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Fractionated radiation therapy stimulates anti-tumor immunity mediated by both resident and infiltrating polyclonal T-cell populations when combined with PD1 blockade

Simon J. Dovedi¹,², Eleanor J. Cheadle¹, Amy L. Popple¹, Edmund Poon², Michelle Morrow², Ross Stewart³, Erik C. Yusko³, Catherine M. Sanders³, Marissa Vignali³, Ryan O. Emerson³, Harlan S. Robins⁴, Robert W. Wilkinson⁵, Jamie Honeychurch¹*, Timothy M. Illidge¹*.

¹ Targeted Therapy Group, Targeted Therapy Group, Division of Molecular and Clinical Cancer Sciences, Manchester Cancer Research Centre, Christie Hospital, Manchester Academic Health Sciences Centre, United Kingdom.

² MedImmune Ltd, Granta Park, Cambridge, United Kingdom.

³ Adaptive Biotechnologies, Seattle, Washington, United States of America.

⁴ Fred Hutchinson Cancer Research Center, Seattle, Washington, United States of America.

* authors contributed equally.

Running title: Fractionated RT modulates the local TCR repertoire

Corresponding authors:
Dr. Simon J. Dovedi. Email: Simon.Dovedi@ics.manchester.ac.uk / DovediSi@MedImmune.com, MedImmune Ltd, Granta Park, Cambridge, CB21 6GH, United Kingdom.

Prof. Tim Illidge. Email: Tim.Illidge@ics.manchester.ac.uk, Targeted Therapy Group, Division of Molecular and Clinical Cancer Sciences, Manchester Cancer Research Centre, Paterson Building, Wilmslow Road, Manchester, M20 4BX, United Kingdom.

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**Translational Relevance.** Radiotherapy (RT) is well documented to be immunogenic; however, systemic anti-tumor immune responses outside of the irradiated tumor field, termed the “abscopal effect”, are rare in patients. The lack of abscopal effect is poorly understood, particularly in the context of low-dose daily fractionated RT, the most common regimen used in clinical practice. We demonstrate that 5 daily fractions of 2 Gy induces a polyclonal T-cell response at the irradiation site which is dominated by the expansion of pre-existing T-cell clones. However, systemic anti-cancer immunity appears to be limited locally by adaptive immunological resistance which can be overcome by blockade of the PD-1/PD-L1 pathway leading to long-term tumor control. Furthermore, whilst T-cells resident in the tumor prior to RT are important, newly-infiltrated polyclonal T-cell populations are required for maximal systemic tumor control. These results provide important new insights for clinical translation to improve outcomes using RT through combination with αPD-1/PD-L1 mAb.
Abstract

Purpose: Radiotherapy (RT) is a highly effective anti-cancer treatment forming part of the standard of care for the majority of patients, but local and distal disease recurrence remains a major cause of mortality. RT is known to enhance tumor immunogenicity; however, the contribution and mechanisms of RT induced immune responses are unknown.

Experimental Design: The impact of low-dose fractionated RT (5 x 2 Gy) alone and in combination with αPD-1 mAb on the tumor microenvironment was evaluated by flow cytometry and next-generation sequencing (NGS) of the T-cell receptor (TCR)-repertoire. A dual-tumor model was used, with fractionated RT delivered to a single tumor site to enable evaluation of the local and systemic response to treatment and ability to induce abscopal responses outside the radiation field.

Results: We show that fractionated RT leads to T-cell infiltration at the irradiated site; however, the TCR landscape remains dominated by polyclonal expansion of pre-existing T-cell clones. Adaptive resistance via the PD-1/PD-L1 pathway restricts the generation of systemic anti-cancer immunity following RT which can be overcome through combination with αPD-1 mAb leading to improved local and distal tumor control. Moreover, we show that effective clearance of tumor following combination therapy is dependent on both T-cells resident in the tumor at the time of RT and infiltrating T-cells.

