Radiotherapy increases the permissiveness of established mammary tumors to rejection by immunomodulatory antibodies

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Abstract

It is becoming increasingly evident that radiotherapy (RT) may benefit from coincident or subsequent immunotherapy. In this study, we examined whether the anti-tumor effects of RT, in established triple-negative breast tumors could be enhanced with combinations of clinically relevant monoclonal antibodies (mAbs), designed to stimulate immunity (anti-CD137, α-CD40) or relieve immunosuppression (α-programmed death (PD)-1). While the concomitant targeting of the co-stimulatory molecules CD137 and CD40 enhanced the anti-tumour effects of RT and promoted the rejection of subcutaneous BALB/c-derived 4T1.2 tumors, this novel combination was non-curative in mice bearing established C57BL/6-derived AT-3 tumors. We identified PD-1 signaling within the AT-3 tumors as a critical limiting factor to the therapeutic efficacy of α-CD137 therapy, alone or in combination with RT. Strikingly, all mice bearing established orthotopic AT-3 mammary tumors were cured when α-CD137 and α-PD-1 mAbs were combined with single- or low-dose fractionated RT. CD8+ T-cells were essential for curative responses to this combinatorial regime. Interestingly, CD137 expression on tumor-associated CD8+ T-cells was largely restricted to a subset that highly expressed PD-1. These CD137+PD-1High CD8+ T-cells, persisted in irradiated AT-3 tumors, expressed Tim-3, granzyme B and Ki67 and produced IFN-γ ex-vivo in response to PMA and ionomycin stimulation. Notably, RT did not deplete, but enriched tumors of functionally active, tumor-specific effector cells. Collectively, these data demonstrate that concomitant targeting of immune-stimulatory and inhibitory checkpoints with immunomodulatory mAbs can enhance the curative capacity of RT in established breast malignancy.
Introduction

Along with surgery and chemotherapy, RT is one of the most important anti-cancer therapies. Indeed, nearly two-thirds of all cancer patients will receive RT during their course of treatment. RT has long been employed for its powerful anti-proliferative and death-inducing capacities. Recent preclinical and clinical data, however, indicate that immunogenic cell-death may also be an important consequence of ionizing radiation (1) and that localized RT can evoke and/or modulate tumor-associated immune responses (2-3). Collectively, these findings have set the stage for examining the therapeutic impact of combined RT and immunotherapy.

Pre-clinical studies evaluating the combined therapeutic effects of RT and immunotherapy have employed the use of virus and dendritic cell (DC)-based vaccines (4-5), toll-like receptor (TLR) agonists, such as CpG oligodeoxynucleotides (6) and recombinant cytokines (7). In addition, RT has been trialed in conjunction with monoclonal antibodies (mAbs) that target co-stimulatory molecules such as CD40, CD137, OX40 (CD134) or the checkpoint inhibitor CTLA-4 (8-12). While these studies have demonstrated that single immunomodulatory agents can enhance the anti-tumor effects of RT, tumor cures were rare, particularly against poorly immunogenic tumors.

Tumor-induced immune-tolerance and immunosuppression (13-14) may restrict the success of radio-immunotherapeutic approaches. In order to overcome such immunological barriers to effectively control established disease, a rational combination of multiple immunomodulatory agents and standard anti-cancer regimens
is likely required. Indeed, we and others have demonstrated that combining mAbs targeting CD40, CD1d and/or CD137, designed to modulate DC and T-cell activity respectively are highly effective, particularly in combination with TRAIL-receptor agonists and histone deacetylase inhibitors (HDACi) in eradicating established solid tumors (15-17). However, given the intrinsic resistance of many tumors to TRAIL-receptor agonists and HDACi, as well as the fact that TRAIL-receptor agonists are still in clinical development and the applicability of HDACi is currently largely restricted to hematological malignancies, the broader application of these combinations is presently limited (18-19). Blockade of immune checkpoint-inhibitory molecules such as CTLA-4 or PD-1, alone or in combination with anti-CD137 therapy also increased the therapeutic activity of vaccine approaches and α-erbB2 therapy (20-21). To date however, the therapeutic benefit of targeting multiple immune stimulatory and/or inhibitory pathways in combination with RT has not been described.

Here, we demonstrate that mAbs to CD137, CD40 and PD-1, which are all in clinical development, combined with single- and low-dose fractionated RT, induced the rejection of established subcutaneous and orthotopically implanted, triple-negative (estrogen receptor (ER), Her2/neu and progesterone receptor (PR)) AT-3 and 4T1.2 mammary tumors. These results are encouraging, given the unmet medical need to treat triple-negative breast cancer in the clinic, as they do not respond to endocrine treatment and other currently available targeted agents. Importantly, we identified the key innate and adaptive immune cells critical to the anti-tumor effects of radio-immunotherapy. These cells expressed CD137 and/or PD-1 and persisted within the irradiated tumors. This study provides strong validation for combining RT with
immunotherapy and justifies the therapeutic application of both immunostimulatory and checkpoint-inhibitory mAbs as adjuvants to RT in breast cancer treatment.
Materials and Methods

Mice

6-12 week-old C57BL/6 (B6) and BALB/c mice were obtained from The Walter and Eliza Hall Institute of Medical Research (WEHI) or Animal Resource Centre (Perth, Western Australia). All mice were maintained under specific pathogen-free conditions and used in accordance with institutional guidelines of the Peter MacCallum Cancer Centre.

