS) for the treatment of patients with a limited number of brain metastases and/or favorable prognostic factors (97). SRS is a single high dose radiation treatment with high accuracy in placing the irradiation field on tumor lesions and improved protection of surrounding tissue. The treatment efficacy of SRS is similar to surgical resection of brain metastases, with local control rates ranging from 80–85% (98). Clinical data also show, that the use of SRS after surgical resection significantly lowers local recurrence compared to surgery alone and that it is associated with a decreased risk of cognitive decline compared to WBRT (99, 100). Controversy remains over potential differences of SRS plus WBRT compared to SRS or WBRT alone. A recent meta-analysis that compared the outcome of patients with one or more brain metastases revealed no differences on survival for patients with multiple metastases, while a WBRT plus SRS improved survival in patients with single metastasis. Moreover, WBRT plus SRS resulted in significantly better local tumor control than WBRT alone (101).

Given the dismal prognosis for GBM and BrM patients, in particular patients with recurrent GBM or patients with multiple BrM it is evident that improved intervention strategies are urgently needed to provide better care for brain cancer patients. The introduction of immunotherapy into the clinics for select cancer types led to new hope for an improved management of primary and metastatic brain cancers.

Immunotherapy

Immune checkpoints are an important component of immune responses to keep cytotoxic activity of T cells under control to prevent autoimmunity. Cancers exploit this safety mechanism by up-regulation of checkpoint components on their cell surface to block T cell activity or by co-opting cells of the tumor microenvironment to establish an immune suppressive environment by dampening T cell responses. Checkpoint inhibitors unleash T cells from their inactive or exhausted state to induce anti-tumor responses (102) (**Figure 2**). To date, the

most prominent examples have been antibodies that block the inhibitory immune checkpoint proteins cytotoxic T lymphocyte antigen 4 (CTLA-4), and PD-1 that are expressed predominantly on T cells, or PD-L1 that is expressed on different immune cells as well as aberrantly on tumor cells (103, 104). Consequently, successful immunotherapy by checkpoint inhibition relies on the natural ability of T cells to recognize and destroy malignant cells. While GBM and BrM are both characterized by highly immunosuppressive environments, GBM is further characterized by T cell exclusion and low mutational burden resulting in minimal neo-antigen generation (105). In contrast, BrM show moderate to high T cell content depending on the primary tumor entity and the majority of tumors that metastasize to the brain show high mutational load. However, it has to be taken into account that mutations that are found in brain metastasis are often not present in matched primary tumors (106). Data on brain metastasis patients are limited since those patients are often excluded from clinical trials. However, immunotherapies have demonstrated survival benefits for patients with tumors that frequently metastasize to the brain such as melanoma and NSCLC (107, 108). The use of checkpoint inhibitors in those patient cohorts allows for retrospective studies to evaluate the efficacy of checkpoint inhibitors against brain metastasis (109), which indicated efficacy of ICB in BrM. Given the potential beneficial effect of ICB in brain metastasis, a limited number of prospective trials have now been initiated to test the efficacy of immunotherapies in the treatment of brain metastases. First clinical trials to evaluate the efficacy of ipilimumab in patients with melanoma brain metastasis reported intracranial responses in 18% of patients with asymptomatic BrM without corticosteroid treatment while only 5% of symptomatic BrM patients on corticosteroid treatment showed intracranial responses (110). This finding further underpins the need that patients are not treated with corticosteroids at the time of ipilimumab treatment. Following clinical trials such as the ABC trial (NCT02374242) (111) and CheckMate-204 (112) aimed to test the efficacy of combining nivolumab and ipilimumab. Both trials report significant intracranial response rates of 46% in the combined treatment group compared to 20% in the nivolumab group in the ABC trial and 57% in the CheckMate-204 trial. Novel combinations are currently explored in clinical trials to further increase the intracranial response and to reduce adverse effects. For example, the activity and safety of the VEGF neutralizing antibody bevacizumab in combination with pembrolizumab or atezolizumab is tested in clinical trial for patients with untreated BrM (NCT02681549; melanoma and NSCLC and NCT03175432 BEAT-MBM; melanoma). Besides the effects of bevacizumab on angiogenesis, there is accumulating evidence, that VEGF blockade leads to reprogramming of the tumor microenvironment from an immunosuppressive to an immune permissive milieu, thus representing a promising combination together with immune checkpoint inhibitors (113). Results from these trials are still pending.

Although multiple factors indicate that GBM harbors intrinsic resistance against checkpoint inhibition as monotherapy, pre-clinical testing showed promising results (114). The CheckMate143 trial (NCT0207717) was the first large-scale randomized clinical trial of PD pathway inhibition in GBM. However, treatment with the PD1 blocking antibody nivolumab failed to extent OS in patients with recurrent GBM, leading to a termination of this trial arm (86). In particular in the context of GBM it is important to take into account that in addition to the immunosuppressive tumor microenvironment, GBM patients often receive TMZ that is known to cause lymphopenia and permanently affect numbers of memory T cells (115). Moreover, corticosteroids such as dexamethasone are commonly used in the treatment of GBM patients to control cerebral edema. However, as already mentioned for BrM patients, corticosteroids are known to adversely affect the efficacy of immunotherapies (116).

In sum, the extent of immune suppression that is established in GBM and BrM together with effects from standard of care treatment that further dampens immune responses might ultimately prevent effective immunotherapies. Therefore, it is important to develop improved intervention strategies that overcome current obstacles to successful immunotherapy in cerebral tumors. In addition to the recently initiated clinical trials that aim to test the efficacy of the combination of different checkpoint inhibitors, there is increasing interest in the potential synergy of radiotherapy and immunotherapy.

Radio-Immunotherapy

Data from retrospective trials suggest that combinations of immunotherapy and radiotherapy significantly increase response rates and show effects on overall survival. For example, Knisely et al. reported that melanoma patients that received ipilimumab plus WBRT achieved longer median survival compared to WBRT alone [21.3 vs. 4.9 months] and a greater 2-year survival rate [47.2 vs. 19.7%] (117). Similarly, ipilimumab plus SRS was shown to increase OS from 5.3 to 18.3 months, while in this study no survival benefits for the combination of ipilimumab plus WBRT was reported (118). Sharverdian et al. reported that within the patient cohort that was enrolled in the KEYNOTE-001 trial (NCT01295827), NSCLC patients that received radiotherapy before pembrolizumab showed better PFS and OS compared to patients who did not receive radiotherapy (119). Ahmed et al. recently reported data from melanoma BrM patients that received nivolumab plus SRS demonstrating high rates of local BrM control of 91% and 85% at the 6 and 12 months follow-up (120). A central question that remains to be addressed in combination trials is the timing of each component for optimal outcome. Possible regimens comprise concurrent, sequential or neo-adjuvant application of the treatment modules (121). So far, the results suggest that the optimal schedule is tumor type and immunotherapy dependent. However, to date, the majority of trials report data that provide evidence for a benefit of concurrent schedules (122) and lowest response rate if radiotherapy is given after the immunotherapy. For example, a study of patients with melanoma brain metastasis showed that concurrent immunotherapy with anti-PD-L1 and anti-CTLA-4 showed improved response rates if immunotherapy was given within a time frame of 4 weeks after radiation compared to treatments that were more than 4 weeks apart (122). Dovedi

et al. demonstrated that acquired resistance to fractionated radiation could be overcome by PD-L1 blockade using syngeneic mouse models of melanoma, colorectal and triple-negative breast cancer. However, the effect was only apparent if treatments were applied either concomitantly with or at the end of radiation. The effect was lost if PD-L1 blockade was given 1 week after radiotherapy (123). In contrast, a retrospective analysis of data from 758 patients suggested an improvement of OS with concurrent ICB and RT and hypo-fractionated RT particularly when immune checkpoint inhibition is started at least 1 month before RT, implying a benefit to commence ICB prior to RT (124). To date, pre-clinical and clinical evidence that optimal scheduling of radio-immuno-therapy critically affects the therapeutic response largely stems from studies on extracranial tumors and must be carefully considered for individual cancer types and different checkpoint inhibitors. Defining the optimal schedule for primary and metastatic brain tumors will require carefully designed prospective clinical trials in combination with systematic preclinical testing or mathematical modeling approaches as recently proposed by Serre et al. (125). While there are several ongoing clinical trials that aim to compare the efficacy of immune checkpoint inhibitors in combination with either WBRT or SRS, there are only few trials that are specifically designed to evaluate how different schedules affect safety and efficacy of combined treatment. One phase II clinical trial at the University of Michigan Cancer Center (NCT02097732) that considers the timing of immunotherapy is evaluating the efficacy of an "induction" of ICB prior to SRS [2 doses of ipilimumab prior to SRS, 2 doses of ipilimumab after SRS] vs. "no induction" [SRS first, followed by 4 doses of ipilimumab (3 mg/kg)]. In the future, more clinical trials that address the question on optimal scheduling will be required for conclusive results.

In sum, data from clinical and pre-clinical studies indicate that radiotherapy can act as a sensitizer for immunotherapy. Conventional fractionation takes advantage of the higher radiosensitivity of tumor cells compared to normal cells with respect to DNA repair and cell cycle regulation. However, whether conventional fractionation represents the optimal strategy to maximize synergy with immunotherapy remains unclear to date. Moreover, it will be critical to evaluate whether radiation dose and fractionation that is optimal to induce an immune response in the CNS, is tolerated by the sensitive brain tissue. In order to optimize radiation dose and fractionation as well as scheduling for therapeutic application, it is essential to gain detailed insight into the molecular basis of genotoxic and immune modulatory effects of radiotherapy. The following paragraph will summarize current knowledge on effects of radiotherapy that result from direct damage on tumor cells (i.e., different forms of cell death) with subsequent effects on the inflammatory response against tumors. Moreover, local and systemic immune responses can also be modulated by radiation-induced changes in different cell populations of the tumor microenvironment (**Figure 3**). Direct and indirect effects on tumor cells and tumor-associated immune cells together determine the extent by which radiotherapy increases immunogenicity of tumors and the synergy between radio- and immunotherapy.

MOLECULAR BASIS OF IMMUNE MODULATORY EFFECTS OF IR

Radiation-Induced Immune Responses Depend on the Type of Cell Death by Which Tumor Cells Are Killed

The central dogma of traditional radiobiology states that effects of radiation on tumor cells are primarily due to the generation of double strand breaks that lead to the induction of different forms of cell death including apoptosis, necrosis, autophagy, or mitotic catastrophe (**Figure 3**). Apart from the notion that necrosis elicits inflammation due to the release of cellular content, radiotherapy has long been regarded as an immunologically inert process. While immunological effects of radiotherapy were neglected for decades, several discoveries established a link between the immune system and the ability of radiotherapy to achieve tumor control. Stone et al. demonstrated already end of the 70s, that the radiation dose that is required to control tumor growth was twice as high in immune-compromised mice compared to immune-competent mice (126). Moreover, the occurrence of abscopal effects in which tumor control is achieved in lesions outside the radiation field provides further proof for the contribution of the immune system in tumor control (127). The discovery of immunogenic cell death (ICD) as a molecularly defined processes that leads to priming and activation of immune cells recently led to a paradigm shift. ICD is characterized by the cell surface translocation of calreticulin (CRT), the extracellular release of HMGB1 (High motility group box 1) and of adenosine triphosphate (ATP) (128). Radiotherapy has been shown to induce all three arms of ICD and is therefore regarded as a potent inducer of ICD (129). Different doses or fractionations are believed to induce different forms of cell death (130), and thus modulate downstream cellular responses. Radiation regimens that induce immunologically silent forms of cell death, i.e., apoptotic cell death are therefore not expected to synergize with ICB, while doses and fractionation that trigger inflammatory responses could be used as immune-modulators to induce additive effects of radiotherapy and immunotherapy (131).

Radiation Increases Recognition of Tumor Antigens by the Immune System

Within the process of immune evasion, tumor cells acquire traits that mask the tumor from immune surveillance and destruction. The immune escape stage is characterized by up-regulation of inhibitory ligands and cytokines, reduced MHCI expression, and increased number of suppressive cell types such as Tregs (132). Radiotherapy has been reported to unmask the tumor and thus make it visible again for the innate and adaptive immune system (133). Radiation can up-regulate MHCI expression on the tumor cell surface to enable better antigen presentation of tumor-specific peptides for recognition by cytotoxic T cells (134). Mutational load of tumors is known to correlate with therapeutic response rates to immunotherapy (135–138). Identification of mutations that drive anti-tumor responses would therefore be of great benefit to identify patients with a high chance of responding to immunotherapy. Moreover, radiation-induced

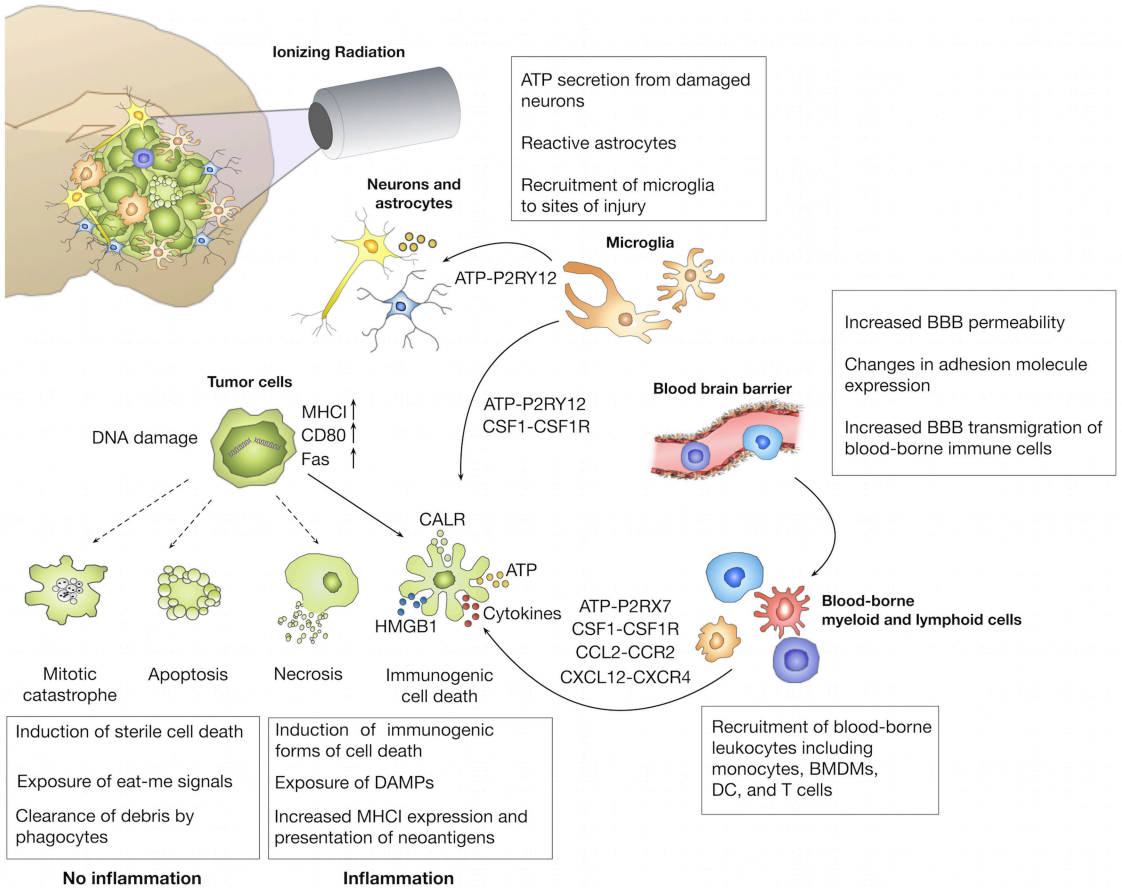

FIGURE 3 | Immune modulatory effects of radiotherapy. The primary aim of radiotherapy is the induction of DNA damage in malignant cells. Depending on the extent of DNA damage, different forms of cell death are induced. The induction of immunogenic cell death (ICD) has been linked to the exposure of danger-associated molecular patterns (DAMP) that induce the recruitment and affect effector functions of brain-resident and recruited immune cells. Radiotherapy can also increase the immunogenicity of tumors by inducing presentation of neo-antigens by upregulation of MHC I molecules. In addition to effects on tumor cells, ionizing radiation also affects tumor-associated stromal cells including cells of the blood vessels and immune cells that further contribute to the establishment of radiation-induced immune responses.

DNA damage can cause an increase in mutational load (139). While an increase in mutational burden might enhance tumor aggressiveness, it might also generate neoantigens that can be recognized and targeted by the immune system (140). Indeed it was demonstrated that IR induces novel peptide synthesis in tumor cells and enhances antigen presentation by MHC class I molecules (134, 141). On the other hand, there is evidence that brain tumors show higher systemic tolerance than tumors at extracranial sites. For example, Jackson et al. employed the B16 melanoma model to compare cytotoxic responses against tumors in the CNS and in the periphery. The study showed that CNS melanomas were more tolerogenic than tumors in extracranial sites due to antigen-specific CD8 T cell depletion leading to impaired systemic antitumor immunity (142). The authors concluded, that the observed T cell dysfunction was mainly caused by elevated levels of microglia-derived TGF-β (142). Interestingly, it was demonstrated that the effect of systemic tolerance was reversible by radiotherapy and vaccination.

The cGas-STING Axis in Anti-tumor Immunity

Radiation-induced DNA damage that causes leakage of DNA into the cytosol is known to be sensed by the stimulator of interferon gens (STING) leading to the activation of innate and adaptive immune responses (143). The STING pathway has originally been described as a host defense mechanism to protect organisms against infection with DNA pathogens. When cytosolic DNA is detected, the product of cyclic GMP-AMP synthase (cGAS), cyclic GMP-AMP (cGAMP) activates STING. STING induces the transcription of type I interferon genes via a cascade that involves the STING downstream factors Tank binding kinase (TBK), interferon regulatory factor 3 (IRF3) and nuclear factor kappa light chain enhancer of B cells (NFkB) (144). A growing body of literature suggests that the STING pathway plays a central role in anti-tumor immunity and its expression is lost in several cancer types including colorectal cancer and melanoma (145, 146). While several studies linked radiation-induced STING activity to type-I-interferon mediated anti-tumor immunity,

there is also evidence that STING activation could drive immune-suppression and radio-resistance via CCR2 mediated recruitment of MDSCs (147). Given the complex cellular and molecular interactions of STING mediated immunological effects, clearly more systemic studies are needed to gain comprehensive mechanistic insight.

Dose and Fractionation Are Critical Parameters for Effective Induction of Immunogenicity

Determining the optimal scheduling for radio-immunotherapy is a major challenge for the field and requires carefully designed prospective clinical studies together with comprehensive studies in animal models to test effects of different treatment regimens (148, 149). As discussed above, data from retrospective and prospective clinical trials suggests that treatment schedules in which radiotherapy was given as concurrent, sequential, or neoadjuvant therapy lead to different therapeutic efficacy. Several preclinical studies compared single high-dose with fractionated radiation for their ability to induce immune responses. For example, in a B16-OVA model, both single dose (15 Gy) and fractionated radiation (5x3 Gy) increased the generation of antigen-specific T cells. However, the single 15 Gy dose generated more tumor-infiltrating T cells than conventional fractionation (150). Later it was demonstrated, that the immune response triggered by ablative radiation doses was abrogated by conventional fractionation (151). Moreover, Camphausen et al. demonstrated in a model of Lewis lung carcinoma that 5 × 10 Gy induced more robust abscopal effects than 12 × 2 Gy (152). However, hypo-fractionation might not be favorable when combined with immunotherapy. Dewan et al. demonstrated in a breast cancer model that an abscopal effect was only induced in response to fractionated radiation, not single dose radiation when combined with CTLA-4 inhibition (153). A potential explanation for dose dependent effects of radiation was recently provided by the balance between activation of cGAS-STING signaling vs. Trex1 activation (154). Extremely high single doses (20-30Gy) were shown to blunt immunogenicity by the induction of the DNA exonuclease Trex1. Trex1-mediated degradation of cytosolic DNA consequently abrogates cGAS-STING activation and downstream IFN type1 production. In this study, CTLA-4 blockade did not synergize with high dose irradiation to induce abscopal effects. However, knockdown of Trex1 reinstated synergistic effects of anti-CTLA-4 in combination with high dose radiation (20Gy) (155).