Conclusions: These data provide evidence that RT can enhance T-cell trafficking to locally-treated tumor sites and augment pre-existing anti-cancer T-cell responses with the capacity to mediate regression of out-of-field tumor lesions when delivered in combination with αPD-1 mAb therapy.
Introduction

Radiation Therapy (RT) is delivered to approximately 50% of all cancer patients, improving local disease control and reducing recurrence (1-3). RT can modulate the tumor microenvironment in several ways to enhance immunogenicity including modulation of class I MHC and novel peptide antigen expression on tumor cells (4); generation of type-I IFN (5-7); activation of the complement pathway (8); induction of immunogenic cell death (9); and direct effects on immune effector cells (10). Irradiated tumor cells may act as an ‘in situ vaccine’ through the provision of increased tumor-associated antigens (TAAs), damage-associated molecular patterns (DAMPs), and modulation of the tumor microenvironment promoting DC recruitment and T-cell priming (4,7,11-14). However systemic anti-tumor immune responses outside of the irradiated tumor field termed the “abscopal effect” are rare in routine clinical practice. Furthermore tumor recurrence frequently occurs following RT and remains the leading cause of patient mortality. Therefore, in the clinic, RT alone appears to be generally insufficient to elicit durable, therapeutic anti-tumor immunity (15).

The PD-1/PD-L1 axis is known to mediate peripheral tolerance and attenuation of acute inflammatory responses through modulation of T-cell receptor (TCR) signal transduction, metabolic reprogramming, anergy and apoptosis (16-18). Furthermore, we and others have previously shown across a range of tumor models that signaling through the PD-1/PD-L1 pathway can limit the ability of RT to generate systemic immune responses (19-21). In most of these preclinical studies enhanced tumor immune responses have been generated by high-doses of hypofractionated RT in combination with a range of immunomodulatory agents (19,21-25). In contrast, the
Fractionated RT modulates the local TCR repertoire potential effects on local and systemic T-cell responses of low-dose daily fractionation as routinely delivered using a series of daily fractionated doses (1.8-2 Gy) are less well studied. Here we have investigated how daily fractionation modulates the TCR repertoire diversity within the irradiated tumor and how this may be modified with RT in combination with αPD-1 mAb to result in a systemic immune response. By sequencing the CDR3 regions of TCRβ in both irradiated and out-of-field tumors, and in peripheral blood, we reveal that RT leads to local expansion of pre-existing T-cell clones within the tumor which dominate the TCR repertoire. In contrast, there was little evidence of T-cell clonal expansion as a consequence of RT-induced DNA-damage with all of the dominant T-cell clones present in both the irradiated and out-of-field tumors following treatment with RT and αPD-1 mAb. In addition, we show that the immunological effects of fractionated RT appear to be limited to the irradiated tumor site through adaptive upregulation of PD-L1. Importantly, blockade of the PD-1/PD-L1 signaling axis circumvents this local immunosuppression facilitating the generation of systemic anti-cancer immunity capable of mediating distal tumor regression. These observations provide new mechanistic insights into the impact of daily fractionated RT on the generation of adaptive anti-tumor immunity.

Materials and Methods

Mice and cell lines

BALB/c and C57Bl/6 mice were obtained from Harlan, U.K. Animal experiments were approved by a local ethical committee and performed under a United Kingdom Home Office license. CT26 murine colon carcinoma cells (ATCC) and 4434 cells isolated from BRAFV600E p16-/- mice (Richard Marais, Cancer Research...
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UK Manchester Institute, Manchester, United Kingdom) were maintained in DMEM, supplemented with 10 % FCS, 1 % L-glutamine (Invitrogen, U.K.). Upon receipt, cells were cultured for up to 4 passages, screened to confirm the absence of Mycoplasma contamination by PCR and aliquots frozen in liquid nitrogen to create a batch of authenticated stock lines. Cell lines were defrosted and cultured to limited passage for one to two weeks prior to implantation with regular re-screening for Mycoplasma contamination. Mice were housed under specific pathogen free conditions in Tecniplast 1284 IVC cages holding a maximum of 7 animals with aspenchips-2 bedding, sizzlenest nesting material, and a cardboard tunnel. Mice were housed on a 12/12 light/dark cycle and were given filtered water and fed ad libitum on Teklad Global 19 % protein extruded rodent diet.