Cell lines

BALB/c-derived 4T1.2 (22) and B6-derived AT-3 (23) mammary carcinoma cell lines were obtained from Dr Robin Anderson in 2005 and Dr Trina Stewart in 2007 (Peter MacCallum Cancer Centre, VIC, Australia). Both cell-lines were periodically authenticated by morphologic inspection and tested negative for Mycoplasma contamination by PCR-tests in 2009-2010. AT-3 cells, retrovirally transduced to stably express ovalbumin (OVA) were generated as described (24). AT-3 cells were cultured at 37°C, 10% CO₂ in DMEM, 10% FBS, 0.1mM non-essential amino acids, 1mM sodium pyruvate, 2mM L-glutamine. 4T1.2 cells were cultured at 37°C, 5% CO₂ in RPMI1640, 10% FBS, 100μg/ml penicillin/streptomycin, 2 mM L-glutamine.

Cell-death and clonogenic assays

For cell-death assays, tumor cells were incubated with 10μM qVD (InSolution Q-VD-OPh, Calbiochem, Darmstadt, Germany) or corresponding DMSO concentrations for 30 minutes. Cells were subsequently treated with 0-30Gy ionizing radiation (IR) (¹³⁷Cs source; at an absorbed dose rate of ~0.6Gy/min) and harvested 24-48h post treatment. Flow cytometry was used to measure (i) cell death by Annexin-V/PI
staining, and (ii) apoptosis by caspase-3 cleavage as described (25). Clonogenic assays were essentially performed as described (26). Briefly, cells were plated at increasing densities of up to 8,000 cells/10cm dish. After adhering (4-6h at 37°C), cells were irradiated (0-10Gy). After 8-11 days of incubation under normal culture conditions, cells were stained with 2.5% glutaraldehyde and 0.05% crystal violet for >15min at room temperature. Colonies (>40 cells) were counted using an inverted microscope and clonogenicity of untreated cells (0Gy) was set to 100%.

**TUNEL staining**

Mice bearing 20-25mm² subcutaneous AT-3 tumors were mock-irradiated or treated with 12Gy RT. Tumors from control and irradiated mice (n=3) were dissected 16, 24 and 48h post RT and processed for immunohistochemical analyses (H&E and TUNEL staining). Fixed and paraffin-embedded tissues were randomly sectioned and stained for apoptotic cells using an ApopTag Peroxidase In Situ Apoptosis-Detection Kit (Chemicon International) as per manufacturers’ instructions. Metamorph Imagin Program Series 7.6.3 was used to quantify single, tumor-cell-specific TUNEL-positive events (Zeiss Microscope (Carl Zeiss Australia); Objective; 10x/0.30 Plan-Neofluar).

**Phenotyping of tumor cells and tumor-infiltrating lymphocytes**

Expression of PD-L1 on *in vitro* cultured and explanted tumor cells, prior to and 12–96h post radiation treatment (0-30Gy *in vitro*; 12Gy *in vivo*) was assessed by flow cytometry as described (27) using biotinylated α-mouse CD274 (PD-L1, BD Pharmingen) mAb and allophycocyanin (APC)-conjugated streptavidin (eBioscience).
CD45.2 (eBioscience) staining of explanted tumor samples distinguished leukocytes from tumor cells.

Analysis of tumor-infiltrating leukocytes was performed prior to and 16, 36 and 84h post RT (12Gy, 5-18 mice/group) for surface expression of CD137 (17B5; eBioscience), PD-1 (J43; eBioscience), CD44 (IM7; BD Pharmingen), CD62L (MEL-14; eBioscience), Tim-3 (RMT3-23; eBioscience) and OVA-tetramer (KbOVA257-264-PE; obtained from SJ Turner, Department of Microbiology and Immunology, University of Melbourne, Parkville, Australia). Intracellular staining for granzyme B (mouse-anti-human Granzyme B; BD Bioscience) and Ki67 (mouse-anti-human Ki67; BD Bioscience) was performed using a Cytofix/Cytoperm™, Fixation/Permeabilization Solution Kit according to the manufacturers’ instructions (BD Bioscience). Spleens from tumor-bearing mice served as background staining controls. Cell viability was assessed using DAPI (4’,6-diamido-2-phenylindole; Invitrogen) or Propidium Iodide (PI) and cells were analyzed on a CantoII or LSRII analyzer (BD Biosciences, San Jose, CA, USA).

**Staining for intracellular IFN-γ following PMA and ionomycin stimulation.**

Untreated, established (20-25mm²) orthotopic AT-3 tumors (n=7) were harvested and processed into single cell suspensions. Whole-cell preparations were plated at 3x10⁵ cells/200μl DMEM/2% FCS in a 96-well U-bottom plate. Cells were treated with 50ng/ml PMA (Sigma Aldrich, St Louis, MO, USA) and 1μg/ml ionomycin (Sigma Aldrich). Control (unstimulated) wells were treated with an equal volume of DMSO. GolgiPlug™ (1μg/ml; BD Biosciences) was added to all wells prior to incubating the cultures for 4h at 37°C, 5% CO₂. Staining for intracellular IFN-γ in CD45.2⁺PD-1⁻low
and PD-1\textsuperscript{High} CD8\textsuperscript{+} T-cells was performed as described above for granzyme B and Ki67 detection and analyzed on a LSRII analyzer.

**Therapeutic Antibodies and Reagents**

The rat mAb to mouse CD137 (3H3, IgG2a) was generated in house as described (28) and protein-G purified or purchased from BioXCell (New Hampshire, USA). The rat \( \alpha \)-mouse CD40 mAb (FGK4.5, IgG2a) was from WEHI. The rat \( \alpha \)-mouse PD-1 mAb (RMP1-14; IgG2a) was prepared as described (29). Depleting antibodies to asialoGM1 (NK-cells; Wako Pure Chemical, Osaka, Japan), mouse CD4 (GK1.5) and CD8\( \beta \) (53-5.8; BioXCell) were prepared as described (30). For both therapy and depletion studies, MAC4 mAb was used as the isotype control (28).