Radiation Modulates the Cellular Composition of the Tumor Microenvironment and Affects Effector Functions of Immune Cells

Another important factor that determines synergy of radio-immunotherapy is the cellular composition of the tumor microenvironment (156). As discussed in the paragraph above, in particular the tumor microenvironment of brain tumors represents a highly complex milieu with brain resident and recruited immune cells (7). Brain tumors are known to establish immune-suppressive environments that are characterized by high myeloid cell content and low percentage of CD8+ effector T cells. Several studies demonstrated that radiotherapy induces increased influx of immune cells into brain tumors. This effect can in part be attributed to effects on the vasculature (157, 158). IR also has profound effects on the secretion of cytokines that serve as chemo-attractants for different immune cells including DC and macrophages (159). In addition, IR has been shown to affect key effector functions such as phagocytosis, antigen presentation, and cytotoxicity and alters activation states of immune cells (160–162). Moreover, radio-sensitivity of T cells has to be taken into account when testing optimal dosage and fractionation. Since immunotherapies rely on functional T cells, their ablation or inactivation is expected to abrogate critical anti-tumor immune responses. Tumor-infiltrating T cells are exposed to radiation and it has been shown that conventional 2 Gy doses given once daily can inactivate T cells (163). This effect is also evidenced by the occurrence of lymphopenia as a common adverse effect associated with whole brain radiotherapy (164) that could significantly dampen anti-tumor immune responses.

Taken together, radiation dose and fractionation have profound effects on the induction of genotoxic and immunogenic effects. Systematic interrogation of the dose dependency of immune responses directed against different cancer types is needed to determine optimal regimens to increase the immunogenicity of tumors and boost the immune system for effective anti-tumor responses that synergize with immunotherapy.

COMBINATION OF IR AND ICB—FUTURE PERSPECTIVES

To date, clinical and pre-clinical data suggest that combining radiotherapy with immunotherapy show higher efficacy compared to mono-therapies. These results represent promising first steps in the quest for improved treatment options for brain cancer patients. However, it is also evident that many hurdles exist that prevent higher response rates and more sustainable anti-tumor reactions. While individual patients show prominent cerebral responses, significant effects on overall survival are rarely reported. This indicates that the pressure of the CNS to establish an immunosuppressive environment is dominant over the attempt to unleash the immune system by immune checkpoint blockade. Based on our current mechanistic understanding of the cellular and molecular drivers of immune-suppression in the steady-state CNS and in the context of cerebral cancers, different approaches appear as viable strategies to overcome the highly immune-suppressive environment in the CNS. Based on the results that TAM-BMDM rather than TAM-MG are implicated in tumor-promotion and immune-suppression, selective depletion or blockade of TAM-BMDM recruitment could lead to more effective T cell activation and execution of anti-tumor effector functions. In addition, systems that would allow more efficient recruitment of T cells into CNS tumors could significantly boost cytotoxic T cell responses directed

against tumor neo-antigens. A recent study by Samaha et al. reported the engineering of T cells with an Activated Leukocyte Cell Adhesion Molecule (ALCAM) homing system (HS) (165). In this approach, CD6 (the ligand for ALCAM) was re-engineered to trigger initial anchorage to ALCAM followed by adhesion to ICAM1 expressed on cancer endothelium. Cytotoxic HS T cells infiltrated brain tumors after intravenous injection and showed potent anti-tumor activity. Other strategies that aim to convert immune-suppressive milieus into inflamed environments might utilize neutralizing antibodies against suppressive cytokines such as TGFβ, IL10, or IL6. Moreover, activation of adenosine signaling has been associated with immune-suppression and acquisition of resistance against immunotherapy in different cancer types including melanoma (166–169). Pharmacological inhibition of enzymes that process ATP into adenosine, i.e., CD39/Entpd1 and CD73/Nt5e or targeting of adenosine receptors are currently evaluated for their potential to block the conversion of a purine-driven, pro-inflammatory environment into an adenosine-driven, immune-suppressive milieu (170–173). Another promising strategy could employ Trex1 inhibitors to prevent the degradation of cytosolic DNA to more efficiently induce cGAS-STING-IRF signaling to trigger innate immune responses (155). Overcoming the immune-suppressive environment appears to be one of the limiting factors for successful immunotherapy against brain cancers. However, it is also important to keep in mind, that immune suppression is an important safety mechanism that protects the brain from excessive inflammation. Inflammatory responses are often associated with swelling that would harm the delicate structures of the CNS and ultimately lead to brain damage. The increased risk of auto-immunity has been reported in several clinical trials with combination of different ICB. The most recent data on the CheckMate143 trial report that 9 of 10 patients treated with a combination of nivolumab and ipilimumab experienced grade 3 or 4 adverse events with 4 of 10 patients discontinuing therapy due to side effects (83). Therapeutic strategies that aim to convert immune suppressive milieus into inflamed environments should therefore be carefully considered and the potential risk of inducing autoimmunity should be evaluated. Detailed mechanistic insight into pathways that are implicated in cancer-associated immune-suppression and inherent or acquired resistance against brain tumors will hopefully lead to the development of novel multimodality intervention strategies that meet the safety and efficacy criteria for the induction of more efficient and long-lasting anti-tumor immune responses in GBM and BrM patients.

CONCLUDING REMARKS

A close link between radiotherapy and the immune system has been proposed already 100 years ago by Russ and Murphy (12). Murphy's observation that "large doses of x-rays, by destroying the immune conditions, will favor the growth of tumors while small doses, by producing immune conditions will help to overcome the tumor" closely resembles the current view on effects of RT on cancer-associated inflammation (174). Yet, immunogenic effects of radiotherapy have been neglected for decades. The introduction of immunotherapy into the clinic and more detailed molecular insights into the underlying mechanism of immunogenic effects of radiation have recently attracted attention to radiotherapy as a potent modulator of cancer-associated inflammation. While immunotherapy is highly effective for patients with inflamed or hot tumor environments, large patient cohort remain unresponsive to immunotherapy due to intrinsic or acquired resistance. Although we are just at the beginning to understand the cellular and molecular basis that distinguish responders from non-responders, accumulating clinical and pre-clinical data indicate that immunogenic effects of radiotherapy convert cold into hot environments and thus sensitize unresponsive tumors toward immunotherapy. Our current knowledge on radio-immunotherapy against brain cancers is largely based on retrospective clinical trials or pre-clinical studies on animal models. The initiation of clinical trials and pre-clinical studies that aim to systematically evaluate the effects of different fractionation and treatment regimens is needed to provide deeper insight into optimal schedules to induce synergy between immunotherapy and radiotherapy. While there are certainly indications that favor specific treatment regimens, it is still too early to draw conclusions on effects on PFS or OS. To further improve the response rate, it will be critical to identify molecular pathways that determine the mode of immune responses and to develop strategies that efficiently induce anti-tumor immune responses without the risk of inducing auto-immunity.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Radiotherapy Both Promotes and Inhibits Myeloid-Derived Suppressor Cell Function: Novel Strategies for Preventing the Tumor-Protective Effects of Radiotherapy

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Cancer immunotherapies aimed at neutralizing the programmed death-1 (PD-1) immune suppressive pathway have yielded significant therapeutic efficacy in a subset of cancer patients. However, only a subset of patients responds to antibody therapy with either anti-PD-1 or anti-PD-L1 antibodies. These patients appear to have so-called "hot" tumors containing tumor-reactive T cells. Therefore, checkpoint blockade therapy may be effective in a larger percentage of cancer patients if combined with therapeutics that also activate tumor-reactive T cells. Radiotherapy (RT) is a prime candidate for combination therapy because it facilitates activation of both local antitumor immunity and antitumor immunity at non-radiated, distant sites (abscopal response). However, RT also promotes tumor cell expression of PD-L1 and facilitates the development of myeloid-derived suppressor cells (MDSC), a population of immune suppressive cells that also suppress through PD-L1. This article will review how RT induces MDSC, and then describe two novel therapeutics that are designed to simultaneously activate tumor-reactive T cells and neutralize PD-1-mediated immune suppression. One therapeutic, a CD3xPD-L1 bispecific T cell engager (BiTE), activates and targets cytotoxic T and NKT cells to kill PD-L1$^+$ tumor cells, despite the presence of MDSC. The BiTE significantly extends the survival time of humanized NSG mice reconstituted with human PBMC and carrying established metastatic human melanoma tumors. The second therapeutic is a soluble form of the costimulatory molecule CD80 (sCD80). In addition to costimulating through CD28, sCD80 inhibits PD-1 suppression by binding to PD-L1 and sterically blocking PD-L1/PD-1 signaling. sCD80 increases tumor-infiltrating T cells and significantly extends survival time of mice carrying established, syngeneic tumors. sCD80 does not suppress T cell function via CTLA-4. These studies suggest that the CD3xPD-L1 BiTE and sCD80 may be efficacious therapeutics either as monotherapies or in combination with other therapies such as radiation therapy for the treatment of cancer.

Keywords: radiotherapy-induced immune suppression, programmed death ligand 1 (PD-L1), myeloid-derived suppressor cells (MDSC), bi-specific T cell engager (BiTE), solubilized CD80

INTRODUCTION

Checkpoint inhibitors that inactivate the programmed death-1/programmed death ligand-1 (PD-1/PD-L1) pathway protect T cells from anergy and apoptosis and have significantly improved the survival of cancer patients with certain types of malignancies. As a result, antibodies to PD-1 and PD-L1 are now FDA-approved for the treatment of Hodgkin's disease, melanoma, merkel cell, non-small cell lung, head and neck, gastroesophageal, bladder, urothelial, renal cell, and hepatocellular cancers, and are being tested in numerous other types of cancer.

Cancers with high mutation rates and *de novo* tumor-infiltrating lymphocytes have response rates of 53–87%, while tumors with lower levels of mutations have response rates of approximately 20% [reviewed in (1)]. Tumor cell mutations render tumor cells immunogenic, resulting in the activation of T cells which traffic to the sites of tumor [tumor-infiltrating T cells (TIL)]. T cell activation and function are characterized by many factors including the expression of PD-1 and by the production of interferon gamma (IFNγ), which is also a potent inducer of PD-L1. Therefore, inherently immunogenic tumors are more likely to be candidates for PD-1/PD-L1 antibody therapy, particularly if the mutations are present in the cancer stem cells and also expressed in the progeny of the stem cells (2).

TIL are a key component for the efficacy of PD-1/PD-L1 therapy; however, not all tumors have a high rate of mutation and do not contain TIL. Therefore, alternative strategies for increasing TIL are being developed. Radiotherapy (RT) is a prime candidate because it facilitates activation of anti-tumor immunity at both locally radiated and distant non-radiated sites (abscopal response) (3, 4). However, RT also promotes tumor cell expression of the checkpoint blockade molecule PD-L1 (5, 6). Multiple studies in mice (6, 7) and patients (8–10) have demonstrated that checkpoint blockade inhibitors (CBI) such as antibodies to PD-1 and PD-L1 delay tumor progression and increase overall survival, thus confirming the suppressive role of PD-1/PD-L1 activity. As a result, there is extensive interest and enthusiasm for combining checkpoint blockade immunotherapy with RT (3, 4, 11–16). Preclinical studies in mice support the concept that the combination of radiotherapy with checkpoint blockade has increased therapeutic efficacy (17, 18), and the few clinical studies completed to date suggest the combination approach will benefit cancer patients (19–23).

However, RT also promotes myeloid-derived suppressor cells (MDSC) (24), another potent immune suppressive mechanism. MDSC use a variety of mechanisms to suppress antitumor immunity; however, they also can express PD-L1, and RT increases MDSC expression of PD-L1 (5, 25). Given that RT enhances immunogenicity but also enhances immune suppression through increased MDSC and PD-L1, this review will summarize how RT induces immune suppression in the context of MDSC and PD-L1 and will describe two novel strategies for neutralizing this RT-induced immune suppression. This information may provide the basis for new approaches for treating cancer in combination with RT.

RADIOTHERAPY ACTIVATES THE IMMUNE SYSTEM BUT ALSO DRIVES IMMUNE SUPPRESSION

Radiotherapy (RT) has been a staple of cancer treatment for some cancers for over a century. Traditionally it was thought that RT controls tumor progression through the induction of DNA damage which results in tumor cell death (26). DNA damage also causes lymphopenia (27) and therefore was considered a deterrent to antitumor immunity. However, T cells contribute to the regression of tumors following radiation (28), and local radiation facilitates the development of tumor-reactive T cells that home to the tumor microenvironment (29). Not only does radiation affect the local radiation site, but it can also limit/prevent progression of distant metastases. This phenomenon is known as the abscopal effect and is mediated by the immune system (30). These studies suggest that RT systemically activates tumor-reactive T cells and makes RT a logical therapy to combine with inactivation of the PD-1/PD-L1 pathway to increase patient responses.

However, RT also inhibits antitumor immunity by facilitating the development of immune suppressive cells, such as T regulatory cells (Tregs) (31), tolerogenic and immune suppressive dendritic cells (DC) (32), tumor-associated macrophages (TAMS) (33), tumor-associated neutrophils (TANs) (34), and MDSC (24), via a series of soluble molecules such as TGFβ (35), adenosine (36), VEGFA (37), CSF1 (24), and CCL2 (38). It is beyond the scope of this article to discuss all of these mechanisms, so the below discussion focuses on MDSC, which are present in virtually all cancer patients and are universally considered a major obstacle to cancer immunotherapies. Descriptions of the effects of RT on other immune suppressive cells and factors have recently been comprehensively reviewed (3, 39–41).

MYELOID-DERIVED SUPPRESSOR CELLS (MDSC)

MDSC are a diverse mixture of cells of myeloid lineage at intermediate stages of differentiation. There are two broad categories of MDSC: monocytic (M-MDSC) and granulocytic or polymorphonuclear (PMN-MDSC). These categories are defined based on their presence in the circulation. In humans M-MDSC are phenotypically $CD11b^{+}CD14^{+}HLA\text{-}DR^{-/low}$ and PMN-MDSC are $CD11b^{+}CD14^{-}CD15^{+}$or $CD66b^{+}HLA\text{-}DR^{-/low}$. Human M-MDSC may also express low levels of CD15. All MDSC are negative for the lineage markers characterizing non-myeloid cells. As apparent from their names, M-MDSC are mononuclear and PMN-MDSC are polymorphonuclear. A third category of human MDSC has recently been defined. These "early-stage MDSC" (eMDSC) are $CD33^{+}HLA\text{-}DR^{-}$ and do not express either CD14 or CD15. Mouse M-MDSC are $CD11b^{+}Ly6C^{+}Ly6G^{-}$ and PMN-MDSC are $CD11b^{+}Ly6G^{+}Ly6C^{-}$. Since the mouse marker Gr1 can include both Ly6C and Ly6G, total mouse MDSC are sometimes phenotyped as $CD11b^{+}Gr1^{+}$ (42). PMN-MDSC and neutrophils

share the same surface markers, so phenotype alone is not sufficient for identifying either human or mouse cells as MDSC. Human PMN-MDSC and neutrophils have different densities so that PMN-MDSC tend to band with mononuclear cells at lower densities in Ficoll gradients, while neutrophils pellet at a higher density (43). However, the definitive characteristic of both M-MDSC and PMN-MDSC is their ability to inhibit the activation and function of T cells (44, 45). Mouse MDSC have been functionally characterized in more detail than human MDSC. In the context of tumor immunity they have also been shown to (i) polarize macrophages toward an M2-like pro-tumor phenotype (46), (ii) inhibit naïve T cell trafficking into lymph nodes and thereby prevent priming (47, 48); (iii) prevent T cell expansion by sequestering cysteine (49); (iv) drive the accumulation of Tregs (50); and (v) inhibit natural killer cell function (51).

MDSC arise in the bone marrow (and spleen of mice) in response to a variety of pro-inflammatory signals produced by tumors and host cells within the tumor microenvironment (44). The dominant driving factors are proinflammatory mediators such as IL-1β (52, 53), IL-6 (54), TNFα (55), prostaglandin E2 (56), high mobility group box protein 1 (57), and indole-amine2,3 dioxygenase (58). The cells traffic through the circulation and are chemoattracted to the tumor microenvironment by a series of chemokines such as CCL2 and CXCL2 that are present in the tumor microenvironment. Once in the tumor, hypoxia increases the suppressive potency of MDSC which is predominantly driven by the transcription factor STAT3 (59). MDSC have a relatively short half-life and M-MDSC can differentiate into non-immune suppressive myeloid cells (45). However, there is strong homeostatic regulation such that MDSC are rapidly replenished (60). A comprehensive discussion of MDSC induction and function can be found in several recent excellent review articles (61–63).

IMPACT OF RT ON MDSC

Since RT induces a local inflammatory response including molecules such as C5a (64) which is a classical inducer of MDSC (65), it is not surprising that RT may induce the accumulation of MDSC. Cervical cancer patients receiving conventional fractionated RT (CFRT) showed an increase in levels of circulating MDSC along with reduced antigen presenting cell activity (66). In a mouse study using several prostate cancer cell lines, fractionated low dose RT caused an increase in MDSC in the blood, spleen, and lymph nodes. The effect was mediated by DNA damage that caused the ABL1 kinase to translocate to the nucleus where it bound to the promoter region of the CSF1 gene. The resulting increase in circulating CSF1 increased myeloid cell levels. Confirming the mouse studies, CSF1 was also elevated in the circulation of prostate cancer patients treated with RT (24). Tumor radioresistance via the induction of MDSC has also been attributed to RT-mediated activation of the Stimulator of Interferon genes (STING) pathway. Local radiation of tumor-bearing mice resulted in tumor cell production of the type 1 interferon IFNβ which, in turn, induced CCL2, CCL7, and CCL12 and chemoattracted $CCR2^+$ M-MDSC to the tumor microenvironment (67).

MDSC levels have also been suggested as potential prognostic indicators of disease outcome. Following CFRT, hepatocellular carcinoma patients with high levels of M-MDSC have a poor prognosis (68).

MDSC have also been reported to have radioprotective activity. MDSC produce high levels of arginase 1 (Arg1). Arg1 promotes tumor progression by degrading arginine, an essential amino acid for T cell activation and function (69). Arginine is also the substrate for the production of nitric oxide (NO) which is generated by NO synthase (iNOS or NOS_2). Under hypoxic conditions within solid tumors NO is a radiosensitizer that acts by reducing mitochondrial respiration (70). In an *in vitro* co-culture/radiation system using mouse and human tumor cells, Arg1-producing MDSC displayed radioprotective activity by reducing arginine and NO (71).

RT can also reduce MDSC levels, an effect that appears to require high dose ablative RT rather than multiple lower dose treatments. In studies with mice, ablative hypofractionated RT (AHFRT), but not CFRT, reduced the levels of intratumoral hypoxia, MDSC, and VEGF, and reduced MDSC expression of PD-L1 and VEGF receptor. Since hypoxia is a driver of PD-L1 expression (72) and VEGF is an inducer and chemoattractant for MDSC (73), the authors concluded that AHFRT reduced MDSC levels and function by decreasing intratumoral hypoxia and VEGF (74). In another mouse study, therapy with a single dose of ablative RT combined with anti-PD-L1 antibody therapy activated $CD8^+$ T cells that subsequently decreased MDSC levels. CD8 T cell-mediated killing was by the production of TNFα (5), which is surprising since TNFα is an established inducer of MDSC (55). Another mouse study using a single high dose radiation treatment similarly resulted in elimination of MDSC. In this system, the high dose irradiation generated $CD40L^+CD4^+$ T cells and $CD8^+$ dendritic cells that through cross-priming activated $CD8^+$ T cells producing IFNγ (75).