**Tumor therapy**

Mice were inoculated sub-cutaneously (s.c.) with 5x10^5 CT26 cells or 5x10^6 4434 cells at one or more distinct sites. Irradiations were performed 7-10 days after the primary tumor was inoculated (when primary tumors were at least 100 mm^3) using a Pantak HF-320 320 kV x-ray unit (Gulmay Medical, U.K.). The machine was operated at 300 kV, 9.2 mA, with filtration fitted in the x-ray beam to give a radiation quality of 2.3 mm Cu half-value layer. Mice were positioned at a distance of 350 mm from the x-ray focus, where the dose rate was 0.80 Gy/min and treated using tangential beam delivery. Administration of αPD-1 (clone RMP1-14), αPD-L1 (clone 10F.9G2) or isotype control mAb (Biolegend, U.K.) commenced on day 1 of the fractionated RT cycle via intra-peritoneal (i.p.) injection 3qw for 1 week at a dose of
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10 mg/kg in a dose volume of 100 μl/10 g in PBS. Tumor volume (up to 1200 mm³) was the primary endpoint for efficacy studies. Peripheral blood was sampled during therapy to confirm cellular depletion. Administration of FTY-720 (Fingolimod; Enzo Life Sciences, UK) commenced either the day prior to tumor inoculation or the day prior to the start of RT and was delivered by oral gavage at a dose of 25 μg/mouse in a dosing volume of 200 μl. Subsequent daily administration was continued for 30 days (after the start of RT) at a dose of 5 μg/mouse in a dosing volume of 100 μl as previously described (26).

For tumor rechallenge experiments, long-term surviving (LTS) mice were implanted with tumor cells at a site distal to the original tumor a minimum of 80 days after previous tumor implantation. Experimental groups contained at least 5 mice/group and are representative of at least 2 independent experiments.

**Measurement of cytokine production by CD8+ T-cells isolated from long-term surviving mice**

Splenocytes were isolated from either LTS or control mice and co-cultured for 5 days with either irradiated tumor cells (50 Gy) or 1 μmol/ml of the H2-Ld restricted peptides SPSYVYHQF (AH1) or TPHPARIGL (β-galactosidase) (Anaspec, U.K.). Cells were restimulated at a 1:1 ratio with 50 Gy irradiated CT26 cells for 16 hours in the presence of 1 μl/ml Brefeldin A (BD Pharmingen, U.K.) and 100 IU/ml human recombinant IL-2 (Chiron, NL) as described previously (27). Experimental groups contained at least 4 mice and are representative of 2 independent experiments.

**Tumor and immune cell phenotyping by flow cytometry**
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To obtain single cell suspensions, tumors were processed using a gentle MACS dissociator and a murine tumor dissociation kit (Miltenyi Biotec, U.K.). For analysis, non-specific binding was blocked as described above and expression of CD4, CD8 (BD Biosciences, U.K.), CD45, CD11b, Gr1, PD-1 and PD-L1 examined by multi-parameter flow cytometry (all eBioscience unless otherwise stated). For analysis, live cells were gated (by vital dye exclusion, Invitrogen, U.K.) and populations phenotyped (as described above). An example of the gating strategy employed for selection of either CD45⁻, CD45⁺ or CD45⁺CD11b⁻Gr1⁺ cells is provided in Supplemental Fig. 1.

**Immunosequencing of the TCR-β expressing repertoire in peripheral blood and tumors.** Immunosequencing of the CDR3 regions of murine TCRβ chains was performed on samples isolated 7 days after the last dose of RT (along with time-matched control cohorts and those treated with αPD-1 mAb) using the ImmunoSEQTM Assay (Adaptive Biotechnologies, Seattle, WA). This assay uses 54 forward V gene primers and 13 reverse J gene primers which are employed in a bias-controlled multiplexed PCR reaction to amplify the variable region of TCRβ chains. Synthetic control templates were also spiked into each sample, thereby enabling quantitation of input TCRβ templates from the read counts (28). Sequences were collapsed and filtered in order to identify and quantitate the abundance of each unique TCRβ CDR3 region for further analysis (28,29).