**Therapy of transplanted tumors**

Mice were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg) or isofluorane and injected with 4T1.2 \((5\times10^4/50\mu l \text{ PBS})\) or AT-3 \((1\times10^6/50\mu l \text{ PBS})\) tumor cells subcutaneously on the right hind leg or orthotopically into the fourth mammary fatpad. Tumor size was measured every 2-3 days using electronic calipers and represented as tumor area \((\text{length} \times \text{width})\). Radio-immunotherapy commenced when tumors reached 20-25mm\(^2\). Mice were divided into groups of 4-7. Irradiation of subcutaneous \((s.c.)\) tumors was performed essentially as described (31). Briefly, for irradiation of s.c. tumors, mice were inserted into perspex jigs and the tumor bearing leg isolated and restrained for exposure to a single dose of radiation \((12 \text{ Gy})\). To minimize exposure of normal tissue to RT, mice were protected by a 3mm thick lead shield containing 1.5 cm\(^2\) holes; permitting exposure of the leg to RT. For irradiation of orthotopic tumors, mice were anesthetized with ketamine/xylazine and positioned...
under a lead shield containing 0.8 cm$^2$ exposure holes. Irradiation was delivered using a 6-MeV electron beam at a dose-rate of 20 Gy/min (Varian Medical Systems, Palo Alto, CA) as a 12 Gy single fraction or in four fractions of 4 Gy or 5 Gy at 24 h intervals. Control mice were placed in radiation jigs (s.c. tumors) or anesthetized (orthotopic tumors), but not exposed to RT.

Immunotherapy consisted of $\alpha$-CD137 (100 μg) and/or $\alpha$-CD40 (100 μg), $\alpha$-PD-1 (100 μg) or MAC4 (100 μg) mAbs, administered intraperitoneally on days 0, 4, 8 and 12 relative to RT. Antibodies depleting CD4$^+$ (100 μg) or CD8$^+$ (50 μg) T-cells were administered intraperitoneally on days -1, 0, then every four days until day 40, $\alpha$-asialo GM1 (100 μg) was administered intraperitoneally on days -1, 0, then every six days until day 40 relative to radio-immunotherapy.

All radio-immunotherapeutic combinations and treatment regimes were well-tolerated. Mice were sacrificed when tumors reached 100-200 mm$^2$.

Statistics

Statistical differences between groups were analyzed with unpaired Student’s $t$-tests (experiments not involving mice) or Mann Whitney $U$-tests using GraphPad Prism (Graph Pad Software, San Diego, CA).
Results

Ionizing radiation induces cell death in triple-negative mammary tumor cells.

In vitro assay systems were initially employed to screen the BALB/c-derived 4T1.2 and C57BL/6-derived AT-3 triple-negative mammary tumor lines for their sensitivity to ionizing radiation. Radiation-induced cell death as determined by Annexin-V/PI staining was evident in both cell-lines and occurred in a time- and dose-dependent manner (Fig.1A,B (top panels)). In 4T1.2 cells, radiation-induced cell death was not affected by the pan-caspase inhibitor qVD (Fig.1A) or over-expression of the anti-apoptotic protein Bcl-2 (data not shown). In addition, caspase-3 cleavage was not detected in irradiated 4T1.2 cultures (data not shown); collectively indicating that apoptosis was not the primary mechanism of radiation-induced 4T1.2 cell death. In contrast, caspase-3 cleavage was detected in irradiated AT-3 cultures (Fig.1B). Over-expression of Bcl-2 (data not shown) or treatment with qVD (Fig.1B, top panels) significantly reduced Annexin-V/PI staining, indicating that apoptosis contributed to radiation-induced AT-3 cell death. Despite these mechanistic differences the radio-sensitivity of 4T1.2 and AT-3 tumor cells, as determined in long-term clonogenic assays, was comparable (Fig.1C).

These findings were subsequently confirmed in a neo-adjuvant setting of established disease, where a single dose of 12Gy RT induced a comparable degree of tumor growth delay in subcutaneous 4T1.2 and AT-3 tumors, without inducing complete regression (Suppl. Fig.1). TUNEL analysis revealed higher levels of RT-induced apoptosis in AT-3 compared to 4T1.2 tumors in vivo (Fig.1D (4T1.2)-E (AT-3)). A modest (<2-fold) but statistically significant increase in the number of apoptotic
events, compared to mock-treatment was detected 24h after RT in 4T1.2 tumors (Fig.1D). Whereas in the AT-3 tumors, at 16h post RT a >10-fold increase in TUNEL+ events, compared to mock-treatment was observed (Fig.1E). Importantly, despite differences in the biological response to RT, the radio-responsiveness of the 4T1.2 and AT-3 tumors was comparable.