In conjunction with the findings of others for T cell responses and antitumor immunity (76), it appears that in contrast to CFRT, AHFRT may generate a more effective abscopal response and better antitumor immunity by limiting the accumulation of MDSC. If AHFRT is sufficient to eliminate MDSC, then additional strategies for reducing MDSC in patients receiving RT may not be needed. However, if AHFRT does not sufficiently eliminate MDSC or prevent MDSC up-regulation of PD-L1 (and potentially other ligands for checkpoint receptors), then additional therapies targeting these cells will be necessary. **Figure 1** summarizes the conditions that drive the accumulation and function of MDSC, and the impact of CFRT and ABHRT on the generation of MDSC.

A BI-SPECIFIC T CELL ENGAGER (BiTE) ACTIVATES T CELLS THAT ARE CYTOTOXIC FOR PD-L1$^+$ TUMOR CELLS

BiTEs are designed to activate T cells via CD3 and simultaneously bind to tumor cells via a tumor antigen. They are single chain recombinant proteins that contain the V_H and V_L regions of an anti-CD3 mAb attached by a short linker to the V_H and V_L regions of a mAb that reacts with a tumor antigen

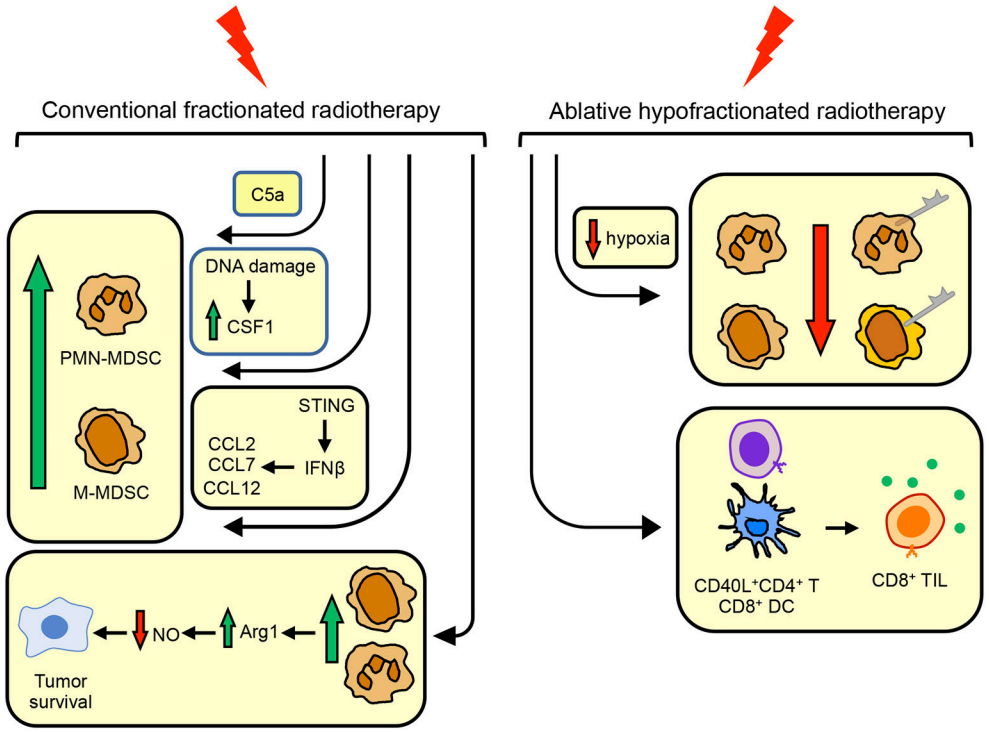

FIGURE 1 | Conventional fractionated radiotherapy (CFRT) increases MDSC while ablative hypofractionated radiotherapy (ABHRT) decreases MDSC. CFRT increases the quantity of MDSC by (i) inducing the complement component C5a; (ii) causing DNA damage resulting in the up-regulation of CSF1; or (iii) signaling through STING to increase IFNβ which up-regulates CCL2, CCL7, and CCL12, chemoattractants for MDSC. MDSC up-regulated by CFRT facilitate tumor cell survival by their production of arginase 1 which decreases nitric oxide, a radiosensitizing molecule. ABHRT enhances antitumor immunity by reducing intratumoral hypoxia which decreases the quantity of MDSC and MDSC expression of PD-L1, resulting in increased levels of $CD40L^+CD4^+$ T cells and $CD8^+$ DC which activate $CD8^+$ TIL.

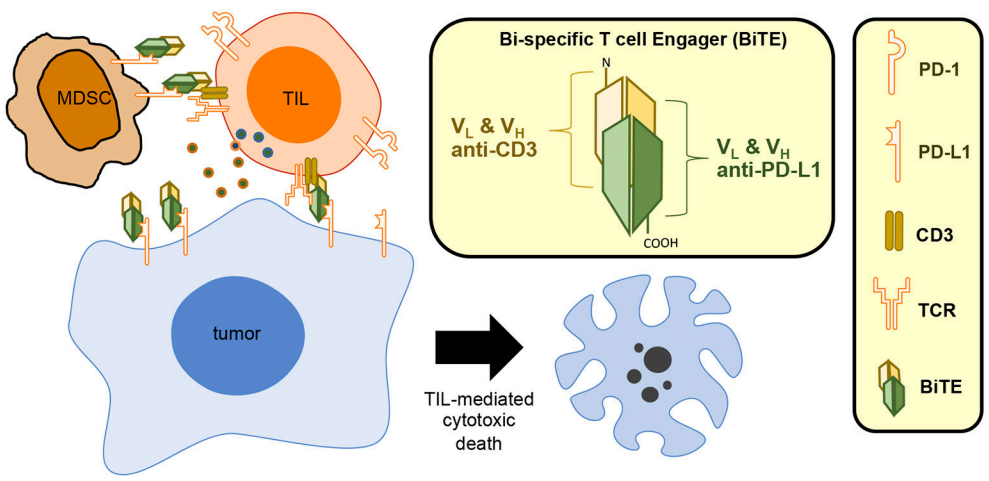

FIGURE 2 | CD3xPDL1 BiTE blocks PD-L1 and induces T cell-mediated cytotoxic death**.** The CD3xPDL1 BiTE consists of the V_H and V_L regions of anti-CD3 and anti-PDL1 linked together to form a 55 kDa single chain structure. The CD3xPDL1 BiTE binds to PD-L1 on $PD\text{-}L1^+$ tumor cells blocking interaction with PD-1 on T cells, thereby preventing PD-1 mediated T cell exhaustion. The BiTE simultaneously binds to CD3 on $CD4^+$ T cells, $CD8^+$ T cells, and NKT cells, activates the cells, and forms a cytotoxic synapse. The activated effector cells then kill the $PD\text{-}L1^+$ tumor cells.

(77, 78). The first BiTE, Blinatumomab, specific for CD19, was FDA-approved for clinical use in 2014 (79). Our CD3xPDL1 BiTE uses the V_H and V_L regions of anti-CD3 mAb in combination with the V_H and V_L regions of the human anti-PD-L1 mAb 4A12 (80) to activate T cells and target them to $PD\text{-}L1^+$ tumor cells. As with other BiTEs, the CD3xPDL1

BiTE has the potential to generate large numbers of cytotoxic $CD3^+$ T cells regardless of T cell receptor expression or MHC genotype, and without costimulation, since the activation occurs via CD3 (81–83).

Binding studies using flow cytometry as the readout demonstrated that the ~55KDa CD3xPDL1 BiTE binds to $CD3^+$ human peripheral blood mononuclear cells (PBMC), and to PD-$L1^+$ human melanoma, chronic myelogenous leukemia, and lung adenocarcinoma cell lines, but not to $CD3^-$ or PD-$L1^-$ human tumor cells. Surface plasmon resonance studies indicated that the BiTE bound to CD3 with a dissociation constant of 2.4×10^{-10} and to PD-L1 with a dissociation constant of 1.28×10^{-11}. The ability of the BiTE to simultaneously bind to $CD3^+$ T cells and to PD-L1 was shown by detecting bound PD-L1-Fc to the BiTE-coated PBMC. When incubated with the BiTE in the presence of PD-$L1^+$ tumor cells, PBMC from healthy human donors were activated as assessed by expression of the activation markers CD69 and CD25, their proliferation, and their production of IFNγ. Importantly, the BiTE-activated healthy donor PBMC were more cytotoxic for PD-$L1^+$ tumor cells than PBMC activated by anti-CD3 mAb by itself, while PD-$L1^-$ cells were not lysed. *In vitro* depletion studies demonstrated that the CD3xPDL1 BiTE not only activated cytotoxic $CD4^+$ and $CD8^+$ T cells, but also activated $CD3^+$ NKT cells (84).

Since cancer patients frequently have MDSC that inhibit T cell activation and function, the CD3xPDL1 BiTE was also tested for its ability to activate cytotoxic cells from small cell (SC) and non-small cell lung cancer (NSCLC) patients. Approximately 24–60% of the PBMC from these patients consisted of M-MDSC ($CD11b^+$HLA-DR^-CD14^+) plus PMN-MDSC ($CD11b^+$HLA-DR^-CD15^+). Despite the high levels of MDSC, the BiTE activated $CD3^+$ cells that specifically lysed PD-$L1^+$, but not PD-$L1^-$ human tumor cells (84). MDSC can express PD-L1 (85), so the ability to lyse tumor cells even in the presence of high levels of MDSC is likely due to BiTE-mediated cytotoxicity of the MDSC. T regulatory cells were not tested in this study. However, since RT induces Tregs (86) and induced Tregs may express PD-L1 (87), the CD3xPDL1 BiTE may also eliminate these cells.

The CD3xPDL1 BiTE was tested for *in vivo* efficacy using immune deficient NSG mice reconstituted with PBMC from healthy human donors ("humanized" mice). Humanized mice were inoculated with a spontaneously metastatic human melanoma and 7 days later the mice were given CD3xPDL1 BiTE for 4 consecutive days and a final dose of BiTE 2.5 weeks later. BiTE treated, but not control mice, had expanded numbers of human $CD3^+$ cells in their spleens, minimal numbers of MDSC, and significantly extended survival times (84).

Collectively, these results suggest that the CD3xPDL1 BiTE might be a useful therapeutic to combine with other cancer immunotherapies and/or with RT. Since MDSC and PD-L1 can be induced by RT (5, 25, 66), and the BiTE expands TIL in response to PD-L1 while inhibiting MDSC, it would be interesting to determine if the CD3xPDL1 BiTE and RT synergize. **Figure 2** shows graphically the structure and function of the CD3xPDL1 BiTE.

THE SOLUBLE FORM OF CD80 (sCD80) NEUTRALIZES PD-L1 MEDIATED IMMUNE SUPPRESSION

PD-L1 not only binds to its receptor PD-1, but also binds to the costimulatory molecule CD80. Mutation analyses demonstrated that PD-1 and CD80 share overlapping binding sites on PD-L1, although the dissociation constant for PD-1/PD-L1 binding is approximately half that of the dissociation constant for CD80-PD-L1 binding (88, 89). This unexpected binding led to the hypothesis that CD80 might bind to PD-L1, thereby interfering with the binding of PD-L1 to PD-1 and facilitating and sustaining antitumor immunity (90). Initial studies of CD80-transfected human melanoma and lung adenocarcinoma cells that constitutively express PD-L1 or are induced by IFNγ to express PD-L1 suggested that CD80 inhibited the plasma membrane expression of PD-L1, despite the transfected cells containing PD-L1 mRNA and protein as assessed by RT-PCR and western blotting. However, the absence of detectable PD-L1 on the plasma membrane was subsequently shown to be due to CD80 sterically blocking the epitope on PD-L1 recognized by the anti-PD-L1 antibodies (91). The ability of CD80 to bind PD-L1 and prevent PD-1 binding was confirmed by assessing the binding of PD-1-Fc molecules to $CD80^+$PD-$L1^+$ and $CD80^-$PD-$L1^+$ human melanoma cells. $CD80^+$PD-$L1^+$ mouse tumor cells similarly did not bind PD-1-Fc, while $CD80^-$PD-$L1^+$ mouse tumor cells bound PD-1-Fc. Flow cytometry using an anti-PD-L1 antibody that recognized a non-CD80-dependent epitope revealed co-localization of PD-L1 and CD80 on the plasma membrane of human tumor cells. Whereas, $CD80^-$PD-$L1^+$ human tumor cells anergized activated PD-1^+ human PBMC and inhibited their production of IFNγ, $CD80^+$PD-$L1^+$ human tumor cells prevented anergy and maintained IFNγ production

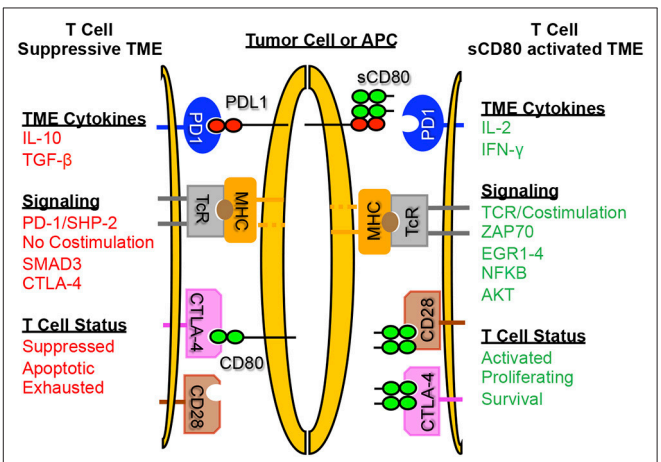

FIGURE 3 | Soluble CD80 activates T cells, blocks PD-1 mediated immune suppression, and promotes anti-tumor immunity. sCD80 acts as a checkpoint inhibitor by blocking PD-L1 on tumor cells and antigen presenting cells while simultaneously binding and activating T cells through CD28. T cells activated by sCD80 have increased IFNγ and IL-2 production and upregulate TCR and CD28 signaling, resulting in an immune-reactive tumor microenvironment with T cell killing of target tumor cells.

(90). Mouse $CD80^+PD\text{-}L1^+$ tumor cells similarly maintained IFNγ production by activated $PD\text{-}1^+$ mouse T cells (91). These results confirmed the hypothesis that CD80 might be a useful therapeutic for preventing the anergizing of any T cells via PD-1.

Since membrane-bound CD80 is not a feasible therapeutic, studies were initiated to determine if a soluble form of CD80 (sCD80 or CD80-Fc) had a similar function. Using four different human tumor cell lines, sCD80, but not an irrelevant Fc-linked protein, maintained IFNγ production by $PD\text{-}1^+$ $CD4^+$ and $CD8^+$ T cells from human donors. A comparison of sCD80 to multiple anti-human-PD-L1 and anti-PD-1 antibodies demonstrated that sCD80 was more effective in maintaining IFNγ-producing activated T cells (91, 92). The latter finding in conjunction with CD80's known costimulatory activity, led to the hypothesis that sCD80 may be a dual agent that simultaneously blocks PD-1 suppression and costimulates through CD28. This hypothesis was confirmed by demonstrating that sCD80 maintained IFNγ production by $PD\text{-}1^+$ activated CD28-deficient mouse T cells, but that the level of IFNγ was significantly higher for $CD28^{+/+}$ $PD\text{-}1^+$ T cells (92). sCD80 costimulation was further confirmed by western blotting and flow cytometry studies demonstrating that sCD80 activates EGR1-4 transcription factors in the CD28 activation pathway and phosphorylates MAPK, and NF-κB in the T cell receptor signaling pathway (93). Thus, sCD80 maintains T cell activation by simultaneously blocking PD-1 suppression and costimulating through CD28. Many tumor and other cells express PD-L1, so sCD80 has the potential to be a generally applicable reagent and is not limited to a specific type of tumor.

In addition to binding to PD-L1 and costimulating through CD28, CD80 also binds to the T cell-expressed co-inhibitory molecule CTLA-4, a receptor that decreases T cell activation and function. The mechanism of CTLA-4-mediated suppression is controversial. Although there is no known inhibitory motif in the cytoplasmic region of CTLA-4, it has been proposed that CTLA-4 functions by negative signaling into activated T cells. Alternatively, it has been suggested that CTLA-4 suppresses T cell function by acting as a "sink" or decoy receptor for CD80 and thereby scavenging CD80 and preventing it from binding to CD28 (94). To resolve if sCD80 suppressed through CTLA-4, $CTLA\text{-}4^+$ activated human T cells were incubated with $PD\text{-}L1^+$ human melanoma cells with or without sCD80 and/or blocking antibody to CTLA-4. Inclusion of anti-CTLA-4 antibody did not increase T cell activation, indicating that CTLA-4 suppression did not occur. Although T cell-expressed CTLA-4 did not impact T cell activation, inclusion of high levels of CTLA-4-Fc did reduce the ability of sCD80 to maintain IFNγ production, suggesting that mechanistically CTLA-4 serves as a decoy receptor (93).

sCD80 injected either intratumorally or systemically delayed tumor progression and extended survival time of syngeneic mice carrying the B16 melanoma or the CT26 renal cell carcinoma. Combination therapy of CT26-bearing mice with intratumoral sCD80 plus CpG further reduced tumor growth. Immunohistochemistry of tumors from systemically-treated mice with CT26 tumors revealed extensive TIL in the tumors of the sCD80-treated mice (93, 95). Studies with C57BL/6 CD28-deficient and PD-1-deficient mice carrying B16 tumors confirmed the earlier *in vitro* findings that sCD80 has the dual functions of inhibiting PD-1-mediated suppression while activating through CD28 (93).

Figure 3 is a graphic depiction of how sCD80 concurrently activates T cells via CD28 and prevents T cell anergy by inhibiting PD-L1/PD-1 binding.

CONCLUSIONS

The use of antibodies to block the PD-1/PD-L1 pathway has been a major advance in the treatment of cancer patients. Since the efficacy of these antibodies depends on patients having tumor-reactive T cells that can be rescued and reactivated by the antibodies, it is essential to combine checkpoint blockade therapy with treatments that activate T cells in patients who do not have constitutively activated lymphocytes. Many cancer patients appear to be in this latter category since checkpoint blockade therapy is only effective in a subset of cancer patients. RT is a natural choice for improving the levels of activated T cells because it induces antitumor immunity both locally and systemically. However, RT can also drive PD-L1 expression and other immune suppressive mechanisms including MDSC. The CD3xPDL1 BiTE and soluble CD80 reagents described here not only inhibit PD-1/PD-L1 suppression, but also activate T cells. Therefore, if combined with RT, the CD3xPDL1 BiTE or sCD80 could synergize with RT to further drive T cell activation while concurrently neutralizing PD-1/PD-L1 immune suppression which may have been induced by the RT. New treatments could be developed where first, ablative hypofractionated RT is utilized to create an immunogenic tumor and reduce MDSC. Next, these novel therapies could be used to simultaneously block PD-L1, eliminate $PD\text{-}L1^+$ tumor cells, and encourage expansion of TILs to eliminate the remaining tumor.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication

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REVIEW
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Radiotherapy in Combination With Cytokine Treatment

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Radiotherapy (RT) plays an important role in the management of cancer patients. RT is used in more than 50% of patients during the course of their disease in a curative or palliative setting. In the past decades it became apparent that the abscopal effect induced by RT might be dependent on the activation of immune system, and that the induction of immunogenic cancer cell death and production of danger-associated molecular patterns from dying cells play a major role in the radiotherapy-mediated anti-tumor efficacy. Therefore, the combination of RT and immunotherapy is of a particular interest that is reflected in designing clinical trials to treat patients with various malignancies. The use of cytokines as immunoadjuvants in combination with RT has been explored over the last decades as one of the immunotherapeutic combinations to enhance the clinical response to anti-cancer treatment. Here we review mainly the data on the efficacy of IFN-α, IL-2, IL-2-based immunocytokines, GM-CSF, and TNF-α used in combinations with various radiotherapeutic techniques in clinical trials. Moreover, we discuss the potential of IL-15 and its analogs and IL-12 cytokines in combination with RT based on the efficacy in preclinical mouse tumor models.