**Statistical Analyses of TCR-β sequencing results**
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The clonality metric is defined as Peilou’s evenness and is calculated as:

$$1 - \frac{\sum_{i=1}^{N} p_i \ln(p_i)}{\ln(N)}$$

Clonality values range from 0 to 1 and describe the shape of the frequency distribution: clonality values approaching 0 indicate a very even distribution of frequencies, whereas values approaching 1 indicate an increasingly asymmetric distribution in which a few clones are present at high frequencies. To estimate the fraction of T-cells in the tissue samples, we considered 6.5 pg of DNA per diploid cell, which is equal to approximately 154 productive TCR loci per ng of DNA, and normalized the total T-cell estimates in each sample to the amount of input DNA multiplied with the value of 154 productive TCR loci per ng of input DNA. Pair-wise comparisons within therapy cohorts (i.e. tumor 1 vs. tumor 2) were performed using a paired t-test. For comparisons across multiple therapy groups we used the non-parametric Kruskal-Wallis test. We identified clones with significantly different abundance in two samples using a Fisher’s exact test with Benjamini-Hochberg corrected p-values such that false-discovery-rates were held at 5 % (30).

Results

Fractionated RT in combination with αPD-1 mAb generates systemic anti-cancer immunity

Low doses of fractionated RT (delivered as 5 daily fractions of 2 Gy) resulted in transient tumor control followed by regrowth in the majority of mice bearing either CT26 or 4434 tumors. In both models, local tumor control following treatment with fractionated RT can be improved when combined with mAb’s targeting the PD-1/PD-L1 pathway (Fig. 1A and B). Moreover, long-term surviving (LTS) mice that undergo a complete response following combination therapy are able to completely reject
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tumors following rechallenge (Fig. 1C. P < 0.01 Log-rank; Mantel-Cox test).
Furthermore, in responding mice we detected an increased frequency of antigen-specific memory CD8\(^+\) T-cells capable of IFN\(\gamma\) expression following co-culture with either irradiated CT26 cells or a CT26 tumor-associated peptide antigen (AH1: SPSYVYHQF) but not with a control peptide (β-galactosidase: TPHARIGL) (Fig. 1D). To address the impact of RT dose and fractionation on therapeutic response RT was also administered as a hypofractionated regimen (12 Gy in 3 fractions) or as a single dose (7 Gy) in combination blockade of the PD-L1/PD-L1 axis (Fig. 1E). Importantly, our data reveal similar combinatorial activity for these different RT dosing regimens.

Dual tumor-bearing mice were used to determine whether fractionated RT delivered to a single tumor could lead to ‘abscopal’ responses in out-of-field tumors. We found that low doses of fractionated RT resulted in transient local tumor control followed by regrowth in the majority of mice treated. However, complete tumor regression was observed in 18.8% (3/16) mice at the irradiated site. Complete regression of the out-of-field tumor following RT was a rare event (observed in 12.5 %, 2/16 mice), occurring only in mice where the response of the irradiated tumor was such that the mice survived to the study endpoint (day 60) (Fig. 2A for experimental schema, 2B and Supplemental Fig. 2A). To determine whether local treatment with an ablative dose of RT would be more effective at generating out-of-field responses we treated mice locally with 10 Gy RT (which led to complete responses in 4/7 (57 %) tumors. Our data demonstrate that treatment with ablative doses of RT alone were insufficient to generate systemic anti-cancer immune responses capable of mediating complete regression of out-of-field tumors with all.
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mice exhibiting progressive tumour growth at the out-of-field tumor site
(Supplemental Fig. 2B).

In contrast, RT delivered concurrently with αPD-1 mAb led to the regression of both the irradiated and out-of-field tumors with >70% of mice undergoing complete responses (Fig. 2B). These data demonstrate that the combination of fractionated RT and αPD-1 mAb generates systemic anti-tumor responses and tumor control in both irradiated and out-of-field tumors.

**Combination therapy leads to convergence of tumoral and systemic TCR repertoires**

In order to understand how these treatments impacted the clonal populations of tumor resident T-cells we sequenced the TCR repertoire 7 days after the last dose of RT. Analysis of the TCR-β sequences confirmed that fractionated RT increases the frequency of T-cells in the irradiated but not out-of-field tumor (Fig. 2C). Whilst TCR quantitation does not distinguish between tumor-resident proliferating T-cells or infiltrating T-cells, increased overlap between the TCR repertoire of the irradiated (but not the out-of-field) tumor and that of peripheral blood suggests that T-cell infiltration occurs after local fractionated RT (Fig. 2D). When delivered as a monotherapy, αPD-1 mAb did not enhance T-cell content in either tumor relative to the NT mice. In contrast, concurrent RT and αPD-1 mAb therapy resulted in increased T-cell infiltration/expansion in both the irradiated and out-of-field tumors relative to NT mice or αPD-1 mAb-treated mice (Fig. 2C, D).