**Therapeutic targeting of PD-1 but not CD40 enhanced the curative capacity of RT/α-CD137 treatment in AT-3 tumors.**

Previously we demonstrated that α-CD137/α-CD40 therapy could enhanced the anti-tumor activity of HDACi or α-DR5 therapy in tumor cells that possessed functional apoptotic pathways, suggesting that drug-induced apoptosis engaged anti-tumor immune responses that could be enhanced with antibody-based immunotherapy (15-16). Based on these results and the ability of RT to induce apoptosis in AT-3 tumors, we initially analyzed novel radio-immunotherapeutic combinations in AT-3 mammary tumors. We first examined whether α-CD137/α-CD40 therapy could be effectively combined with RT to control and eradicate subcutaneous AT-3 tumors. Predetermined doses of α-CD40 and/or α-CD137 mAbs were used alone (Fig.2A, top panels) or in combination with RT (12Gy; Fig.2A, lower panels). Anti-CD137 therapy, alone or in combination with α-CD40 treatment significantly enhanced RT-induced growth inhibition of AT-3 tumors (Fig.2A). However, tumors grew out in all mice (Fig.2A). Although a percentage (40%) of tumor-bearing mice were found to be highly responsive to RT and α-CD40 treatment (Fig.2A), α-CD40 therapy did not further enhance the therapeutic response of AT-3 tumors to RT/α-CD137 treatment (Fig.2A). Interestingly, however, in established subcutaneous 4T1.2 tumors RT/α-CD40/α-CD137 therapy was found to be curative in >80% of mice (Suppl. Fig.2A).
tumor effects of this triple-combination in 4T1.2 tumors were critically dependent on CD8+ T and NK-cells (Suppl. Fig.3A), which expressed CD137 (Suppl. Fig.3B) and could induce immunological memory capable of controlling the outgrowth of a secondary tumor challenge (Suppl. Fig.2). Importantly, these findings demonstrate that this novel radio-immunotherapy has the potential to eradicate established, triple-negative mammary tumors.

Given the inability of RT/α-CD137/α-CD40 treatment to eradicate AT-3 tumors, tumor-cell associated factors that might inhibit the development of an effective anti-tumor immune response were examined. One candidate was the checkpoint-inhibitory ligand, programmed death-ligand 1 (PD-L1) that has been reported to inhibit T-cell activation on PD-1-expressing T-cells, by blocking IL-2 production following TCR stimulation (32). Interestingly, while PD-L1 expression was not detected on in vitro cultured AT-3 cells (Fig. 3A, left panel), explanted AT-3 tumors expressed PD-L1 (Fig.3A, right panels) and were unaffected by RT. Based on these observations we examined the therapeutic activity of an antagonistic α-PD-1 mAb in combination with RT and α-CD137 treatment. While α-PD-1 therapy had no single-agent activity, nor did it enhance the response of AT-3 tumors to RT (Fig.3B), α-PD-1 treatment significantly enhanced the responsiveness of AT-3 tumors to RT/α-CD137 therapy, achieving a rejection rate of up to 40% (Fig.3B,C). These combined effects were lost in mice depleted of CD8β+ T-cells and significantly affected by depletion of NK-cells (Fig.3D), but unaffected by depletion of CD4+ T-cells (Fig. 3D). Importantly, in mice cured of primary tumors, the outgrowth of secondary AT-3 tumors was significantly impaired; indicating the establishment of immunological memory (Fig.3E). Taken together, these data suggest that PD-1 signaling within AT-3 tumors limited the
capacity of $\alpha$-CD137/$\alpha$-CD40 immunotherapy to support the immuno-adjuvant properties of RT and that inhibition of PD-1 signaling was therefore required to optimally promote CD8$^+$ T-cell and NK-cell function to reject AT-3 tumors.

**Rejection of orthotopic AT-3 tumors with RT/$\alpha$-CD137/$\alpha$-PD-1 therapy.**

We next determined whether $\alpha$-CD137/$\alpha$-PD-1 therapy promoted the rejection of irradiated AT-3 tumors in the physiologically relevant tissue microenvironment of the mammary fatpad. Notably, orthotopic AT-3 tumors expressed PD-L1 at levels similar to subcutaneous tumors (data not shown). Combined effects were observed with RT and $\alpha$-CD137, $\alpha$-PD-1 and $\alpha$-CD137/$\alpha$-PD-1 therapy (Fig.4A,B). Strikingly, up to a 100% rejection-rate was achieved when RT was combined with both $\alpha$-CD137 and $\alpha$-PD-1 mAbs. Consistent with our observations in subcutaneous AT-3 tumors, $\alpha$-CD137 therapy showed single-agent activity, while $\alpha$-PD-1 treatment did not. However, $\alpha$-PD-1 treatment prolonged the therapeutic response of orthotopic AT-3 tumors to $\alpha$-CD137 therapy and significantly enhanced the anti-tumor effect of RT in a small percentage of mice (Fig.4A,B). These results reaffirm the importance of PD-1 as a possible immunotherapeutic target in breast cancer.

**Characterization of a persistent CD137$^+$PD-1$^{\text{High}}$CD8$^+$ T-cell subset in irradiated AT-3 tumors.**

Given the profound therapeutic efficacy of $\alpha$-CD137/$\alpha$-PD-1 therapy in irradiated AT-3 tumors, we next examined the AT-3 tumor-associated lymphocytes for expression of CD137 and PD-1. We focused on the characterization of CD8$^+$ T-cells since they accounted for $>40\%$ of the tumor-associated CD45.2$^+$ cells ($44.8\pm3\%$; 20-25mm$^2$ control tumor, n=7) and were the primary mediators of the anti-tumor activity.
of RT/α-CD137/α-PD-1 therapy in subcutaneous AT-3 tumors (Fig.3D). Interestingly, CD137 expression was only detected on a subset of PD-1^{High}-expressing CD8^{+} T-cells (Fig.5A). The majority of CD8^{+} T-cells in AT-3 tumors expressed low levels of PD-1 and were negative for CD137 (Fig.5A). Notably, CD137 and PD-1 expression was also observed on tumor-associated NK and CD4^{+} T-cells (data not shown). Of the PD-1-expressing CD8^{+} T-cells, the PD-1^{Low} subset was most affected by RT. Indeed the frequency of cells within the PD-1^{Low} population was significantly reduced post RT, resulting in a temporary enrichment of the PD-1^{High}CD137^{+} population of CD8^{+} T-cells (Fig.5A) and an increased ratio of PD-1^{High} to PD-1^{Low} cells in tumors at 12h post RT (Fig.5B).