Keywords: radiotherapy, cytokine, immunocytokine, immunotherapy, immunogenic cell death

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INTRODUCTION

The radiation therapy or radiotherapy (RT) started to be used as a cancer treatment modality soon after the discovery of X-rays in 1895 by Wilhelm Röntgen and Marie Curie's discovery of the radioactive elements polonium and radium in 1898. More than 100 years later, RT plays an important role in the therapy of cancer patients and represents a part of the management of more than 50% of patients during the course of their disease. RT is generally used as a primary therapy of localized tumors and regional lymph nodes in a curative setting but also as a palliative treatment to alleviate symptoms or for local control of metastasis. Ionizing radiation is frequently administered in combination with other treatment modalities such as surgery, chemotherapy, hyperthermia, hormone therapy, or immunotherapy (1, 2). RT can be administered as a neoadjuvant intervention to decrease the tumor size, intra-operatively to gain access to neoplastic lesions in a particularly complicated anatomic location or as an adjuvant treatment to prevent disease relapse (3). The most common cancer indications for RT include tumors of breast, lung, cervix uteri, endometrium, stomach, prostate, leukemia, lymphomas, skin, brain, or head and neck (4).

RT utilizes ionizing radiation that delivers its energy via photons, protons, and electrons. High doses of ionizing radiation are employed to kill tumor cells or slow their growth by inducing DNA damage and block the cell division. This process may take days and weeks of treatment before DNA is damaged enough for cancer cells to die, and the cancer cells keep dying over weeks after the termination of radiation treatment. The amount of absorbed radiation in photon RT is measured as joules per kilogram, expressed in the unit gray (Gy) and applied doses vary depending on the cancer type and stage of cancer being treated. The curative use of local ionizing radiation aims at achieving the cancer cells elimination while causing the least toxicity to normal adjacent tissues. The total dose of ionizing radiation is applied in fractions which refers to the delivery of the prescribed dose during separate radiation sessions, usually once per day (5). This provides time to normal healthy cells to recover, while tumor cells are generally less efficient in repair between fractions. Similarly, fractionation can sensitize tumor cells to RT by inducing reoxygenation or shifting the tumor cells to a radiation-sensitive phase of the cell cycle. Fractionation regimens are individualized for different clinical applications. Nevertheless, the standard fractionation schedule ("normofractionation"), which is based on extensive clinical empirical evidence, involves doses of 1.8–2 Gy per day, 5 days a week. The total cumulative dose can differ based on the tumor radiosensitivity and can range from 20 to 40 Gy for lyphomas, from 45 to 60 Gy for most tumor types to control microscopic disease after surgical resection or preoperatively in a neoadjuvant approach and from 60 to 80 Gy for curative purposes in some types of solid epithelial tumors (5). Modified fractionation schedules such as hyperfractionation or hypofractionation are also used. Hyperfractionation involves increasing the number of fractions per day while the dose per fraction becomes lower. This was shown to be beneficial in fast growing tumors such as head and neck squamous cell carcinoma (HNSCC) (6). Hypofractionation means lowering the number of fractions per weak while increasing the dose. Hypofractionation schedules are used in palliative treatments of i.e., bone metastasis (7). Similarly, radiation schedules applying single high radiation doses are employed in stereotactic radiosurgery (SRS) for brain metastasis (8).

RT comprises a wide range of various techniques which are used in dependence on the type of cancer, size of the tumor, anatomic location of the tumor, proximity of the tumor to normal tissue sensitive to radiation, or how radiation is applied to target. The use of RT techniques also depends on the patient's medical history and general health. RT can be broadly divided into 2 groups in dependence on how the radioactivity is applied to target malignant lesions: external-beam radiotherapy (EBRT) and internal radiotherapy (1). EBRT is the most common RT, which is applied on malignant lesions through the intact skin. The internal radiotherapy can be further divided into brachytherapy and systemic RT. In brachytherapy, the radiation source is placed directly at the site of the tumor. Tumors can be treated with very high doses of localized radiation with low probability of damage to the surrounding healthy tissues. This might provide an advantage over EBRT in certain clinical settings. Systemic radioisotope therapy is based on the distribution of a radionuclide or on radioisotopes attached to a tumor-targeting antibody or another tumor-targeting molecule (1). RT has several side effects which are, except for fatigue, associated with the anatomical location of irradiated volumes of the RT fields (4). RT-induced side effects can be broadly divided into early toxicities, occurring during or shortly after the end of RT treatment and late toxicities. Late toxicities occur at least 6 month after the end of RT treatment and are often irreversible (4). Over the last two decades, new RT techniques in the field have been developed and made accessible to cancer patients in routine clinical practice to improve the therapeutic efficacy of RT and to lessen the RT-related toxicities. This involves the introduction of intensity-modulated radiotherapy (IMRT), image-guided radiotherapy (IGRT), stereotactic radiotherapy (SRT), or proton or carbon beam therapy (4). These techniques can greatly increase the therapeutic efficiency by localizing the radiation effect to the target volume, steeper dose gradient, and utilization of imaging methods which leads to lower toxicity to normal tissue and shorter treatment duration (4, 9).

RADIATION-INDUCED EFFECTS ON TUMORS AND IMMUNE SYSTEM

Historically, it was thought that RT exerts immunosuppressive effects. However, in the light of recent research it has been shown that the interaction with immune system is much more complex (10). Unfortunately, the immune response against tumor cells elicited by local RT alone is mostly insufficient to eliminate all tumor cells. In successful RT treatments, beside the direct effect on the irradiated cells, there has been also observed tumor regression in sites distant to the irradiated field called abscopal effect (from the Latin *ab scopus*—away from the target) (11). The abscopal response following radiation is rare in the clinic. Despite millions of patients treated worldwide between 1969 and 2014, the abscopal effect of RT was reported only in 46 cases (11). Recently, more frequent abscopal responses were observed in patients refractory to immunotherapy with checkpoint inhibitors (ICIs) alone, who then received RT in combination with ICIs, e.g., with ipilimumab as reported by Postow et al. (12). The abscopal effect was also observed in patients undergoing RT in combination with other immunotherapeutic approaches such as cytokine therapy, Toll-like receptor (TLR) agonists or adoptive cell transfer therapy (2, 13–15).

On the molecular level, radiation causes DNA damage directly and indirectly by means of induced free radicals. Cytoplasmic double-stranded DNA (dsDNA) is detected by a cytosolic dsDNA sensor cyclic GMP-AMP synthase (cGAS). cGAS is a pattern recognition receptor that triggers IFN-I production via the downstream adaptor stimulator of interferon genes (STING) and is critical for activation of immune response to viruses (16). The RT-induced damage to the cells leads to the exposure and/or release of several damage-associated molecular pattern (DAMP) molecules such as plasma membrane-exposed calreticulin, HMGB1, and ATP during the radiation-induced immunogenic cell death (17). These molecules attract and activate dendritic

cells to phagocytose dying tumor cells, to process and present released tumor antigens to T cells (17). Particularly, BATF3-tumor-infiltrating dendritic cells are stimulated by autocrine production of interferon β (IFN-β) upon detecting cell-derived dsDNA via the cGAS-STING pathway (18). Activated BATF-3-dendritic cells then migrate to tumor draining lymph nodes where they can prime $CD8^+$ T cells to initiate cytotoxic T cell response. Cytotoxic $CD8^+$ T cells migrate to the irradiated tumor and eliminate the residual cancer cells as well as to distant metastatic sites which can lead to a systemic tumor regression, the abscopal effect (10). Besides increasing immunogenicity of tumor cells by inducing immunogenic cell death, RT improves also the access of chemotherapeutic agents and leukocytes into the tumor sites. RT can change the immunosuppressive tumor environment by triggering expression of MHC class I, NKG2D ligands, or FAS/CD95 on tumor cells. RT can stimulate secretion of various proinflammatory cytokines or release of biologically active molecules such as reactive oxygen species and nitrogen species that can act locally to promote cell death of bystander cells (10, 19, 20). On the other hand, RT can hinder the development of anti-tumor immunity by promoting the immunosuppressive tumor microenvironment. Several mechanisms have been documented. Some cytokines, chemokines or growth factors such as tumor growth factor-β (TGF-β), the chemokine C-C motif ligand 2 (CCL2), colony-stimulating factor 1 (CSF-1), C-X-C motif chemokine ligand 12 (CXCL12), or insulin-like growth factor 1 (IRF1) are induced by RT in the tumor environment. These molecules can attract and drive differentiation of immunosuppressive populations such as M2 macrophages, myeloid-derived suppressor cells (MDSC) or directly inhibit the function of immune cells (10, 21). RT can also activate the hypoxia-inducible factor 1α (HIF-1α) which upregulates genes that control angiogenesis, metabolism and metastasis (22). Similarly, some RT regimens lead to upregulation of DNA exonuclease three-prime repair exonuclease 1 (TREX1) which cleaves RT-induced dsDNA and limits the cGAS/STING/IFN-β pathway induction (23). Interestingly, the upregulation of TREX1 was dependent on the RT regimen used. Whereas, 6 Gy and 8 Gy doses enhanced dsDNA without increasing TREX1, 20 Gy induced a prominent upregulation of TREX1 (24). This suggests that the upregulation of TREX1 is determined by the dose of a single RT fraction and not by the total dose applied. The more detailed mechanisms of RT-induced immune activation or suppression are summarized in **Table 1**. Radiation, specifically in combination with immunotherapy, alters the balance between immune-activating and immune-suppressive signals in the tumor microenvironment, however it seems that the success of RT is determined by the intrinsic immunogenicity of the tumor, the type of radiation dose and fractionation regimen and the type of immunotherapy agent used (10).

In 2016, there were 95 clinical trials reported which examined the combinatorial effect of RT and immunotherapy. These trials included mainly ICIs, but also immunostimulatory antibodies, anti-cancer vaccines, oncolytic viruses, TLR agonists, indoleamine 2,3-dioxygenase 1 (IDO1) inhibitors, recombinant cytokines, adoptively transferred cells, and several small molecules with immunostimulatory effects (2, 46). So far, the clinical data of the effective synergy of RT and immunotherapy and reported abscopal effects are largely limited mainly to the combination of anti-CTLA-4 and RT in melanoma (2, 20, 47). Beside the ICIs, clinically significant abscopal effects were observed in a study where the combination of RT and GM-CSF in various tumor types resulted in overall response rate of 26% (11 patients out of 41) (14). The interest in cytokine therapy has been recently renewed mainly by the development of improved IL-2 analogs and IL-15 agonist being currently tested in clinical trials showing lower toxicity and improved therapeutic window (48, 49). Therefore, the main focus of this review is to summarize the data on the anti-tumor effects of combining RT and recombinant cytokines obtained in preclinical testing, but mainly in clinical trials. We discuss the effectivity of RT and cytokine treatment, and potential pitfalls and benefits together with future directions for research on RT and cytokine combinatorial treatment.

CYTOKINES IN CANCER IMMUNOTHERAPY

Cytokines belong to a large diverse family of small glycoproteins that regulate a plethora of physiological functions in a paracrine, autocrine and endocrine manner. They play a crucial role in regulation of the innate and adaptive immunity. The development of recombinant protein technology allowed their use in modulating various pathophysiological conditions including their use in cancer treatment. Despite efforts to develop systemic anti-cancer treatment with cytokines as a standalone therapy, there are several limitations in the form of severe dose-limiting toxicities and generally low objective response rates (durable responses are approximately 10% for a systemic high dose IL-2 therapy). To circumvent this, cytokines are being investigated clinically using novel engineered cytokine mutants (superkines) or chimeric antibody-cytokine fusion proteins (immunocytokines) (50, 51).

To date only few cytokines have been licensed for clinical use in a limited number of oncologic indications. Namely, recombinant IFN-α2a, IFN-α2b, interleukin (IL)-2, granulocyte colony-stimulating factor (G-SCF), granulocyte monocyte colony-stimulating factor (GM-CSF), and tumor necrosis factor α (TNF-α) (50). IFN-α2a is approved for the treatment of hairy cell leukemia and chronic myelogenous leukemia, IFN-α2b for follicular lymphoma, multiple myeloma, AIDS-related Kaposi's sarcoma, melanoma, cervical intraepithelial neoplasms, and hairy cell leukemia, and IL-2 for the treatment of metastatic melanoma and renal cell carcinoma. G-CSF and GM-CSF are approved as immunoreconstituting agents and TNF-α as an oncotoxic factor rather than to augment the anti-tumor immune responses (50). Besides the approved cytokines, there are other cytokines such as IL-12, IL-21, IL-7, IL-15, IFN-γ, IL-8, and IL-18 tested in anti-cancer treatment in clinical trials. These cytokines were tested either as monotherapy but mostly to use their immunoadjuvant potential to boost the effectivity of other therapeutic agents (50, 51). Data on the combination of RT and

TABLE 1 | RT-induced mechanisms that promote or limit the anti-tumor immunity.

Immune-stimulatory effects of RT	Immune-suppressive effects of RT
Induction of DAMPs during immunogenic cancer cell death (17) • dsDNA activates cGAS/STING pathways which leads to Interferon-β (IFN-β) production by irradiated tumor cells as well as tumor-infiltrating dendritic cells (DC), promoting the cross-presentation of antigens to T cells (18)	**Upregulation of three-prime repair exonuclease 1 (TREX1)** • leads to dsDNA degradation and impaired cGAS/STING/IFN-β pathway activation (23)
Reoxygenation of hypoxic tumors • an effect attributed to increased perfusion and decreased oxygen consumption (25)	**Upregulation of HIF-1α transcription factor (25)** • increases PD-L1 expression on tumor cells and myeloid-derived suppressor cells (MDSCs) (26) • induces VEGF-A production from tumor cells which recruits Tregs and MDSC and suppresses DC maturation (27) • increases shedding of NKG2D ligand MICA from tumor cells reducing NK cell killing (28)
Increased secretion of chemokines and upregulation of adhesion molecules • CXCL9, CXCL10, and CXCL16 recruit primed effector T cells to the tumor microenvironment (TME) (24, 29, 30) • adhesion molecules on tumor vascular endothelium contribute to improved T cell infiltration (31)	**Increased production of chemokines and growth factors** • CCL2 recruits monocytes to the tumor site which can be differentiated into suppressive macrophages (32) • CSF-1 enhances recruitment of MDSC (33) • CXCL12 recruits suppressive myeloid cells to TME (34)
Upregulation of MHC class I molecules and NKG2D ligands • in cancer cells to enable recognition by cytolytic T cells (35, 36)	**Conversion of ATP** • released from dying cells to suppressive adenosine by CD39 and CD73 expressed in TME. This leads to a suppression of DC and effector T cells while promoting Tregs and M2 macrophages (37)
Release of tumor antigens from dying cells • processed by DC and presented to T cells in lymph nodes (36) • presented on tumor infiltrating myeloid cells which become sensitive to cytotoxic T cell-mediated killing (38)	**Induction of senescence-associated secretory phenotype (SASP) in cancer-associated fibroblasts (CAFs)** • drives chronic inflammation and protumorogenic TME (39) • activation of insulin-like growth factor-1/receptor (IGF1/IGF-1R) promotes cancer cell growth (40) and M2 macrophage polarization (41)
Upregulation of FAS/CD95 • to facilitate receptor-mediated apoptosis of cancer cells (42)	**Conversion of inactive TGF-β to an active form in TME** (43) • promotes DNA repair (44) • converts $CD4^+$ T cells to Tregs, polarizes M2 macrophages and inhibits priming of $CD8^+$ T cells (45)

cytokine treatment are available only for IFN-α, IL-2, IL-15, GM-CSF, TNF-α, and IL-12, out of which the potential effects of IL-15 and IL-12 with RT have not yet been explored in patients. The summary of clinical trials combining cytokine treatment with some form of RT is shown in **Table 2**. The simplified immune cell-enhancing mode of action of these cytokines in combination with RT treatment is depicted in **Figure 1**.

INTERLEUKIN 2 (IL-2)

IL-2 is a 15.5 kDa glycoprotein composed of four amphipathic α-helixes. IL-2 mediates signaling via three subunits of its IL-2 receptor which include the γ chain (γ_c) (CD132) shared with IL-4, IL-7, IL-9, IL-15, and IL-21, the IL-2Rβ (CD122) shared with IL-15 and the IL-2Rα (CD25) chain. IL-2 signals via its high affinity receptors IL-2Rαβγ and via its intermediate affinity receptors, IL-2Rγβ. IL-2 promotes expansion of antigen-activated $CD8^+$ T cells, acts as an important $CD4^+$ T cell and NK cell growth factor and boosts antibody production in B cells. It activates NK cells and promotes differentiation and proliferation of memory $CD8^+$ T cells. On the other hand, IL-2 plays a crucial role in negative regulation of T cell responses by maintaining and activating regulatory T cells and by inducing Fas-mediated activation-induced cell death (AICD) of T cells (51, 71). At low doses, IL-2 activates mainly regulatory T cells via its high affinity receptors and this might be convenient for treatment of autoimmune diseases (72). At high doses IL-2 induces cytolytic activity of T cells and NK cells involving also signaling via its intermediate affinity receptors on target cells (73). IL-2 was approved by FDA for immunotherapy of metastatic renal cell carcinoma in 1992 and metastatic melanoma in 1998 (48).

Several studies mainly in metastatic melanoma or renal cell carcinoma have been conducted over the last two decades combining IL-2 and various doses and techniques of RT, however, generally with a low efficacy or partial responses.

The combination of rapid fractionation radiation up to 20 Gy followed within 24 h by IL-2 treatment in 28 metastatic patients showed a good tolerability. Four patients showed a significant shrinkage of the tumor at the irradiated site and 2 patients showed an abscopal effect outside the irradiation field (54). Low-dose total body irradiation exhibited a synergistic immune-mediated anti-tumor effect when used in combination with IL-2 in a murine metastatic malignant melanoma model (74–76). Based on this preclinical data a phase II clinical trial combining IL-2 with RT was conducted in metastatic melanoma (52). Forty-five patients received a maximum of 2 cycles of high dose

TABLE 2 | The summary of clinical trials combining cytokine therapy with radiotherapy.