We next examined the impact of these treatments on the clonality of TCR repertoires at the different tumor sites. We found that monotherapy with αPD-1 mAb did not alter T-cell clonality in either the local or distal tumor when compared to NT
Fractionated RT modulates the local TCR repertoire controls. In contrast, RT-treated mice had increased TCR repertoire clonality in the irradiated but not out-of-field tumor when compared to either NT or αPD-1 mAb-treated mice (Fig. 2E). Moreover, RT increased the number of unique TCRs (thereby increased the diversity of the TCR repertoire) in the irradiated but not out-of-field tumor when compared to NT controls (Fig. 2F). The largest increase in TCR diversity in both the irradiated and out-of-field tumors occurred in mice treated with RT/αPD-1 mAb therapy, demonstrating that this combination generated an immunologic response that extended beyond the RT-treatment field. However, the precise contribution of these individual T-cell clones to tumor control remains unknown.

Analysis of clone sharing and dynamics between the irradiated and out-of-field tumors (tumor #1 and #2 respectively) revealed that most TCR clones and all high abundance clones (>10 copies detected) were present in both tumors in the NT mice (Fig. 3A, first panel). Moreover, the frequencies of individual clones in both tumor repertoires showed a strong concordance (slope = 0.85, \( R^2 = 0.94 \)). These observations indicate that similar TCR repertoires, derived from common progenitor clones were established in both tumors prior to therapy. Similar results were observed in the mice treated with αPD-1 mAb as a monotherapy (slope = 0.90, \( R^2 = 0.95 \)) (Fig 3A, second panel), which is consistent with the lack of T-cell infiltration/expansion observed in either tumor (Fig. 2C). In contrast, fractionated RT led to preferential clonal expansion in the irradiated tumor and infiltration of many unique clones (clones shown below the dotted line; slope = 0.12, \( R^2 = 0.84 \), Fig. 3A, third panel. median = 3,015, IQR: 2,759 – 3,124) which corresponded to 80% of unique clones but only 13.3% of T-cells (IQR = 5.7 – 16.3%). Interestingly, a large portion of the clones identified as having a significantly greater frequency in tumor 1 than in tumor 2 (median: 90%, IQR: 92.6 – 89.1%) were also detected at low abundance in the out-
Fractionated RT modulates the local TCR repertoire of-field tumor. Treatment with RT and αPD-1 mAb restored some of the concordance of T-cell clones observed in the tumors of NT mice and led to greater convergence in expanded T-cell clones present in both the irradiated and out-of-field tumors (slope = 0.5, $R^2 = 0.66$) (Fig. 3A, last panel and Fig. 2D). Moreover, the majority of high abundance clones in the irradiated tumor were also observed in the out-of-field tumor (median = 99.53%). These data demonstrate that there is a high degree of concordance in the TCR clonotypes infiltrating into both the irradiated and out-of-field tumors.

Whilst RT led to an increase in the TCR repertoire overlap between the tumors, the number of clones shared between the irradiated and out-of-field tumors only increased in response to combination therapy (Fig. 3B and C). Correspondingly, few T-cell clones had higher abundance in the out-of-field tumor than in the irradiated tumor except in mice that received combined therapy (Fig. 3D). These results are consistent with T-cell trafficking either from the irradiated tumor and/or the periphery to the out-of-field tumor. Analysis of the top 25 clones identified in the irradiated tumor and tracking the frequency of these clones in the out-of-field tumor and in peripheral blood, reveals that expansion of these principal clones in the second tumor only happens following combination therapy with RT and αPD-1 mAb (Fig. 3E and Supplemental Fig. 3). Importantly, these data demonstrate that fractionated RT stimulates a polyclonal T-cell response that is restricted to the irradiated tumor site and ultimately fails to control tumor growth. When RT is delivered in combination with PD-1 blockade, this polyclonal response extends outside of the irradiated field with propagation to the out-of-field tumor and to generation of a systemic therapeutic anti-tumor response. Furthermore, dose-scheduling was critical for anti-tumor
Fractionated RT modulates the local TCR repertoire activity with concomitant but not sequential administration of αPD-1 mAb required to mediate tumor regressions in the non-irradiated tumor (Supplemental Fig. 4).