Further characterization of the tumor-associated PD-1^{High} and PD-1^{Low} subsets revealed that both were CD44^{+} (Fig.5C) and CD62L^{-} (data not shown), two hallmarks of an activated T-cell phenotype. However, expression of the activation marker CD44 was highest on the PD-1^{High} subset, indicative of a more antigen-experienced population (Fig.5C). In support of this, expression of the immune-regulatory molecule T-cell immunoglobulin mucin (Tim)-3, which has been linked to TCR engagement and proliferation (33) was also found to be tightly associated with this PD-1^{High} subset (Fig.5C). Notably, RT did not alter the expression status of either of these molecules on the PD-1^{High} and PD-1^{Low} CD8^{+} T cell subsets (Fig.5C). Assessment of their proliferative status by Ki67 staining demonstrated that both PD-1^{High} and PD-1^{Low}-expressing cell populations were actively proliferating (Fig.5D). Ki67 staining was highest in the PD-1^{High} subset and was unaffected by RT. In contrast, Ki67-expression within the PD-1^{Low}CD8^{+} T-cell population increased at 36h post RT. This observation may account for the normalization of the PD-1^{High}/PD-1^{Low} CD8^{+} T-cell ratio seen
between 12 and 36h post RT (Fig.5B). Despite these differences, both the PD-1\textsuperscript{High} and PD-1\textsuperscript{Low} CD8\textsuperscript{+} T-cell subsets possessed similar levels of Granzyme B, which was increased at 36h post RT (Fig.5E). Further to this, the PD-1\textsuperscript{High} and PD-1\textsuperscript{Low} CD8\textsuperscript{+} T-cell subsets were equally capable of producing IFN-γ in response to PMA and ionomycin (Fig.5F), suggesting that both were functionally active \textit{ex vivo}.

Importantly, these functionally responsive populations of CD137\textsuperscript{+}CD8\textsuperscript{+} T-cells persisted post RT, highlighting the fact that RT does not deplete, but can enrich tumors for critical effector cells, capable of driving immune responses to dying tumor cells.

**Tumor-specificity is associated with PD-1\textsuperscript{High}CD137\textsuperscript{+}CD8\textsuperscript{+} T-cells.**

To investigate whether RT enriched AT-3 tumors for tumor-reactive CD8\textsuperscript{+} T-cells, we examined the tumor-specificity of the PD-1\textsuperscript{High} and PD-1\textsuperscript{Low} CD8\textsuperscript{+} T-cell subsets in explanted OVA-expressing orthotopic AT-3 tumors by OVA-tetramer staining. Similar to what was observed in the parental AT-3 tumors, CD137 expression was restricted to the PD-1\textsuperscript{High}CD8\textsuperscript{+} T-cell subset (Fig.6A), which was enriched for at 12h following RT (Fig.6A,B). Importantly, only cells within the PD-1\textsuperscript{High}CD137\textsuperscript{+}CD8\textsuperscript{+} T-cell population were OVA-tetramer reactive and thus tumor antigen-specific (Fig.6C), which indicates that RT enriches the tumor microenvironment for tumor-specific CD8\textsuperscript{+} T-cells.

**Fractionated RT in combination with immunotherapy induces regression of orthotopic mammary tumors.**

Although our data so far highlight the therapeutic potential of radio-immunotherapy for the treatment of breast cancer, in the clinic, patients typically receive a series of
low-dose fractionated treatments of RT to limit normal-tissue toxicity. We therefore examined whether concomitant targeting of CD137 and PD-1 could reject established orthotopic AT-3 tumors treated with fractionated RT. Certainly this approach seemed viable given that single-dose 12Gy RT did not deplete the AT-3 tumors of CD137+CD8+ T-cells. Based on the clonogenic survival curve in Fig. 1C, mice were treated with four fractions of 4Gy, achieving a dose close to 12Gy, or four fractions of 5Gy. Anti-CD137/α-PD-1 therapy in combination with either 4x4Gy or 4x5Gy was more effective in controlling tumor outgrowth compared to either treatment alone; achieving rejection rates of 40% and 60%, respectively (Fig.7A,B). The finding that antibody-based immunotherapy enhanced the anti-tumor response to low-dose fractionated RT demonstrates the clinical potential of this radio-immunotherapeutic approach for treating established breast cancer.
Discussion

To our knowledge, this is the first preclinical study to examine the therapeutic impact of combining multiple stimulatory and/or inhibitory mAbs with RT and the cellular mechanisms underlying these responses in established breast cancer. We demonstrate in two distinct models of triple-negative mammary cancer that unique combinations of radio-immunotherapy are capable of curing mice of established disease. Radio-immunotherapy was well-tolerated and effective when used in a clinically-relevant fractionated radiotherapy regime against orthotopically implanted tumors. The profound therapeutic efficacy of RT in combination with $\alpha$-CD137/$\alpha$-PD-1 therapy in established AT-3 tumors is likely linked to the capacity of RT to enrich the tumor microenvironment of critical tumor-reactive CD8$^+$ T-cells that co-express CD137 and PD-1.