Agent	Indication	Phase	Status/Results	Radiotherapy	References
IL-2	Metastatic melanoma	II	Completed. 2 out of 45 patients PR, 13 patients SD up to 3 months (52)	Low dose total body irradiation	ND
IL-2	Metastatic melanoma, renal cell carcinoma	I	Completed. 1 out of 12 patient CR, 7 patients PR (53)	SABR	ND
IL-2	Metastatic tumors	I	Completed. 6 out of 28 patients showed significant shrinkage of tumor (54)	Fractionated radiotherapy	ND
IL-2	Metastatic renal cell carcinoma	II	Active	SABR	NCT01896271
IL-2	Metastatic renal cell carcinoma	II	Active	SABR	NCT02306954
IL-2	Metastatic renal cell carcinoma, metastatic melanoma	II	Recruiting (55)	Booster radiotherapy	NCT01884961
IL-2	Metastatic melanoma	II	Active	SABR	NCT01416831
L19-IL2	Oligometastatic solid tumors	I	Completed. Results not published.	SABR	NCT02086721
L19-IL2	NSCLC Stage IV	II	Withdrawn (Not yet submitted, unclear timelines)	SABR	NCT02735850
NHS-IL2	Lung cancer, NSCLC	I	Completed. No objective response, 2 out of 13 patients achieved long-term survival (56)	Fractionated radiotherapy	NCT00879866
IL-2, pembrolizumab	NSCLC, metastatic melanoma, metastatic renal cell carcinoma, head and neck carcinoma	I/II	Not yet recruiting	Hypofractionated radiotherapy	NCT03474497
IL-2, ICB	Metastatic NSCLC	I	Recruiting	Hypofractionated radiotherapy	NCT03224871
IL-2, autologous DC vaccine	Renal cell carcinoma	II	Recruiting	Booster radiotherapy	NCT03226236
GM-CSF	Metastatic cancers	II	Completed. 11 out of 41 patients showed abscopal responses (14)	Not specified	NCT02474186
GM-CSF	Hepatocellular carcinoma	II	Recruiting	Carbon ion RT	NCT02946138
GM-CSF, temozolomide	Glioblastoma multiforme	II	Recruiting	Hypofractionated IMRT	NCT02663440
GM-CSF, thymosine 1 alpha	Stage IV NSCLC	II	Recruiting	SABR	NCT02976740
GM-CSF, Poly I:C	Recurrent glioblastoma	I	Not yet recruiting	Not specified	NCT03392545
Oncolytic virus expressing GM-CSF, cisplatin	Squamous cell head and neck cancer	I/II	Completed. 4 out of 17 patients CR, 10 patients PR (57)	Fractionated radiotherapy	ND
GM-CSF, vaccine therapy	Liver metastases	I	Completed. No results published.	External beam radiotherapy	NCT00081848
IL-2, GM-CSF, poxviral vaccine encoding PSA	Prostate cancer	II	Completed. 13 out of 17 patients had increases in PSA-specific T cells compared to RT alone (58)	Not specified	NCT00005916
GM-CSF, IMA950 multi peptide vaccine	Glioblastoma multiforme	I	Completed. 36 out of 40 patients had tumor antigen-specific T cells (59)	Not specified	NCT01222221
GM-CSF, pembrolizumab	Follicular lymphoma	II	Recruiting	Local radiotherapy (1 × 8 Gy)	NCT02677155
GM-CSF, pembrolizumab, GVAX	Pancreatic cancer	II	Recruiting	SBRT	NCT02648282

(Continued)

TABLE 2 | Continued

Agent	Indication	Phase	Status/Results	Radiotherapy	References
IFN-α, DC vaccine	Metastatic melanoma stage III–IV	II	Recruiting	IMRT-IMAT	NCT01973322
IFN-α, retinoic acid	Cervical cancer	II	Completed. No survival benefit compared to RT alone (60)	Not specified	NCT01276730
IFN-α	Melanoma	I/II	Completed. No results published	Not specified	NCT00005615
IFN-α	Melanoma	III	Completed. No results published.	Not specified	NCT00003444
IFN-α, busulfan, cellular therapy	Multiple myeloma and plasma cell neoplasm	II	Completed. No results published.	Not specified	NCT00003195
IFN-α, cisplatin,	Malignant mesothelioma	I	Completed. No results published.	Not specified	NCT00003263
IFN-α, cisplatin, 5-fluorouracil	Pancreatic cancer	II	Completed. Improved 2-year OS (61)	External-beam radiotherapy	NCT00059826
IFN-α, cisplatin, 5-fluorouracil	Esophageal cancer	I/II	Completed. 33 out of 41 patients had pathological response. Improved median survival in responders (62)	External-beam radiotherapy	ND
IFN-α, cisplatin, 5-fluorouracil	Pancreatic cancer	II	Completed. 2-year OS 84% compared to 54% in chemoradiation alone (63)	External-beam radiotherapy	ND
TNF-α	Soft tissue sarcoma	I	Completed. 2 patients out of 13 CR, 9 PR, 1 patient SD (64)	Fractionated radiotherapy	ND
TNF-α	Solid tumors	I	Completed. 5 patients out of 30 CR, 9 PR, 7 MR (65)	External beam radiation	ND
TNF-α, 5-fluorouracil, hydroxyurea	Head and neck cancer	I	Completed. 5 patients out of 12 CR, 5 PR, 2 patient SD (66)	3D conformal or IMRT	NCT00496535
TNF-α	Locally advanced, recurrent, or metastatic solid tumors	I	Completed. Only a protocol available at https://www.liebertpub.com/doi/pdf/10.1089/104303401750214320	Not specified	ND
TNF-α, 5-fluorouracil, cisplatin	Esophageal cancer	I	Completed. 6 patients out of 24 CR (67)	Fractionated radiotherapy	NCT00051480
TNF-α	Metastatic melanoma	II	Completed. No results published.	Not specified	NCT00261404
TNF-α	Rectal cancer	II	Completed. No results published.	Not specified	NCT00137878
TNF-α	Head and neck cancer	I/II	Completed. No results published.	Not specified	NCT00496236
TNF-α, 5-fluorouracil	Pancreatic cancer	III	Completed. 8 patients out of 97 PR, 72 patient SD. No survival benefit compared to Standard of Care group (68)	Fractionated radiotherapy	NCT00051467
TNF-α	Solid tumors	I	Completed. 2 out of 16 patients PR, 5 MR, 4 patients SD (69)	Not specified	ND
TNF-α, 5-fluorouracil	Pancreatic cancer	I/II	Completed. 1 patient out of 50 CR, 3 PR, 12 patients SD (70)	External-beam radiotherapy	ND

ND, no data.

subcutaneous IL-2 and low-dose total body irradiation (single radiation fraction of 0.1 Gy on days 1, 8, 22, and 30). Of note, 0.1 Gy total body irradiation is not comparable to clinical RT. The treatment was well-tolerated but the clinical efficacy was low. In this study, an increase in percentage of cells expressing IL-2Rβ (CD122), an increase in NK cells and a decrease of B cells and monocytes was observed (52). A pilot study assessing the safety and response rate combination of SBRT followed by a high-dose IL-2 regimen in patients with metastatic melanoma and renal cell carcinoma showed complete response or partial response in 8

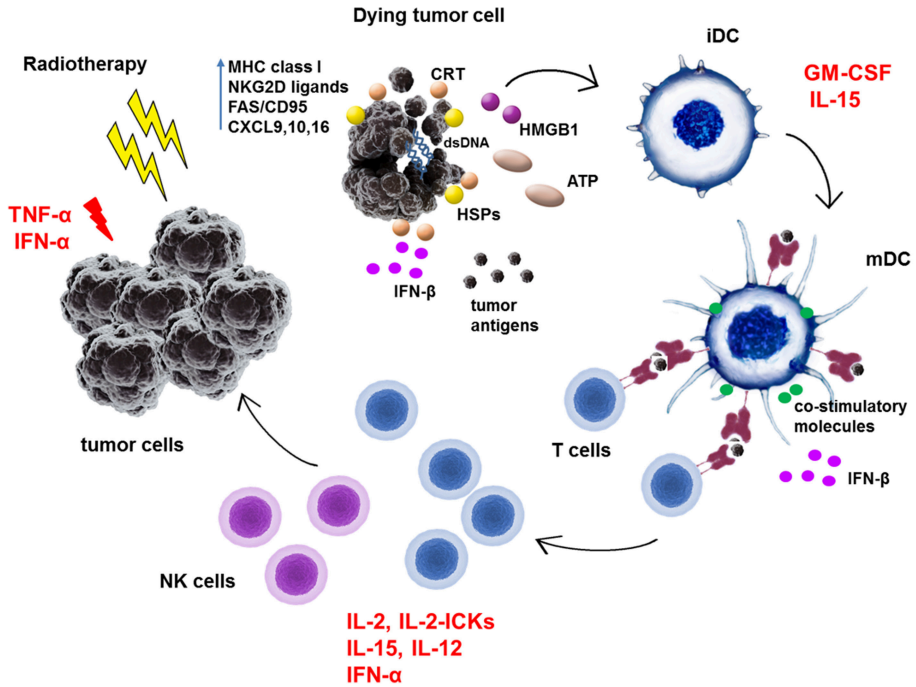

FIGURE 1 | Schematic representation of immunoadjuvant effects of cytokines in combination with radiotherapy treatment of tumors. Radiotherapy was shown to stimulate anti-tumor immunity by increasing expression of MHC class I molecules, NKG2D ligands or FAS/CD95 in tumor cells. RT induces production of chemokines such as CXCL9, 10, and 16 in tumor cells which attract effector T cells to tumor site. RT induces immunogenic cell death and exposure and release of danger-associated molecular patterns (DAMPs) such as calreticulin, HMGB1, ATP, or heat-shock proteins (HSPs). Moreover, RT-generated dsDNA activates via cGAS/STING pathway the IFN-β production in tumor cells as well as in dendritic cells (DC). All of these molecules facilitate the phagocytosis of dead tumor cells and uptake of released tumor antigens by DC. They activate immature dendritic cells (iDC) to process antigens, enhance the expression of MHC class I and II molecules and co-stimulatory molecules such as CD80, CD86, CD83 on its surface to become mature dendritic cells (mDC). In lymph nodes, mDC prime T cells to become effector cells which then exert direct cytotoxic effects on tumor cells or generate the proinflammatory milieu in tumors. TNF-α and IFN-α can directly exert apoptotic effects on tumor cells. GM-CSF mainly activates dendritic cells. IL-2, IL-2 immunocytokines (IL-2-ICKs), IFN-α, and IL-15 activate directly T cells and NK cells to become cytotoxic effector cells. Moreover, IL-15 can bind to its high affinity receptor IL-15Rα on DC and as a part of immunological synapse can enhance T cell functions.

out of 12 patients (53). Currently, there are two ongoing phase II studies to assess the treatment with SABR in combination with a high dose IL-2 regimen for patients with metastatic renal carcinoma (NCT01896271, NCT02306954), one phase II trial for patients with metastatic melanoma (NCT01416831) and one phase II study for both patients with metastatic melanoma and renal cell carcinoma (55) (NCT01884961). Several additional clinical studies examining SABR in combination with IL-2 in metastatic melanoma and renal cell carcinoma are underway (**Table 2**).

Triple combinations of IL-2 administration, RT and immune checkpoint blockade is currently designed in two clinical trials as well as IL-2 and RT combined with autologous DC vaccine (NCT03226236) (**Table 2**). Nivolumab and Ipilimumab with IL-2 and RT will be tested in pilot phase I trial for patients with metastatic NSCLC (NCT03224871) and pembrolizumab in phase I/II trial for patients with various metastatic tumors (NCT03474497).

In recent years, new derivatives of IL-2 such as PEGylated IL-2 (NKTR-214) or IL-2 conjugated with tumor-targeting antibodies (IL-2-based immunocytokines) have found their way to the clinical testing (77, 78). More preclinical data is needed to evaluate the potential of NKTR-214 and RT combination. Currently there are no clinical trials in progress to combine NKTR-214 with RT, but some data have been already collected for IL-2 based immunocytokines.

IL-2-BASED IMMUNOCYTOKINES

IL-2-based immunocytokines tested with RT involve L19-IL2 and NHS-IL2. Both immunocytokines showed promising results in preclinical models (79, 80). L19-IL-2 (Darleukin) is a conjugate of IL-2 and L19 an antibody fragment targeting extracellular domain B of fibronectin (ED-B). L19-IL-2 in combination with RT showed efficacy in preclinical mouse models (81–83). A long-lasting synergistic effect was observed in C51 colon tumor model with 75% of tumors cured (82). The induction of an abscopal effect was observed as well as an increase in memory $CD44^{+}CD127^{+}$ T cells. These preclinical findings set base for the initiation of phase I clinical trial in patients with metastatic solid tumors (NCT02086721), and phase II trial for stage IV NSCLC patients (currently withdrawn) (NCT02735850) (**Table 2**).

NHS-IL2 (selectikine) is an IL-2-based conjugate of a human antibody (NHS76) targeting necrotic tissue (non-membrane-enclosed DNA/histone complexes) fused to genetically modified human IL-2 which selectively targets the high affinity IL-2 receptor (84). Similarly to L19-IL-2, NHS-IL2 was tested in LLC lung carcinoma animal model to examine the efficacy when combined with RT and cisplatin. Mice were treated with NHS-IL2 alone (5 mg/kg; days 7–9), fractionated radiation (3.6 Gy; days 0–4) plus cisplatin (4 mg/kg; day 0), or the triple combination. Tumor regression was observed in 80% of mice when treated with RT and NHS-IL2 and in almost 100% mice when treated with the triple combination (84). Based on these results, a phase I clinical trial in patients with stage IV NSCLC (NCT00879866) was conducted. Patients received local irradiation (5 × 4 Gy) of a single pulmonary nodule. Dose-escalated NHS-IL2 was administered as 1 h intravenous infusion on three consecutive days every 3 weeks. The treatment was well-tolerated and in 2 out of 13 patients it achieved long term survival (84).

INTERLEUKIN 15 (IL-15)

Interleukin I5 (IL-15) is a 15 kDa cytokine structurally similar to IL-2. It belongs to the four-α-helix bundle family of cytokines. The IL-15 receptor involves the γ_c subunit, IL-15Rβ shared with IL-2 and IL-15 specific subunit IL-15Rα. Mainly monocytes, macrophages and dendritic cells produce IL-15. This cytokine induces proliferation of various effector cells including NK cells and $CD8^+$ T cells via mechanism called trans-presentation (85, 86). Soluble IL-15 binds to its IL-15Rα subunit located on the surface of antigen presenting cells, mainly dendritic cells, and then it is ligated to IL-15Rβγ receptors on target cells. IL-15 also supports the IgG production from B cells and activation and maintenance of memory $CD8^+$ T cells. Even though IL-15 displays a similar effect on immune cells as IL-2, there are major differences. Unlike IL-2, IL-15 exerts anti-apoptotic effects on cells and does not expand regulatory T cells (51). IL-15 is the only cytokine found to correlate with the progression-free survival in colorectal cancer patients and with immune cell density within tumors (87). The NCI review listed IL-15 as the most promising cytokine among 12 other immunotherapeutic agents that could potentially cure cancer (88).

Recombinant human IL-15 has been tested in clinical trials as a monotherapy (89, 90), but no patient data are available on its combination with RT. In preclinical research it has been shown that IL-15 can potentiate immune activation induced by RT (91). Poorly immunogenic TSA breast cancer tumors were treated with RT (locally in 8 Gy fractions on days 13, 14, and 15), IL-15 (2 μg/mouse daily for 10 days starting on day 12), or a combination of RT and IL-15. The highest survival was observed in the RT and IL-15 combination group (median 102 days) with 1 of 6 mice showing complete tumor rejection and a development of a long-lasting immunity. Moreover, a significant infiltration of T cells was detected (91).

Similarly to IL-2, there have been various analogs of IL-15 developed to increase the anti-tumor efficacy and lower the toxicity (49). ALT-803 is a mutated IL-15 (N72D) to enhance its biological activity bound to an IL-15RαSu/Fc fusion protein (92, 93). ALT-803 has been evaluated for safety and efficacy in a few clinical trials (94, 95) including combinatorial clinical trials with ICIs. However, the data on the combination of ALT-803 and RT are available only from one preclinical study (96). Here ALT-803 was combined with a SRS in murine glioblastoma model. However, no synergistic effect was observed. More data is necessary to evaluate the effectivity of IL-15 or IL-15 analogs in cancer treatment in combination with RT.

GRANULOCYTE MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF)

Granulocyte macrophage colony-stimulating factor (GM-CSF) is a 23 kDa glycoprotein that binds to a heterodimeric receptor which consists of subunits belonging to the type 1 cytokine receptor family (51). GM-CSF stimulates production of monocytes, neutrophils, and eosinophils. It is produced by various types of cells including T and B lymphocytes, neutrophils, eosinophils, epithelial cells, fibroblasts, and other cells (97). GM-CSF stimulates antigen presentation to the immune system by directly acting on dendritic cells and macrophages (98). GM-CSF was also shown to stimulate the capacity of neutrophils, macrophages and monocytes to mediate antibody-dependent cytotoxicity (51). In contrast to data from preclinical mouse models, the adjuvant effects of GM-CSF in human trials were inconsistent. This may be explained by the capacity of GM-CSF on one hand to stimulate dendritic cells, and on the other hand also to induce myeloid suppressor cells (99).

However, preclinical data show that GM-CSF in combination with RT can help boost the abscopal effect (100). Similarly, the abscopal effect of GM-CSF and RT in a patient with metastatic pancreatic cancer has been documented (101). Based on the preclinical data there had been several clinical trials conducted investigating combination of RT and GM-CSF. A proof-of-principle trial showed that 11 out of 41 patients with various metastatic diseases, developed abscopal responses (14) (NCT02474186). This trial set base for an ongoing phase II trial combining carbon ion RT and GM-CSF for patients with hepatocellular carcinoma (NCT02946138). GM-CSF in combination with RT is also combined or planned to be combined with additional agents (**Table 2**). GM-CSF is planned to be combined with pembrolizumab and RT (1 × 8 Gy) in follicular lymphoma (NCT02677155) or with pembrolizumab, GVAX (GM-CSF gene-transduced tumor cell vaccine) and SBRT in patients with locally advanced pancreatic cancer (NCT02648282). There is a phase II study investigating combination of GM-CSF with hypofractionated IMRT and temozolomide for patients with glioblastoma multiforme (NCT02663440). Another immune enhancer, thymosine-α, is investigated with this combination of GM-CSF and SABR in phase II trial for patients with stage IV

NSCLC (NCT02976740). Similarly, in glioblastoma there is a study planning the intratumoral addition of polyI:C together with GM-CSF and RT (NCT03392545). A phase I/II study combining chemoradiotherapy with a herpes simplex type 1 oncolytic virus expressing GM-CSF in patients with HNSCC was conducted (57). The treatment was well-tolerated, 14 out of 17 patients showed response to the treatment and pathologic complete remission was confirmed in 93% of patients at neck dissection. GM-CSF treatment was also used in combination with RT and PSA tumor antigen-encoding poxviral vaccines (58) or with multipeptide vaccines (59) where an increase of antigen-specific T cells was detected in comparison to control arms.

INTERFERON ALPHA (IFN-α)

Interferon alpha (IFN-α) is member of the type I interferon family and acts as immune modulator with antiviral and anti-proliferative properties (102, 103). Twenty IFNs have been identified in humans, out of which the most subtypes belong to the IFN-α group. Type I IFNs signal via a common pair of receptors, IFNAR1 and IFNAR2. IFN-α is produced mainly by plasmacytoid dendritic cells but also by most of other cell types in response to encounter with DAMPs by pattern recognition receptors (PRRs) which can be produced by virus-infected or cancer cells (104, 105). IFN-α induces MHC class I expression on tumor cells, induces apoptosis of tumor cells, mediates maturation of dendritic cells and activation of B and T cells, and displays antiangiogenic properties (51).