The checkpoint-inhibitory receptor PD-1 plays an important role in the regulation of immune-responses and maintenance of peripheral immune-tolerance (34). However, in the context of tumor-immunity, the inhibitory actions of PD-1 can be deleterious to the development of robust anti-tumor immune-responses (reviewed in (35)). While antibody-mediated blockade of PD-1 signaling had minimal impact on the growth of irradiated AT-3 tumors, it significantly enhanced the curative capacity of RT/$\alpha$-CD137 therapy in this model; highlighting PD-1 signaling as a potential limiting factor to the therapeutic success of radio-immunotherapy. Interestingly, $\alpha$-CD137/$\alpha$-PD-1 therapy was equally as effective as RT in suppressing orthotopic tumor growth, highlighting the capacity of $\alpha$-CD137/$\alpha$-PD-1 mAbs to re-engage the anti-tumor activity of tumor-associated lymphocytes. Consistent with this, CD137 expression on
AT-3 tumor-associated CD8\(^+\) T-cells, which were critical for the anti-tumor activity of RT/\(\alpha\)-CD137/\(\alpha\)-PD-1, was restricted to a PD-1\(^{\text{High}}\)-expressing subset. These CD137\(^+\)PD-1\(^{\text{High}}\)CD8\(^+\) T-cells also expressed the immune-regulatory molecule Tim-3. The co-expression of PD-1 and Tim-3 has been associated with an exhausted T-cell phenotype (36). However, the similarities between the PD-1\(^{\text{High}}\)Tim3\(^+\) and PD-1\(^{\text{Low}}\)Tim3\(^-\) CD8\(^+\) T-cell populations in terms of IFN-\(\gamma\) production, proliferative status and cytolytic potential, suggests that the PD-1\(^{\text{High}}\)CD8\(^+\) T-cell subset is not an exhausted population but comprises of terminally-differentiated, antigen-responsive cells. Interestingly, in mice bearing OVA-expressing AT-3 tumors, all tumor-associated OVA-tetramer\(^+\)CD8\(^+\) T-cells co-expressed CD137 and PD-1. While endogenous AT-3 antigens and antigen-specific CD8\(^+\) T-cells have yet to be characterised, these results strongly suggest that tumor-specific CD8\(^+\) T-cells reside within the tumor-associated CD137\(^+\)PD-1\(^{\text{High}}\) subset and thus could be the primary targets of \(\alpha\)-CD137/\(\alpha\)-PD-1 therapy.

The temporary enrichment of PD-1\(^{\text{High}}\) CD8\(^+\) T-cells in irradiated AT-3 tumors and concomitant loss of PD-1\(^{\text{Low}}\)-expressing CD8\(^+\) T-cells is intriguing and further favors the PD-1\(^{\text{High}}\)-expressing cells as immunotherapeutic targets of \(\alpha\)-PD-1 therapy. The reasons for the differential responsiveness of PD-1\(^{\text{High}}\) and PD-1\(^{\text{Low}}\)-expressing CD8\(^+\) T-cell subsets to RT are still unclear. However, it is tempting to speculate, based on their CD44\(^{\text{High}}\)CD62L\(^-\) phenotype that the antigen-experienced PD-1\(^{\text{High}}\) subset comprises of more committed or terminally-differentiated tumor-specific T-cells and thus are more radio-resistant. Indeed, studies have reported the acquisition of radio-resistance by T-cells upon encountering tumor-antigen in vivo (37). Furthermore, within the PD-1\(^{\text{Low}}\)CD8\(^+\) T-cell pool, a population of suppressor T-cells may exist,
which are characteristically more radio-sensitive than other T-cells (38-39). Alternatively, RT may promote a more rapid and/or preferential recruitment of antigen-experienced PD-1\textsuperscript{High} T-cells into the tumor microenvironment compared to ‘nonspecific’ or newly engaged tumor-reactive CD8\textsuperscript{+} T-cells.

In 4T1.2 tumors, which in contrast to the AT-3 model, supported a necrotic core (Suppl. Fig.4) and died in response to RT in a non-apoptotic manner, α-PD-1 therapy alone was sufficient to reject irradiated tumors (Suppl. Fig.5). These data suggest that the inflammatory nature of the tumor microenvironment can impact upon the potency of radio-immunotherapy. In line with this, while RT/α-CD137/α-CD40 therapy was non-curative in established subcutaneous AT-3 tumors, this novel combination increased the survival rate achieved with RT alone by greater than 70% in 4T1.2 tumors. These results are significant given the high metastatic potential of these tumors (40) and demonstrate the immunogenic nature of non-apoptotic radiation-induced cell death (reviewed in (41-42)).

Given the efficacy of α-CD137 therapy in irradiated AT-3 and 4T1.2 tumors, we predicted that antibody-based targeting of other co-stimulatory receptors might mediate similar therapeutic effects. The CD137-family member OX40 has been reported to enhance CD8\textsuperscript{+} T-cell-dependent anti-tumor effects of RT in highly immunogenic OVA-expressing Lewis lung carcinoma and MCA205 sarcoma (8, 43) and was therefore of particular interest. Surprisingly however, α-OX40 therapy did not enhance the therapeutic efficacy of RT in 4T1.2 (Suppl. Fig.6A) and AT-3 tumors (Suppl. Fig.6B). The reasons for the lack of α-OX40 activity in these models remain unresolved; however α-CD137 therapy can also directly alter expression of adhesion
molecules on tumor endothelial tissue, which can in turn promote infiltration of activated T-cells (44). It is therefore possible that other factors prior to or post RT, account for the different levels of α-CD137 and α-OX40 mAb activity in 4T1.2 and AT-3 tumors.

In summary, we found that single-dose and fractionated RT can be safely and effectively combined with multiple immune-stimulatory and/or -inhibitory mAbs to cure mice of established triple-negative mammary tumors. Importantly, functionally responsive, tumor-reactive lymphocytes, expressing clinically viable immunological targets such as CD137 and/or PD-1 persisted within irradiated tumors. Collectively, these results provide strong validation for combining RT with multiple immune-stimulatory and inhibitory signaling pathways in breast cancer patients treated with neo-adjuvant RT. We predict that cancers of other tissue origins, in which RT is employed as the primary course of treatment will also be responsive to such therapeutic strategies.
Acknowledgements

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References


Figure legends

**Figure 1. Radiation-induced cell death in mammary tumor cells.** Dose-response curves to ionizing radiation (IR) in presence (qVD; squares) or absence (Ctr; circles) of 10μM qVD are shown for 4T1.2 (A) and AT-3 (B) cell-cultures. Annexin-V/PI staining and Caspase-3 cleavage were used to quantify cell death and apoptosis. Data-points represent mean ±SEM of 3-6 independent experiments; *P<0.05, **P<0.01, ***P<0.001, between Ctr and qVD-treated samples. (C) Clonogenic survival of 4T1.2 and AT-3 cells following treatment with the indicated doses of IR. Data-points represent mean ±SEM of 3 independent experiments. (D-E) Mice bearing s.c. 4T1.2 (D) or AT-3 (E) tumors were left untreated or exposed to 12Gy RT. At the indicated time-points, tumors were harvested and processed for TUNEL-analysis (left panels; Scale bar: 50μM). Data-points represent mean ±SEM of TUNEL+-events per field; *P=0.0132, ***P=0.0006 and ****P<0.0001 at corresponding time-points compared to t=0h.

**Figure 2. Response of subcutaneous AT-3 tumors to treatment with RT and α-CD40/α-CD137 mAbs.** (A) AT-3 tumor-bearing mice were treated with 12Gy RT (bottom panels) or mock irradiated (upper panels) in combination with α-CD40 and/or α-CD137 mAbs as indicated. Individual tumor-growth curves (grey lines) and mean tumor-growth (black line) are shown. Numbers in parentheses indicate the fraction of tumor-free mice at 50 days post tumor inoculation. Results are representative of 2 independent experiments. (B) Mean tumor sizes in each group from Fig. 2A at day 28 post tumor inoculation; *P<0.05, **P<0.01 and ***P<0.001.
Figure 3. Rejection of established subcutaneous AT-3 tumors with RT and α-CD137/α-PD-1 mAbs. (A) Surface expression of PD-L1 on live, *in vitro* cultured AT-3 tumor cells and explanted, live CD45.2^+ AT-3 tumor cells, prior to (narrow line) and at the indicated time-points post (bold line) IR. Isotype controls 0Gy: Solid histogram, Dashed histogram: 12Gy. (B) AT-3 tumor-bearing mice were treated with 12Gy RT (bottom panels) or mock-irradiated (upper panels) in combination with α-CD137 and/or α-PD-1 mAbs as indicated. Individual tumor-growth curves (grey lines) and mean tumor-growth (black line) are shown. (C) Mean tumor sizes in each group in Fig.3B at day 28 post tumor inoculation; *P<0.05, **P<0.01 and ***P<0.001. (D) AT-3 tumor-bearing mice were treated with cIg (Ctr) or depleting antibodies to CD4, CD8β or asialoGM1, prior to mock-irradiation and MAC4 (Ctr), or radio-immunotherapy (RT/α-CD137/α-PD-1 mAbs, (IR+IT)). Results represent mean tumor-growth ±SEM. Differences between growth curves in the radio-immunotherapy-treated groups are indicated: Control versus CD8 T-cell depletion (from day 23 post tumor inoculation) **P<0.01, Control versus NK-cell depletion (days 23, 26, 33, 37, 49) *P<0.05, (days 30, 35, 40, 42, 44, 46) **P<0.01. (E) Left panel: tumor-free, IR/α-CD137/α-PD-1 treated mice were rechallenged with 10^6 AT-3 cells (grey lines) >80 days post primary AT-3 tumor clearance. Growth of secondary s.c. tumor inocula were assessed against primary AT-3 tumor-growth in naive B6 mice (black lines). Right panel: mean tumor sizes in naïve and cured mice at day 50 post tumor inoculation; *P=0.013. Results are pooled data from 2 independent experiments. (B, D-E) Numbers in parentheses indicate the fraction of tumor-free mice 40 days post tumor inoculation.
Figure 4. Rejection of established orthotopic AT-3 tumors with RT and α-CD137/α-PD-1 mAbs. (A) Mice bearing orthotopic AT-3 tumors were treated with 12Gy RT (bottom panels) or mock-irradiated (upper panels) in combination with α-CD137 and/or α-PD-1 mAbs as indicated. Individual tumor-growth curves (grey lines) and mean tumor-growth (black line) are shown. Numbers in parentheses indicate the fraction of tumor-free mice 40 days post tumor inoculation. (B) Mean tumor sizes in each group from Fig. 4A at day 29 post tumor inoculation; **P<0.01.