IFN-α has been approved by FDA for treatment of several malignancies including hairy cell leukemia, chronic myelogenous leukemia, follicular lymphomas, malignant melanoma, multiple myeloma, or renal cell carcinoma (106, 107) but displays also significant toxicity and side effects such as flu-like symptoms, anorexia, fatigue, depression. It has been shown in *in vitro* models that IFN-α has a synergistic cytotoxic effect with chemotherapy and RT (108, 109). This synergistic effect as well as radiosensitizing effect of 5-fluorouracil was also observed in patients with small cell lung cancer and anal cancer (110, 111). A phase I/II study combining 5-fluorouracil, cisplatin, IFN-α and concurrent EBRT before resection in patients with advanced esophageal cancer resulted in 80% of the patients responding to the therapy but the authors claimed that the contribution of IFN-α to the treatment was uncertain (62). In a preliminary phase II study, patients with pancreatic cancer underwent similar adjuvant therapy of 5-fluorouracil, cisplatin, IFN-α and RT after pancreaticoduodenectomy (63). The study showed better survival in the group of patients receiving IFN-α in comparison with patients with similar adjuvant therapy without IFN-α. In a similar multicenter phase II study (NCT00059826), patients with pancreatic cancer undergoing this adjuvant therapy had better overall survival results but the study had to be terminated prematurely due to the high toxicity of treatment (61). Acceptable toxicity was observed in phase II clinical trial (NCT01276730) where patients with stage III cervical cancer were treated with RT in combination with IFN-α and retinoic acid (60). Unfortunately, there was no survival advantage in comparison with the group receiving RT and cisplatin. In melanoma, there have been several trials examining treatment with IFN-α and various forms of RT with mixed outcomes involving high toxicity of the combinatorial treatment as well as generally low efficacy. These studies are summarized extensively in the review of Barker and Postow (112).

A phase II study investigating the combination of RT and IFN-α with a dendritic cell-based vaccine for patients with metastatic melanoma is currently ongoing (NCT01973322). Two other clinical studies combining chemotherapy and/or cellular therapy and IFN-α have been completed (NCT 00003195 and NCT00003263) (**Table 2**).

TUMOR NECROSIS FACTOR α (TNF-α)

Tumor necrosis factor alpha (TNF-α) is a strong proinflammatory cytokine with anti-tumor activity both *in vitro* and *in vivo* (113, 114). TNF-α is produced mainly by macrophages, granulocytes and epithelial cells but also by other types of cells and its anti-tumor properties are due to direct cytotoxic and antiangiogenic effects (70). Also it has been shown that TNF-α acts as a radiosentitizer and enhances cytotoxic effect of radiation (115). Despite of its anti-tumor properties, the systemic administration of TNF-α in a sufficient dose is associated with a high toxicity (116, 117). Thereby the use of TNF-α in cancer therapy is limited to isolated limb perfusion (ILP) of advanced melanoma and soft tissue sarcoma (118, 119). The only clinical testing of TNF-α and RT involves gene therapy delivering TNF-α gene to cancer cells—TNFerade™ (120, 121). TNFerade is an adenovector containing TNF-α gene with early growth response gene (Egr-1) radiation activated promoter that is injected intratumorally. In preclinical models this combination showed remarkable anti-tumor effects with minimal toxicity (122). Several trials combining TNFerade and RT or chemoradiotherapy were conducted in patients with various types of tumors including breast, lung, pancreatic, head and neck, rectal cancer, melanoma, esophageal cancer, or soft tissue sarcoma (64–67, 69, 70, 123, 124). These studies showed that the treatment is well tolerated with complete or partial tumor responses and complete tumor regressions in some patients (**Table 2**). Despite these favorable results a phase III clinical trial randomizing patients with locally advanced pancreatic cancer to 2:1 groups standard of care (SOC) plus TNFerade vs. SOC alone showed no survival benefit of the patients in SOC plus TNFerade group (68). This dampened the enthusiasm of using this approach and there are currently no open clinical trials (**Table 2**).

INTERLEUKIN 12 (IL-12)

IL-12 is a four-bundle α-helix heterodimeric cytokine encoded by two genes: *IL-12A* (p35 subunit) and *IL-12B* (p40 subunit). The active IL-12 forms a heterodimer of p35 and p40 subunits referred as p70. The receptor for IL-12 consists of IL-12Rβ1 and IL-12Rβ2. IL-12 is produced by macrophages, dendritic cells and B cells. IL-12 induces proliferation of T cells and NK cells as well as their IFN-γ production. IL-12 polarizes Th1 immune

response and displays antiangiogenic properties (51). The anti-tumor efficacy of IL-12 was shown in several animal models (125–127). IL-12 has shown promising results in preclinical studies but the clinical trials did not result in satisfactory outcome. As IL-12 displays a high systemic toxicity, the local treatment in the form of gene or viral therapy was tested in combination with RT in preclinical models (128, 129). A non-viral murine IL-2 and IL-12 gene therapy and external beam radiation (2 × 1 Gy) was tested in HNSCC in an orthotopic murine model (130). A significant increase in anti-tumor effects and T lymphocyte infiltration was detected in comparison to single therapies and the control. Furthermore, the anti-tumor and anti-metastatic activity of the oncolytic adenovirus expressing IL-12 and GM-CSF injected intratumorally in combination with RT was investigated in a murine hepatic cancer (HCa-I) model (131). This combinatorial therapy was effective in suppressing primary tumor growth and an increased immune cell infiltration was observed. The therapeutic effect of the naked IL-12 cytokine combined with fractionated RT was investigated in Lewis lung carcinoma mouse model (132). The treatment was effective against primary tumor and the number of lung metastasis decreased. A pronounced tumor growth delay was observed when GM-CSF was added together with IL-12 and fractionated RT. IL-12, similarly to IL-2, has been fused to an antibody tumor-necrosis targeting IgG1 (NHS76) to create a novel immunocytokine NHS-IL12 (133). NHS-IL12 immunocytokine exhibited a longer half-life and a selective tumor targeting *in vivo*. NHS-IL12 showed a superior anti-tumor effect when combined with RT in MC38 mouse colorectal cancer model (133). Currently there are no clinical trials combining IL-12 cytokine therapy or NHS-IL12 immunocytokine with RT.

CONCLUSIONS AND FUTURE PERSPECTIVES

Cancer immunology has made a remarkable progress, which led to the development of various immunotherapies that can be combined with ionizing radiation. The combination of RT and immunotherapy represents a growing field of clinical investigation with an increasing number and various types of clinical trials (2). Despite partial therapeutic success of the combination of RT and immunotherapy including the rare abscopal effect, most patients do not respond to RT and immunotherapy, and the same goes for using cytokine adjuvant treatment and RT. For RT itself, there are important challenges to overcome and there is a need to conduct rigorous research. These include the selection of appropriate radiation dose, fractionation, appropriate technique for RT, sequencing of therapies and selection of meaningful endpoints in clinical trials (134). Radiation dose and regimen is likely to be a critical determinant in successful generation of an anti-tumor response. Radiation dose and regimen largely affect both the immunomodulatory and cytotoxic effects of RT. These might attenuate the immunosuppressive environment but might not induce the immunogenic cell death of cancer cells to elicit strong anti-tumor responses. In the same line, although the cytokine therapy as documented with IL-2 can induce significant durable responses in patients, there are strong limitations, which lie mainly in the toxicity after the systemic administration. This can be mitigated by intratumoral administration of cytokines, which on the other hand, might represent technical challenges for the clinicians, or targeted versions of cytokines (e.g., immunocytokines). Out of the clinically tested cytokines IL-2, IFN-α, GM-CSF, and TNF-α, overall only IL-2 and GM-CSF combinatorial treatment with RT showed some even clinically relevant efficacy with acceptable toxicity. Novel analogs of IL-2 engineered to mitigate the toxic effects and to reduce the induction of immunosuppressive T regulatory cells might increase the efficacy of treatment with radiation. Similarly, analogs of IL-15 showing promising results in clinical trials when combined with ICIs hold the potential to boost the immunogenic effect of RT. From this summary, it becomes clear that multiple combinations using cytokines and RT together with some other immunotherapeutic approaches might hold the promise to increase the clinical benefit of cancer patients. As we are only beginning to explore the possibilities of multiple immunotherapeutic combinations, the understanding how to best integrate the scientific rationale, mode of actions and the most effective therapeutic regimens remains of urgent need.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Tumor Cell-Based Vaccine Generated With High Hydrostatic Pressure Synergizes With Radiotherapy by Generating a Favorable Anti-tumor Immune Microenvironment

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Dendritic cell (DC)-based vaccines pulsed with high hydrostatic pressure (HHP)-inactivated tumor cells have been demonstrated to be a promising immunotherapy for solid tumors. We focused on sole injection of tumor cells that were inactivated by HHP and their combination with local radiotherapy (RTx) for *in vivo* induction of anti-tumor immune responses. HHP-treatment of tumor cells resulted in pre-dominantly necrotic cells with degraded DNA. We confirmed that treatments at 200 MPa or higher completely inhibited the formation of tumor cell colonies *in vitro*. No tumor growth was seen *in vivo* after injection of HHP-treated tumor cells. Single vaccination with HHP-killed tumor cells combined with local RTx significantly retarded tumor growth and improved the survival as shown in B16-F10 and CT26 tumor models. In B16-F10 tumors that were irradiated with 2 × 5Gy and vaccinated once with HHP-killed tumor cells, the amount of natural killer (NK) cells, monocytes/macrophages, CD4+ T cells and NKT cells was significantly increased, while the amount of B cells was significantly decreased. In both models, a trend of increased CD8+ T cell infiltration was observed. Generally, in irradiated tumors high amounts of CD4+ and CD8+ T cells expressing PD-1 were found. We conclude that HHP generates inactivated tumor cells that can be used as a tumor vaccine. Moreover, we show for the first time that tumor cell-based vaccine acts synergistically with RTx to significantly retard tumor growth by generating a favorable anti-tumor immune microenvironment.

Keywords: radiotherapy, immunotherapy, tumor cell-based vaccine, high hydrostatic pressure, malignant melanoma, colorectal carcinoma, tumor-infiltrating leukocytes, tumor microenvironment

INTRODUCTION

In recent years, cancer immunotherapy has revived. It comes in a variety of forms, including checkpoint inhibitors, targeted antibodies, adoptive cell transfer, tumor-infecting viruses, cytokines, adjuvants, and cancer vaccines. Cancer vaccines aim specifically to activate the immune system in cancer patients (1). As dendritic cells (DCs) link the innate and adaptive immune system as powerful antigen-presenting cells, they were used as cancer vaccines in several clinical trials. DC-based immunotherapy has been demonstrated to be safe and capable of inducing anti-tumor immunity. Long-term survival in advanced melanoma patients undergoing DC vaccination is similar to ipilimumab-treated patients (2). Nevertheless, the response rates are often low. Improved vaccines with higher immunogenicity and particularly combination with other tumor therapies should therefore be implemented (3).

High hydrostatic pressure (HHP)-treatment is an innovative method for the generation of whole cell-based tumor vaccines. Although HHP has been mainly used in the food industry for processing and preserving meat and other food to avoid thermal treatment (4). Though HHP is known to denature proteins, it doesn't affect covalent bonds, meaning that the proteins' primary and secondary structure is maintained, whereas their tertiary and quaternary structure is changed (5). Urbanova et al. showed that HHP-treatment of tumor cells affects the antigenic pool and that loading of DCs with HHP-killed tumor cells can induce CD8+ T cell responses *in vitro* (6). Fucikova et al. demonstrated that HHP-treatment induces immunogenic cancer cell death in human tumor cells and that interaction of HHP-killed cancer cells with DCs results in phagocytosis of the tumor cells and activation of the DCs (7). DCs pulsed with HHP-killed cancer calls can be used as cancer vaccine (8). Based on these data, *ex vivo* HHP-killed tumor cell-loaded DCs are currently being tested in clinical trials as therapeutic cancer vaccines. For this, patient's monocyte-derived DCs pulsed with HHP-killed allogeneic tumor cell lines (DCVAC) are used to treat prostate, ovarian and lung cancer (NCT03514836, NCT03905902, NCT02470468). One has to stress that such tumor vaccination is well-combinable with chemotherapy (9).

We have aimed to test whether sole injection of HHP-killed tumor cells without DCs can also be used as a cancer vaccine in a multimodal approach together with RTx, hypothesizing that under distinct *in vivo* micro-environmental conditions such inactivated tumor cells are taken up by endogenous DCs. We already demonstrated in previous work that murine CT26 tumor cells are effectively inactivated by HHP-treatment and that specific IgG antibodies against tumor cells were significantly increased after immunization of mice with HHP-treated tumor cells (10). This work gave first hints that sole injection of HHP-killed tumor cells is capable of triggering anti-tumor immune responses *in vivo*. In variance to the approach of DC pulsed vaccines, we use syngeneic rather than allogeneic tumor cells for vaccination. This syngeneic vaccine mimicking in cancer patients autologous vaccine from their own tumor cells should contain all potentially relevant tumor-associated antigens (TAAs) for a particular patient (11).

It should be stressed that HHP treatment fulfills the main requirements for clinical vaccine: it effectively inactivates tumor cells, it has no intrinsic toxicity, it does not destroy the immunogenicity of the tumor cells and it can be applied with legal and GMP-compliant requirements. Further, it is further a highly reproducible and easy to apply method (12). Therefore, HHP is advantageous to other preparation methods such as heat killing, radiation, or freeze-thaw approaches.

We performed our pre-clinical studies with two broadly used B16-F10 melanoma and CT26 colorectal cancer models. Although malignant melanoma is an aggressive disease with rising incidence and high resistance to classical therapy, targeted therapies and immune therapy have significantly improved the treatment of patients with advanced malignant melanoma in recent years (13). In colorectal cancer, the proportion of patients with an immunosuppressive tumor microenvironment is high, again calling for combination therapies that modulate the immune system (14).

Emerging evidence suggests that radiotherapy (RTx) is capable of activating the patient's immune system by acting as an *in situ* cancer vaccine (15, 16). RTx modifies the phenotype of the tumor cells and the tumor microenvironment (17). It however results in both, immune activation and immune suppression (18). Therefore, the combination of RTx with immunotherapy has the potential to induce regression of tumors, even outside of the radiation field (19).

It has become evident that in established cancers anti-tumor vaccines will require co-treatments to overcome immune evasion (20). RTx might act as adjuvants for the vaccine and this combination might be effective in generating anti-tumor immune responses. Here we show for the first time that a single vaccination with HHP-killed tumor cells combined with local RTx significantly retards tumor growth and improves survival of tumor-bearing mice by generating a favorable anti-tumor immune environment as analyzed in B16-F10 and CT26 tumor models.

Abbreviations: AnxA5, AnnexinA5; CTLA-4, cytotoxic T-lymphocyte-associated Protein 4; DC, dendritic cell; FELASA, Federation of European Laboratory Animal Science Associations; GM-CSF, granulocyte-macrophage colony-stimulating factor; GV-SOLAS, Gesellschaft für Versuchstierkunde; HHP, high hydrostatic pressure; HLA, human leukocyte antigen; NK, natural killer; PI, propidium iodide; PD-1, programmed cell death protein 1; RTx, radiotherapy; SD, standard deviation; SEM, standard error of the mean; TAA, tumor-associated antigen; TIL, tumor-infiltrating leukocytes.

MATERIALS AND METHODS

Cell Lines and Cell Culture

B16-F10 melanoma and CT26 colon carcinoma cells were both obtained from ATCC (Manassas, VA, USA). The tumor cells were grown up to a maximum confluence of 80% at 37°C, 5% CO_2, and 95% humidity, in RPMI 1640 (Sigma Aldrich, Munich, Germany) with the addition of 10 % fetal bovine serum (FBS, Biochrom AG, Berlin, Germany) and 1% penicillin-streptomycin (PenStrep, Gibco, Carlsbad, USA).

High Hydrostatic Pressure Treatment

After detaching of the adherent tumor cells, the cell suspension was transferred into cryovials (Greiner Bio-one, Frickenhausen, Germany). The vials were filled completely (2.5 ml) and closed tightly by avoiding any air bubbles. Afterwards the vials were sealed with Parafilm^{TM} (American National Can, Chicago, USA) to prevent leaking.

The equipment for HHP-treatment (**Supplemental Figure 1**) was provided by the "Institut für Prozessmaschinen und Anlagentechnik" (iPAT, Friedrich-Alexander-Universität Erlangen-Nürnberg). For pressurizing the tumor cells, the cryovial with the cell suspension was put into the autoclave (1). Pressure that was built up at a velocity of around 5 MPa/s by a manual spindle press (2) in addition to a pneumatic pump (3) was transmitted to the autoclave via a system of metal tubes (4) containing pressure transmitting fluid (hydraulic oil Ultra-Safe 620, Petrofer, Hildesheim, Germany). According to Pascal's law, pressure which is generated and transmitted by the transmitting fluid acts to the same amount on the cells filled in the cryovials. The fluid is stored in a reservoir (5) that is attached to the aperture and the pressure can be recorded via a digital manometer (6). The pressure is maintained and released by several switches (7) in the aperture. Since different pressure levels showed promising results for inactivation of tumor cells in earlier studies (7, 10, 21–23), we also first tested pressure from 100 to 500 MPa at a compression time of 300 s for some *in vitro* examinations of the vaccine. According to the 3Rs concept for more ethical use of animals in testing, namely replacement, reduction and refinement, we focused on whole tumor cell-based vaccines generated with 200 MPa for the *in vivo* tumor models. Generally, after pressurizing, the tumor cells were first re-cultivated in cell culture flasks (Greiner Bio-one, Frickenhausen, Germany).

Cell Death Detection by AnnexinA5/Propidium Iodide Staining

For analyses of cell death forms by flow cytometry (EPICS XL MCL, Beckman Coulter, Brea, USA), HHP-treated tumor cells were suspended in 400 µl Ringer (B. Braun, Melsungen, Germany) and stained with FITC-labeled AnnexinA5 (AnxA5; 0.2 µl, Geneart, life technologies, Regensburg, Germany) and propidium iodide (PI; 0.4 µl, Sigma Aldrich, Munich, Germany) according to the protocol of Vermes et al. (24). AnxA5-negative/PI-negative cells were considered as viable ones, AnxA5-positive/PI-negative as apoptotic cells and AnxA5-positive/PI-positive cells as necrotic ones.

Cell Cycle and SubG1 DNA Content Analyses With Propidium Iodide

1×10^6 tumors cells were fixed in 70 % ethanol and incubated at −20°C for at least 20 min. Afterwards, a solution containing Triton X-100 (Sigma Aldrich, Munich, Germany), 200 µg/ml RNase (Biochemica, Buchs, Germany), and 5 µg/ml PI was added at room temperature for at least 30 min. The cell cycle phases were consecutively analyzed by flow cytometry. Apoptotic and secondary necrotic cells that lost their nuclear DNA content due to DNA fragmentation show subG1 DNA content (25).

Monitoring of the Clonogenicity of HHP-Treated Cells *in vitro* and *in vivo*

In vitro, the pressurized tumor cells were plated in multiplicates at increasing concentrations in petri dishes (BD Falcon, New York, USA) and cultivated for 10 days. After staining the cells with 3 ml methylene blue (Sigma Aldrich, Munich, Germany), colonies consisting of more than 50 cells were scored (26). For *in vivo* analysis, a suspension of 2×10^6 treated tumor cells in Ringer's solution was injected subcutaneously into mice. The subsequent tumor growth was analyzed up to 39 days after injection of the tumor cells.

Multimodal Treatment of Tumor-Bearing Mice

All animal experiments were conducted according to the guidelines of the "Federation of European Laboratory Animal Science Associations" (FELASA) and the "Gesellschaft für Versuchstierkunde" (GV-SOLAS) and were authorized by the government of Mittelfranken/Unterfranken. C57BL/6 mice were inoculated subcutaneously with 1×10^6 viable B16-F10 melanoma cells. After 8 days, when a visible and vascularized tumor was established, the mice were either locally irradiated with 2 × 5Gy at day 8 and 10, subcutaneously vaccinated next to the tumor with 5×10^6 24 h-aged HHP-treated cells without any additional adjuvant on day 11, locally irradiated plus vaccinated, or left untreated. For the induction of CT26 tumors, Balb/c mice were injected subcutaneously with 1.2×10^6 viable CT26 colon carcinoma cells. In this tumor model, palpable tumors were established after 14 days. Beginning on that day, the treatment was conducted in the same scheme as for the B16-F10 cells. Since pressure of 200 MPa showed promising results *in vitro* and in former studies (10, 12, 23) and according to the 3Rs concept for more ethical use of animals in testing, this pressure level was used for the *in vivo* experiments. Tumor growth was determined with an electronic caliper. The tumor volume was calculated by the formula $VTumor = ½ \cdot (L \cdot B2)$ (27). Mice were sacrificed whenever the tumor volume exceeded 1,600 mm^3 or the well-being of the mouse was reduced according to approved criteria. A PRIMART linear accelerator (Siemens, Munich, Germany) was used for RTx. The local irradiation of the tumor-bearing mice was performed closely resembling the clinical situation as previously established and applied by our group (28, 29).

Immune Phenotyping of Tumors and Blood

Tumor samples and whole blood for immune phenotyping by multicolor flow cytometry were taken in a group of mice on day 7 after first irradiation. Erythrocyte lysis of blood samples was performed with a TQ-Prep^{TM} Workstation (Beckman Coulter, Brea, USA) prior to the antibody staining. Single cell suspensions from tumors were obtained with the Tumor Dissociation Kit and the gentleMACS^{TM} Dissociator according to the manufacturer's instructions (Miltenyi Biotec, Bergisch

Gladbach, Germany). To enrich tumor samples for tumor-infiltrating leukocytes (TIL), CD45 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for subsequent separation with MACS® Technology according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) were used. The following antibodies were applied for the staining of both, tumor and blood samples: CD3e V450, CD4 FITC, Ly-6C FITC, CD11c BV510, CD19 APC-Cy7, and Ly6G PE-Cy7 (all from BD Biosciences, Franklin Lakes, USA), CD49b APC, PD-1 PE/Dazzle 594, CD8a BV605, Zombie NIR and Zombie Aqua (all from Biolegend, San Diego, USA), CD11b APC, CD45.2 PerCP-Cy5.5, MHC-II (I-A/I-E) eFluor 450 and γδTCR PE (all from eBioscience, San Diego, USA). All samples were acquired with a CytoFLEX S flow cytometer (Beckman Coulter, Brea, USA) and analyzed with the Kaluza software (Beckman Coulter, Brea, USA). To calculate the concentration of tumor infiltrating leukocytes (cells/g tumor), tumors were weighed prior to dissociation.

Statistical Analysis

The types of statistical test for data analyses are depicted in the figure legends. Results were considered statistically significant for $p < 0.05$ (*) and highly significant for $p < 0.01$(**).

RESULTS

High Hydrostatic Pressure Treatments at 200 MPa or Higher Pre-dominantly Induce Necrosis in Tumor Cells

While viability of B16-F10 melanoma cells was hardly affected by pressurizing with 100 MPa, treatments at 200 MPa or higher

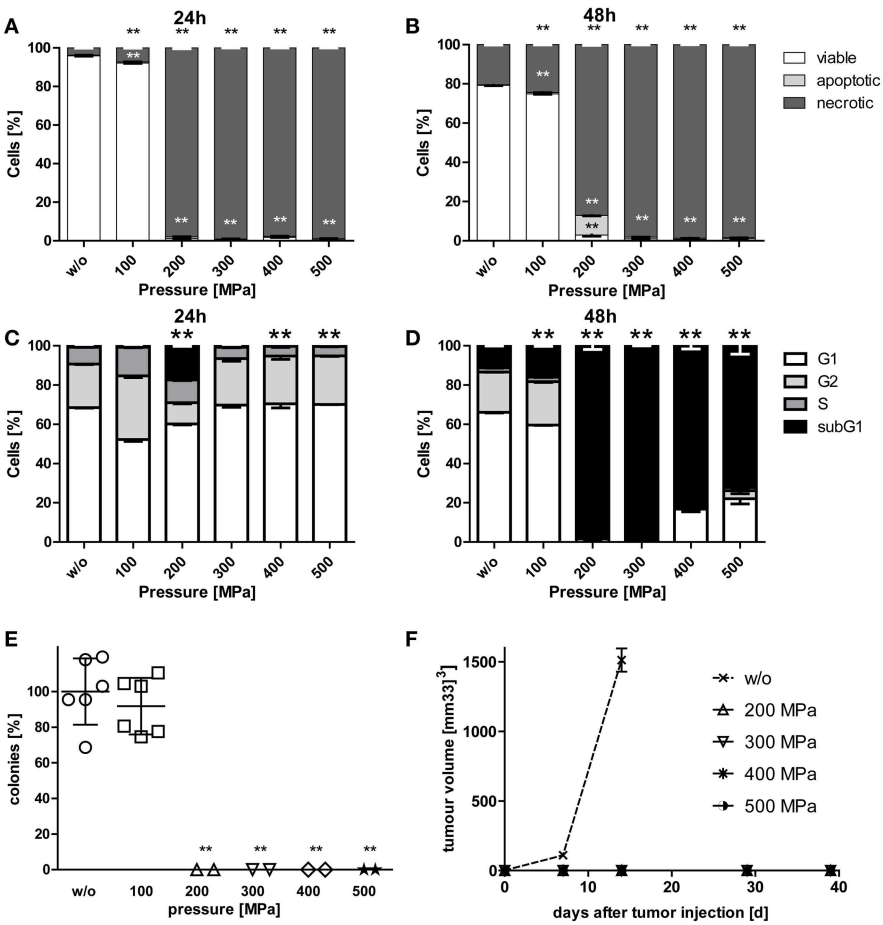


FIGURE 1 | Cell death and clonogenicity of B16-F10 melanoma cells following treatment with HHP. B16-F10 melanoma cells were treated with pressure at 100 MPa up to 500 MPa for 5 min and afterwards cultured for 24 **(A,C)** and 48 h **(B,D)**, respectively. The cells were either stained with AnxA5-FITC/PI **(A,B)** or PI in the presence of detergent **(C,D)** and cell death as well as cell cycle phases were analyzed by flow cytometry. The percentages of viable (AnxA5$^-$/PI$^-$), apoptotic (AnxA5$^+$/PI$^-$), and necrotic (AnxA5$^+$/PI$^+$) cells are displayed in **(A)** and **(B)**. The percentages of cells in the G1-, G2-, and S-phase as well as the subG1 DNA content are displayed in **(C)** and **(D)**. Data of three independent experiments are presented as mean ± SD. **(E)** shows the *in vitro* colony formation of HHP-treated B16-F10 melanoma cells. Single values, means, and SDs are presented. **(F)** displays the growth of syngeneic B16-F10 tumors in C57BL/6 mice after subcutaneous injection of 2×10^6 HHP-treated tumor cells. Three mice were used for each treatment condition. Data are presented as mean ± SEM. w/o: mock-treated control. Significant values are determined by an unpaired, one-tailed Student's *t*-test with Welch's correction for unequal variances; $^{**}p < 0.01$ related to w/o.

resulted in mostly necrotic tumor cells. Small percentages of apoptotic melanoma cells were observed when the tumor cells were treated with 200 MPa (**Figures 1A,B**). The latter pressure already resulted in degraded tumor DNA as early as 24 h after treatment. Almost all melanoma cells had degraded DNA 2 days after pressurizing if they were treated with pressure above 100 MPa (**Figures 1C,D**).

High Hydrostatic Pressure Treatments at 200 MPa or Higher Effectively Inactivate Tumor Cells

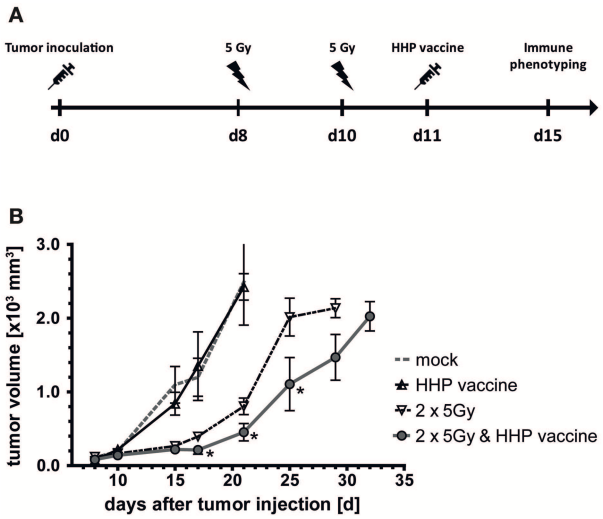
To prove the inactivation of B16-F10 melanoma cells after HHP-treatment, their potential to form colonies *in vitro* (**Figure 1E**) and their potential for progression *in vivo* after having been injected into C57BL/6 mice was analyzed (**Figure 1F**). Treatments at 200 MPa or higher completely inhibited the formation of colonies *in vitro*. Further, no tumor growth was seen *in vivo* after tumor cell injection. Notably, pressure of 100 MPa is not sufficient to suppress colony formation of melanoma cells. Similar results were already previously observed for CT26 cells (23).

Combination of RTx With Whole Tumor Cell-Based Vaccine Generated by HHP Significantly Retards Tumor Growth in C57BL/6 Mice and Increases Their Survival

Eight and 10 days after tumor inoculation, the tumors were locally irradiated with 2 × 5Gy and vaccination with HHP-treated tumor cells was performed at day 11 (**Figure 2A**). Vaccination with HHP-treated cells was not sufficient to significantly slow-down the tumor growth (**Figure 2B**). At day 21 after tumor inoculation, all mice of the vaccination and control group had to be euthanized, because the tumor volume had exceeded 1,600 mm^3. RTx resulted in significantly retarded tumor growth when compared to vaccinated or mock-treated animals. Vaccination with HHP-treated cells in addition to RTx at day 11 resulted in further significant tumor growth retardation and even at day 32 after tumor inoculation three animals could still be monitored. Similarly to the tumor growth reduction, the survival of the mice could be significantly improved by RTx alone, and was further significantly improved when RTx was combined with vaccination (**Figures 2C,D**).

Combination of RTx With Whole Tumor Cell-Based Vaccine Generated by HHP Generates a Beneficial Immune Cell Infiltrate for Melanoma

A sole vaccination of the mice with HHP-killed tumor cells did not affect infiltration of cells of the innate and adaptive immune system into B16-F10 tumors. RTx with 2 × 5Gy slightly, but not significantly enhanced the infiltration of NK cells, monocytes/macrophages, DCs and NKT cells. Only the combination of RTx with HHP vaccine significantly increased the total number of immune cells (CD45+) per gram of tumor, which were almost 3 fold higher compared to mock-treated controls



D

Log Rank Test	mock	HHP vaccine	2 x 5Gy	2 x 5 Gy & HHP vaccine
mock		n.s.	0.0004	0.0002
HHP vaccine			0.0003	0.0003
2 x 5 Gy				0.0425
2 x 5 Gy & HHP vaccine				
number of animals	N = 15	N = 11	N = 11	N = 11

FIGURE 2 | Impact of radiotherapy and whole tumor cell-based vaccine generated by HHP on B16-F10 melanoma growth and survival. **(A)** 1 × 10^6 viable B16-F10 melanoma cells were injected subcutaneously into syngeneic C57BL/6 mice. The tumor-bearing mice were either left untreated (mock), vaccinated at day 11 with 5 × 10^6 B16-F10 tumor cells that had been inactivated with 200 MPa (HHP vaccine), locally irradiated at day 8 and 10 after tumor implantation with a single dose of 5Gy per fraction (2 × 5Gy), or treated with both RTx (2 × 5Gy) and HHP vaccine. The tumor growth is displayed in **(B)** and the survival time of C57BL/6 mice after B16-F10 tumor implantation in **(C)**. When the tumor volume exceeded 1,600 mm^3, the mice were euthanized. The number of mice in the treatment groups, as well as the results of the Log-Rank (Mantel-Cox) test concerning the survival **(C)** is displayed in **(D)**. Significant values are determined by an unpaired, one-tailed Student's *t*-test with Welch's correction for unequal variances. The normality of the values was confirmed by a Kolmogorov-Smirnov and Shapiro-Wilk test for each time point. Four animals of each group were sacrificed on day 15 to analyze the infiltrating immune cells; $^*p < 0.05$.

(**Figure 3A**). The immune infiltrates primarily consisted of NK cells (CD3–, CD49b+; **Figure 3B**), monocytes or macrophages (CD11b+, Ly-6C+; **Figure 3C**) and T cells (CD3+; **Figure 3F**); about half of the latter being NKT cells (CD3+, CD49b+;

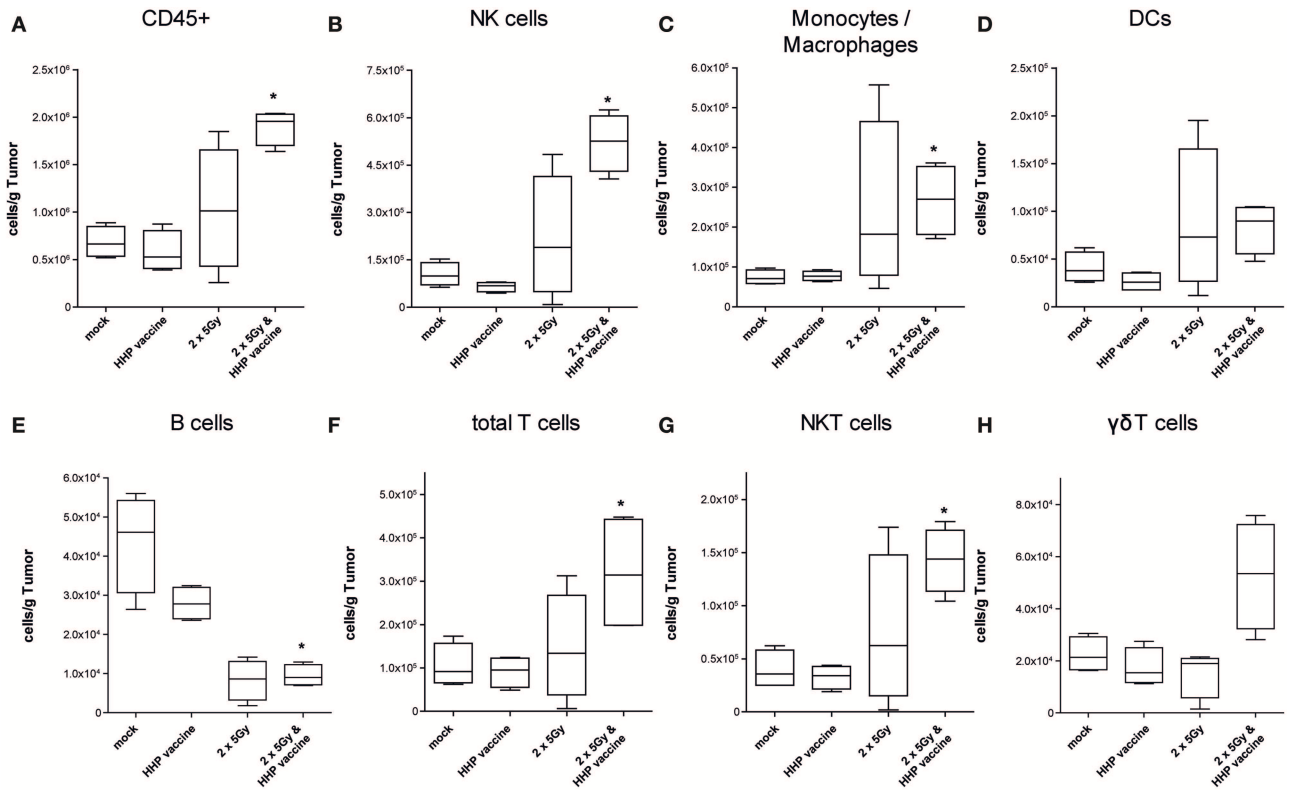


FIGURE 3 | Impact of radiotherapy and whole tumor cell-based vaccine generated by HHP on infiltration of immune cells into B16-F10 tumors. Single cell suspensions of tumors from mice that had been treated with RTx (2 × 5Gy) and/or HHP vaccine (B16-F10 tumor cells that had been inactivated with 200 MPa) were prepared on day 15 after tumor inoculation. Multicolor flow cytometry was performed to detect tumor-infiltrating immune cell subtypes. The latter were identified as follows: all immune cells CD45+ **(A)**; natural killer (NK) cells CD49b+, CD3– **(B)**; Monocytes/Macrophages CD11b+, Ly-6C+ **(C)**; dendritic cells (DCs) MHC-II+, CD11c+ **(D)**; B cells CD19+ **(E)**; total T cells CD3+ **(F)**; NKT cells CD3+, CD49b+ **(G)**; γδT cells CD3+, γδTCR+ **(H)**. Data are presented as box plots showing the median and minimum to maximum values. $n = 4$; Mann-Whitney U test was used for statistical analyses compared to the control group; $^*p < 0.05$.

Figure 3G). Although not as prominent and in lower absolute numbers, also DCs (MHC-II+, CD11c+; **Figure 3D**) and in particular γδT cells (CD3+, γδTCR+; **Figure 3H**) tended to be present in higher numbers after the combined treatment. In contrast to the other immune cell types, B cell (CD19+; **Figure 3E**) numbers were significantly reduced after RTx plus vaccination. No major alterations were found for neutrophil, eosinophil, basophil, and pDC infiltration (data not shown).

For further characterization of the T cell response we determined the CD4/CD8 composition of the infiltrating T cells as well as the expression of the immune checkpoint molecule programmed cell death protein 1 (PD-1). In absolute numbers, T cells were pre-dominantly CD8+ T cells (**Figure 4B**). However, RTx combined with HHP vaccine particularly promoted CD4+ T cell infiltration (**Figure 4A**). Most tumor-infiltrating CD4+ T cells expressed PD-1 in response to RTx and combination of RTx with HHP vaccine enhanced it further significantly (**Figure 4C**). In contrast, the majority of tumor-infiltrating CD8+ T cells expressed PD-1 irrespective of the treatments (**Figure 4C**). When focusing on T cells of the peripheral blood of the mice, expression of PD-1 was observed only in very few CD4+ and CD8+ T cells (**Figure 4D**).

Combination of RTx With Whole Tumor Cell-Based Vaccine Generated by HHP Induces Retardation of CT26 Tumor Growth in Balb/c Mice and Increases Their Survival

To investigate if the efficiency of RTx in combination with HHP vaccination is only a melanoma-specific phenomenon, CT26 colon carcinoma-bearing Balb/c mice were treated similarly as the B16-F10 melanoma-bearing C57BL/6 mice (**Figure 5A**). RTx significantly retarded tumor growth compared to mock-treated or vaccinated mice. This was also reflected by the prolonged time until tumors reached a volume of 750 mm^3 (**Figures 5B–D,F**). The combined treatment with RTx plus vaccination further delayed tumor growth in about half of the mice (**Figure 5E**). In accordance with that, the survival of the mice was further prolonged (**Figures 5G,H**).