Figure 5. Characterization of the immune-cells subsets contributing to the rejection of orthotopic AT-3 tumors. (A) Mice bearing orthotopic AT-3 tumors were treated with 12Gy RT or mock-irradiated. At the indicated time-points, CD45.2+CD8+ tumor-infiltrating T-cells were analyzed for CD137 and PD-1 expression. The contour plots represent a concatenated analysis of CD137 and PD-1 expression for all mice within each group (n=6-18). The concatenated frequency of CD45.2+PD-1Low and PD-1HighCD8+ T-cells is shown for each time-point in each group (B) Ratio of CD45.2+CD8+PD-1High/CD45.2+CD8+PD-1Low cells in the control and irradiated tumors analyzed in (A) at all indicated time-points. **P<0.01 between control and IR groups at 12h. Results are representative of 2 independent experiments. (C) Surface expression of CD44 (top panel) and Tim-3 (bottom panel) on gated tumor-associated CD45.2+, CD62L−, PD-1Low and PD-1High CD8+ T-cells from control (mock irradiated) and irradiated AT-3 tumors, 12h post treatment. Spleen (Sp): solid histogram, PD-1High subset: solid line histogram, PD-1Low subset: dashed histogram. Concatenated analysis (n=6) (D-E) Quantification of intracellular Ki67 (D) and Granzyme B (E) expression in tumor-associated PD-1High and PD-1Low expressing CD8+ T-cell subsets at 12, 36 and 84h post 12 Gy RT (n=6-18/group) or concatenated
analysis of mock treatment from all these time-points (t=0h; n=18). In (D), **P<0.01, ***P<0.001 between PD-1^{High} and PD-1^{Low} subsets at the indicated time-points; #P<0.05 at 36h post RT for the PD-1^{Low} T-cell subset compared to t=0h. In (E), **P<0.01 at corresponding time-points compared to t=0h for the PD-1^{High} T-cell subset and #P<0.05, ##P<0.01 at corresponding time-points compared to t=0h for the PD-1^{Low} T-cell subset compared to t=0h. (F) Flow cytometric analysis of intracellular IFN-\gamma in CD45.2^{+}PD-1^{Low} and PD-1^{High} CD8^{+} T-cells in unstimulated and PMA/ionomycin-stimulated cultures from untreated, established AT-3 tumors. Contour plots represent concatenated analysis of intracellular IFN-\gamma in the PD-1^{Low} and PD-1^{High}CD45.2^{+} gated CD8^{+} T-cells for all mice (n=7). Frequency of CD45.2^{+} cells is shown for each quadrant.

**Figure 6. Antigen specificity of CD137^{+}PD-1^{High} and PD-1^{low} CD8^{+} T cells. (A)** Mice bearing orthotopic OVA-expressing AT-3 tumors were treated with RT. CD45.2^{+}CD8^{+} tumor-infiltrating T-cells in explanted AT-3 tumors were analyzed and contour plots generated as described for Fig.5A (n=6-18 mice/group). The concatenated frequency of CD45.2^{+}PD-1^{Low} and PD-1^{High} CD8^{+} T-cells is shown for each time-point in each group. (B) Ratio of CD45.2^{+}CD8^{+}PD-1^{High}/CD45.2^{+}CD8^{+}PD-1^{Low} cells in control and irradiated tumors analyzed in (A) at all indicated time-points; **P<0.01. (C) Flow cytometric plots showing OVA-tetramer-staining within PD-1^{High} and PD-1^{Low}CD8^{+} T-cell populations from mice analyzed in (A). Spleen from tumor-bearing mice served as background staining control.

**Figure 7. Fractionated RT in combination with immunotherapy induces regression of orthotopic AT-3 tumors. (A)** Mice bearing orthotopic AT-3 tumors
were treated with four fractions of 4Gy or 5Gy RT (middle and right panels) or mock-irradiated (left panels) in combination with α-CD137/α-PD-1 mAbs (bottom panels) or cIg (upper panels). Individual tumor-growth curves (grey lines) and mean tumor-growth (black line) are shown. Numbers in parentheses indicate the fraction of tumor-free mice 40 days post tumor inoculation. (B) Mean tumor sizes in each group in Fig.6A at day 34 post tumor inoculation; **P<0.01.
Figure 2

A

B

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Figure 3

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

A

in vitro

ex vivo

Time post inoculation (days)

B

Control

α-CD137

α-PD-1

α-CD137 + α-PD-1

Time post inoculation (days)

C

Day 28

Mean tumor size (mm²)

D

Control

CD4⁺ depleted

CD8⁺ depleted

NK depleted

Time post inoculation (days)

E

Rechallenge

Day 50

Tumor size (mm²)

Mean tumor size (mm²)
Figure 4

A

Control
α-CD137
α-PD-1
α-CD137 + α-PD-1

IR
IR + α-CD137
IR + α-PD-1
IR + α-CD137 + α-PD-1

Time post inoculation (days)

Tumor size (mm²)

(0/6) (0/6) (0/6) (0/6)
(0/6) (3/6) (1/6) (6/6)

B

Day 29

Mean tumor size (mm²)

**

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Figure 6

A) CD8+ T cells

Control  12 h  84 h

Irradiated

B) PD-1High/PD-1Low Ratio

Time post irradiation (h)

C) Events (% of max)

PD-1High

PD-1Low

Control

Spleen

PD-1High

PD-1Low

OVA-tet
Figure 7

A

Control  4x4 Gy  4x5 Gy

IT  4x4 Gy + IT  4x5 Gy + IT

Tumor size (mm²)

Time post inoculation (days)

B

Day 34

Mean tumor size (mm²)

Ctr  IT  4x4 Gy  4x4 Gy/IT  4x5 Gy  4x5 Gy/IT

*p  **
Radiotherapy increases the permissiveness of established mammary tumors to rejection by immunomodulatory antibodies

Inge Verbrugge, Jim Hagekyriakou, Leslie L Sharp, et al.

Cancer Res Published OnlineFirst May 8, 2012.