Compared to B16-F10 tumors, the amount of tumor-infiltrating CD8+ T cells varied more (**Supplemental Figure 2A**), but a trend of reduced tumor size with higher infiltration of CD8+ T cells was observed (**Supplemental Figure 2B**). As seen in the B16-F10 model, most tumor-infiltrating CD4+ T cells expressed PD-1 in response

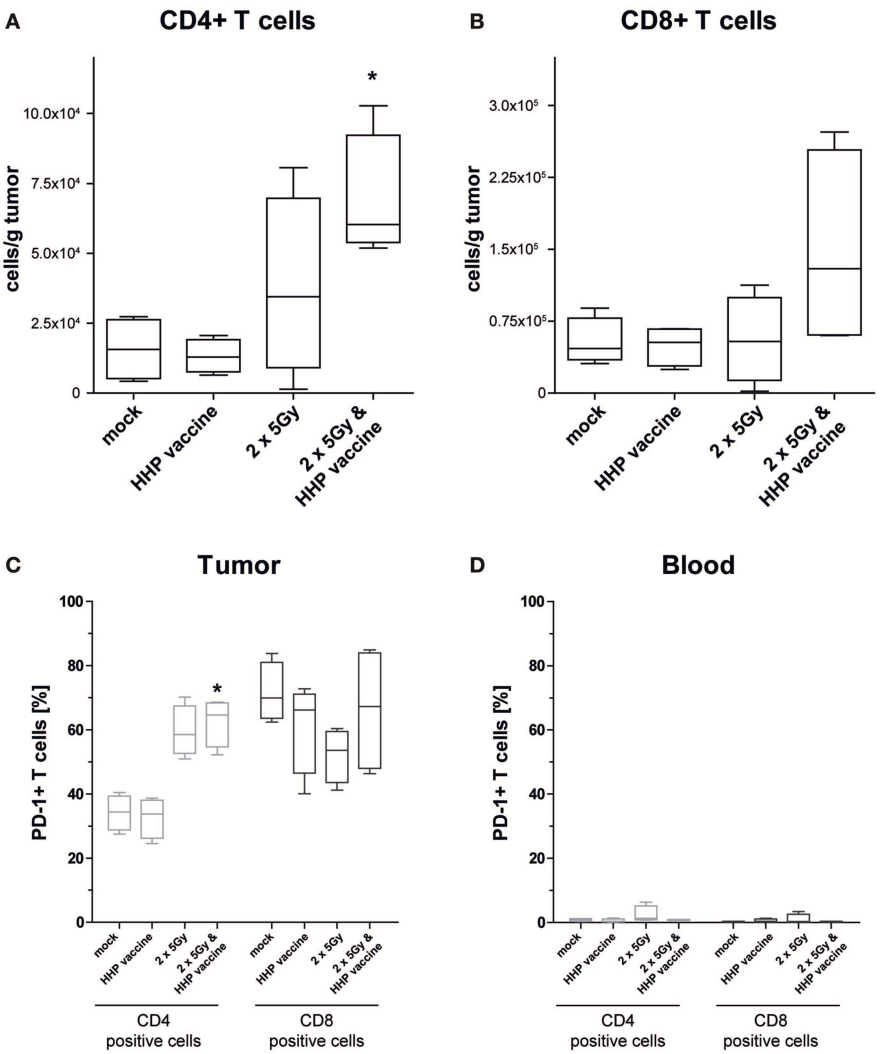


FIGURE 4 | Impact of radiotherapy and whole tumor cell-based vaccine generated by HHP on infiltration of T cells into B16-F10 tumors and on PD-1 expression of T cells. Single cell suspensions of tumors from mice that had been treated with RTx (2 × 5Gy) and/or HHP vaccine (B16-F10 tumor cells that had been inactivated with 200 MPa) were prepared on day 15 after tumor inoculation. Multicolor flow cytometry was performed to detect tumor infiltrating CD4+ **(A)** and CD8+ T cells **(B)**, respectively. Further, expression of PD-1 on T cells being present in the tumors **(C)** and those circulating in blood **(D)** is shown. Data are presented as box plots showing the median and minimum to maximum values. $n = 4$; Mann-Whitney U test was used for statistical analyses compared to the control group; $^*p < 0.05$.

to RTx, but combination with HHP vaccine did not enhance it further (**Supplemental Figure 2C**). Again, the majority of tumor-infiltrating CD8+ T cells expressed PD-1 irrespective of the treatment. But in contrast to B16-F10 tumors, RTx in combination with vaccination further slightly enhanced PD-1+ CD8+ T cells (**Supplemental Figure 2C**). As observed in the B16-F10 model (**Figure 4D**), only very view CD4+ and CD8+ T cells of the peripheral blood did express PD-1 (**Supplemental Figure 2D**).

DISCUSSION

Promising results have recently been achieved using immunotherapy such as checkpoint inhibitors to treat a range of different tumor entities. However, therapeutic cancer vaccines as sole immune therapy for solid cancer encounter three key challenges: immunogenicity of the vaccine, established diseases burden, and existing immune suppressive tumor microenvironment (30). Autologous whole tumor cell-based vaccines have the advantage that target antigens do not have to be prospectively identified and they deliver many TAAs, which are however aberrantly expressed self-antigens. In contrast to neo-antigens, the latter should only be able to activate remaining low affinity T cells and have to break self-tolerance. Several additional treatments have been developed and are discussed to overcome this hurdle, as e.g., repeated vaccination, addition of adjuvants or co-stimulators (31). Regarding the latter, RT might come into play. It has been demonstrated that besides immune suppressive mechanisms, ionizing radiation has additionally immune stimulatory priorities that enhance

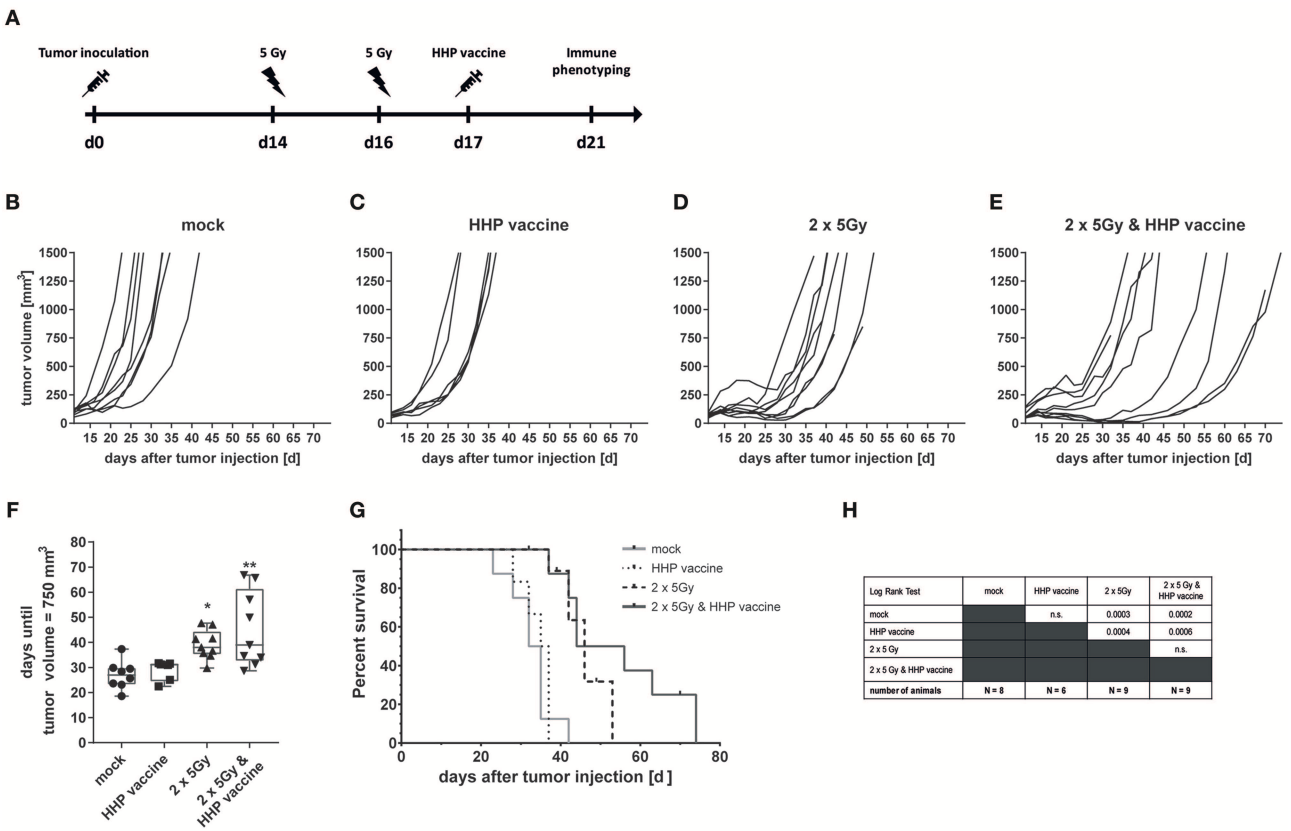


FIGURE 5 | Impact of radiotherapy and whole tumor cell-based vaccine generated by HHP on CT26 colon carcinoma growth and survival. **(A)** 1.2×10^6 viable CT26 cells were injected subcutaneously into syngeneic Balb/c mice. The tumor-bearing mice were either left untreated (mock), vaccinated at day 17 with 5×10^6 CT26 tumor cells that had been inactivated with 200 MPa (HHP vaccine), locally irradiated at day 14 and 16 after tumor implantation with a single dose of 5Gy per fraction (2 × 5Gy), or treated with both RTx (2 × 5Gy) and HHP vaccine. The tumor growth is displayed as individual growth curves **(B–E)** and days until the tumor reached a volume of 750 mm^3 **(F)**. The survival time of Balb/c mice after CT26 tumor implantation is depicted in **(G)**. When the tumor volume exceeded 1,500 mm^3, the mice were euthanized. Significant values are determined by a Kruskal-Wallis test with Dunn's correction for multiple testing. The number of mice in the treatment groups, as well as the results of the Log-Rank (Mantel-Cox) test concerning the survival **(G)** is displayed in **(H)**; $^*p < 0.05$, $^{**}p < 0.01$.

activation of DCs and improve antigen presentation, both being pre-requisites for induction of anti-tumor immune responses (16, 20, 32).

It was already demonstrated for many human cancer cells lines that HHP treatment with 200 MPa results in apoptotic and necrotic tumor cells that activate DCs following their phagocytosis (7, 21). Sipuleucel-T as therapeutic cancer vaccine against castration-resistant prostate cancer has been proven to show efficiency (33). This depicts that therapeutic vaccination with enriched DCs that are stimulated and loaded with antigen can work. In a pre-clinical setting, just recently Hradilova et al. demonstrated that HHP-killed lung cancer cell lines as source of TAAs in combination with the adjuvants poly(I:C) act as DC maturation signal. They further showed that DC-based HHP lung cancer vaccine generated from monocytes of NSCLC patients induces tumor-antigen specific CD8+ and CD4+ T cells (34). Currently a Phase I/II clinical trial for NSCLC is ongoing that uses DC-based active cellular immunotherapy (DCVAC/LuCa) in combination with chemotherapy and immune enhancers (NCT02470468). In an orthotopic mouse model of prostate cancer, the same group demonstrated that DC-based vaccines are as effective as chemotherapy to retard tumor growth. In this setting, however, no difference between un-pulsed DCs and those pulsed with HHP-killed tumor cells was seen. However, a tendency of increased numbers of CD8+ T cells and NK1.1 cells in the spleen of the animals was detected when DCs were pulsed with HHP-killed tumor cells (35).

We have aimed to focus on another cellular vaccine approach (36) using HHP-killed tumor cells alone as vaccine instead of tumor cell-loaded DCs. This approach aims to stimulate and deliver TAAs to DCs *in vivo* when the vaccine is combined with local stimulation of the tumor tissue by irradiation. A major mechanism for the observed synergistic effects is most likely that HHP-killed tumor cells are phagocytosed by the endogenous DCs and antigens are presented to T cells for T cell stimulation.

We here show that murine tumor cells, which are necessary to be applied if consecutive *in vivo* testing of multimodal therapies is performed in syngeneic mice (29), are killed in the same way as human tumor cells (7) by HHP. HHP-treatment was already

included in the list of immunogenic cell death inducers (37). We additionally observed that murine tumor cells continue to degrade DNA following HHP-treatment. This might additionally impact on the immunogenicity of the vaccine, as it was already shown for DNA exonuclease Trex1 that it regulates RTx-induced immunogenicity of tumor cells (38, 39). Therefore, cytosolic DNA following treatment of the tumor cells with HHP might contribute via STING-dependent cytosolic DNA sensing to the immunogenicity of the HHP-vaccine (40). This has however to be proven in future work.

We focused on the new fact if RTx can be combined with syngeneic whole tumor cell-based vaccine without previous co-cultivation of the killed tumor cells with DCs and without any additional adjuvants. Combining vaccination with therapies that modify the tumor and its micro-environment should be promising approaches to enhance the vaccine's efficacy (41). We observed significantly reduced tumor growth and significantly improved survival of B16-F10 tumor-bearing C57BL/6 mice that had been treated with RTx plus HHP vaccine in comparison to RTx alone. Just vaccination did not impact on tumor growth and survival at all. In recent small phase I trials that combine vaccines with other immunotherapy evidence increases that boosting the immune system before vaccination can generate a better response (30). Targeting CTLA-4 in combination with a poxviral-based vaccine targeting prostate-specific antigen resulted in a small number of patients with increased frequency of antigen specific T-cells (42). In another phase I trial for prostate cancer, a vaccine containing two irradiated prostate cancer cell lines that express GM-CSF (GVAX-PCa) again in combination with targeting the immune suppressive immune checkpoint molecule CTLA-4 by ipilimumab, induced an increased expression of CD40 by DCs. This again suggests an enhanced DC function in these cancer patients (43). To exclude that a synergistic effect of RTx and HHP vaccination is only melanoma-specific, we additionally used the CT26 colon carcinoma model. In accordance with the B16-F10 tumor model, also CT26 tumor growth was further retarded when RTx was combined with HHP vaccination. This was seen in about half (4/9) of the mice.

Since the specific T cell numbers needed for an efficient cancer vaccine are unknown to date and do vary between tumor type, antigens and T cell receptor affinity (31), we here focused on analyses of number and quality of tumor-infiltrating immune cells following vaccination, RTx and combination of vaccination and RTx. The immune phenotyping data of B16-F10 tumors demonstrated an enhanced tumor infiltration of a variety of immune cells of the innate as well as the adaptive immune system after combination of RTx and the HHP vaccine. Although different immune cell subtypes are suspected to have diverse impact on tumor progression, the infiltration of immune cells is generally associated with good prognosis for melanoma patients (44) and for most of the solid tumors (45).

We revealed that combination of RTx with HHP vaccine generates a favorable anti-tumor immune microenvironment for melanoma. γδT cells are known to infiltrate into melanoma and are capable of killing melanoma cells (46). We identified that only combination of RTx with HHP vaccine increased the number of γδT cells in the tumor. Further, NK cells were significantly enhanced. These innate immune cells are key players in mediating anti-tumor immunity (47). We also previously observed that NK cell depletion after immunization results in a significant acceleration of melanoma growth (48). NKT cells were also significantly enhanced and may lead to downstream activation of both innate and adaptive immune cells in the tumor microenvironment (49). Since B cells might foster tumor-promoting humoral immunity in melanoma (50), decreased numbers following RTx plus HHP vaccine treatment should also contribute to a beneficial therapy-induced tumor microenvironment.

They et al. demonstrated that a favorable modulation of the melanoma microenvironment fosters the infiltration of CD4+ and CD8+ T cells (51). However, tumor escape by upregulation of PD-1 is frequent and additional treatment with anti-PD-1 antibody restored effector functions of CD4+ and CD8+ T cells as well as of NK cells and γδT cells. We demonstrate that combination of RTx with HHP vaccine also fosters infiltration of CD4+ and CD8+ T cells as well as that of NK cells and γδT cells into B16-F10 melanomas. In the CT26 model, combination of RTx plus HHP vaccine resulted in heterogeneously responding tumors. Here, high CD8+ T cell infiltration tended to result in smaller tumors (**Supplemental Figure 2**). We further observed a high expression of PD-1 on infiltrating T cells. This depicts both that activation of the T cells against the tumor has taken place and that subsequently immune suppressive checkpoint molecules such as PD-1 are expressed to regulate the immune response and that re-stimulation of the immune system by anti-PD-1 treatment will be necessary. Dyck et al. demonstrated in the CT26 model that anti-PD-1 treatment reduced regulatory T cell induction and enhanced CD8+ T cell mediated tumor killing. Combined treatment of tumor-bearing mice with a vaccine, comprising heat-shocked irradiated tumor cells and a TLR 7/8 agonist, significantly reduced tumor growth and enhanced survival (52). This calls for further improvement of induction of anti-tumor immune responses by combining RTx plus HHP vaccine with immune checkpoint-inhibition in the future (16, 18, 53). While almost absent in blood, the majority of CD8+ T cells infiltrating into B16 tumors and almost all CD8+ T cells in CT26 tumors expressed PD-1. This enrichment of PD-1+ T cells in the tumor was already reported for patients with metastatic disease (54), indicating that the up-regulation of the inhibitory receptor PD-1 is driven by the tumor microenvironment. Nevertheless, PD-1 expression can also be considered as favorable marker for an effectively primed T cell response, as suggested by Fernandez-Poma et al. Only the fraction of T cells selected for positive PD-1 expression exhibited anti-tumor reactivity when adoptively transferred into mice and combination with anti-PD-L1 further enhanced tumor control (55).

Future work will focus on a triple combination of RTx with HHP vaccination and checkpoint inhibition for the induction of anti-tumor immune responses to primary and abscopal tumors (16). Furthermore, one should think about to modify the radiation dose that has most likely to be adapted very individually in the future for optimization of immune stimulation by RTx. However, one has to be aware that too high single dose might again decrease immunogenicity of the tumors (38). Additionally, the HHP-vaccine could be injected multiple times to break self-tolerance with appropriate adjuvants. We demonstrated in

another pre-clinical setting that repeated vaccination of tumor cells that had been killed by RTx in combination with heat is superior to single vaccination with regard to induction of tumor growth retardation (48). Even though many hurdles still will have to be overcome for most beneficial combination of RTx with tumor cell-based vaccines, such approaches are particularly important for patients who harbor weak spontaneous immune responses to their cancer. Furthermore, development of cancers vaccines have to respect that standard of care for most cancer patients involves chemotherapy and/or RTx (31). The here presented pre-clinical work give first hints that RTx is well-combinable with tumor-cell based vaccines generated by HHP and provides a basis for continuing work on optimization of multimodal cancer therapies.

ETHICS STATEMENT

All animal experiments were conducted according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) and the Gesellschaft für Versuchstierkunde (GV-SOLAS) and were authorized by the government of Mittelfranken/Unterfranken.

AUTHOR CONTRIBUTIONS

CS carried out most of the *in vitro* experiments and parts of the *in vivo* work and wrote the manuscript together with MR, UG, and BF. MR carried out most of the *in vivo* work and wrote the manuscript together with CS, BF, and UG. LD contributed to the *in vivo* experiments and to the drafting of the manuscript. E-MW contributed to drafting the *in vitro* experiments and parts of the Balb/c *in vivo* experiments. SU performed parts of the *in vitro* experiments. MI optimized the HHP treatment procedure together with NE. ES drafted the vaccine preparation experiments together with BF. RF contributed to the design of the work. UG drafted the whole study including most of the *in vitro* and *in vivo* experiments together with BF. UG further drafted the manuscript and wrote it together with BF, CS, and MR. BF drafted the whole study including most of the *in vitro* and *in vivo* experiments together with UG. BF further wrote the manuscript together with UG, CS, and MR. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00805/full#supplementary-material

Supplemental Figure 1 | Technical sketch of the high hydrostatic pressure aperture that was used for the generation of the tumor cell-based vaccine. The numbered properties of the aperture are explained in the main text.

Supplemental Figure 2 | Impact of radiotherapy and HHP vaccination on infiltration of T cells into CT26 tumors and on PD-1 expression of T cells. Single cell suspensions of tumors from mice that had been treated with RTx (2 × 5Gy) and/or HHP vaccine (CT26 tumor cells that had been inactivated with 200 MPa) were prepared on day 21 after tumor inoculation. Multicolor flow cytometry was performed to detect tumor infiltrating CD4+ and CD8+ T cells **(A)**. The infiltration of CD8+ T cells into the tumor in relation to the tumor weight is depicted in **(B)**. Further, expression of PD-1 on CD4+ and CD8+ T cells being present in the tumors **(C)** and those circulating in blood **(D)** is shown. Data are presented as box plots showing the median and minimum to maximum values. $n = 6$; Mann-Whitney *U* test was used for statistical analyses; $^*p < 0.05$.

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