The effects of fractionated RT rarely extend beyond the treatment site

To provide further context to the TCR sequencing data we also characterized the changes in the tumor microenvironment by flow cytometry (experimental schema outlined in Fig. 4A). Profiling of tumor-infiltrating effector T-cells revealed that fractionated RT leads to an acute reduction in the number of CD8+ and CD4+ T-cells (at day 1 and day 1 and 3 respectively) in the irradiated but not out-of-field tumors when compared to time-matched NT controls (Fig. 4B, left and middle panels). By day 7 there is a small expansion in CD8+ (1.47 fold compared to time-matched controls) but not CD4+ T-cells in the tumor that received RT. In contrast, RT led to a significant increase in the number of CD11b+ Gr1+ cells infiltrating into the in-field, but not the out-of-field tumors when compared to time-matched NT controls (Fig. 4B, right panel). This change was also transient and by day 7 post RT no difference in CD11b+ Gr1+ cell numbers was observed between the in- and out-of-field tumors. Given that tumor cell expression of PD-L1 may represent a biomarker of a local effector T-cell response we evaluated the impact of local RT on tumor cell PD-L1 expression both in and out of the RT field. We have previously shown that low-dose fractionated RT can lead to CD8+ T-cell dependent expression of PD-L1 at an irradiated tumor site (27). Here we demonstrate that expression of PD-L1 is elevated on CD45+ tumor cells only in the irradiated, but not out-of-field lesion (at all timepoints tested) (Fig. 4C and Supplemental Fig. 5A) and on CD11b+Gr1+ cells (transiently at day 3) (Fig. 4D and Supplemental Fig. 5B). We have previously demonstrated that this increase in PD-L1 following local fractionated RT is mediated
Fractionated RT modulates the local TCR repertoire by CD8^+ T-cell issued IFNγ (20). In this context, tumor cell expression of PD-L1 may be a biomarker of local effector T-cell responses suggesting that these responses are restricted to the site of treatment.

**Combined treatment with fractionated RT and αPD-1 mAb facilitates CD8^+ T-cell expansion in the irradiated and out-of-field tumors**

As we previously observed with RT alone, combination therapy with RT and αPD-1 mAb (experimental schema outlined in Fig. 5A) also led to a significant reduction in the number of CD8^+ T-cells (but not CD4^+ T-cells) infiltrating the tumor (when compared to out-of-field lesions) (Fig. 5B). However, this reduction in CD8^+ T-cells was acute, and by day 7 both the irradiated and out-of-field tumors had significantly greater numbers of CD8^+ T-cells (2.15 and 1.96 fold respectively) when compared to the time-matched NT controls. Moreover, the numbers of CD11b^+ Gr1^+ cells in the irradiated tumor were increased on day 1 post combined RT/αPD-1 mAb therapy in the irradiated tumor when compared to the out-of-field tumor (Fig. 5B). However, in contrast to our observations after RT alone, this increase was transient and followed by a significant reduction in CD11b^+ Gr1^+ cell numbers in the irradiated tumor, when compared to the out-of-field tumor at days 3 and 7. In contrast to treatment with RT alone, combined therapy with RT and αPD-1 mAb leads to the upregulation of PD-L1 expression in both the irradiated and out-of-field tumors at all timepoints tested (Fig. 5C and Supplemental Fig. 5A). A similar pattern of PD-L1 expression was also observed on tumor infiltrating CD11b^+ Gr1^+ cells (Fig. 5D and Supplemental Fig. 5B). Together these data demonstrate that fractionated RT, when delivered in combination with αPD-1 mAb leads to dynamic changes in tumor-
Fractionated RT modulates the local TCR repertoire infiltrating CD8+ effector T-cell populations capable of mediating regression of both irradiated and out-of-field tumors.

**Both tumor resident and infiltrating T-cells contribute to therapeutic activity following combination therapy**

To determine the relative contribution of tumor resident versus infiltrating T-cells on the therapeutic response following combined RT/αPD-1 mAb therapy we used FTY-720 (a sphingosine 1-phosphate receptor agonist) which prohibits T-cell emigration from lymphoid tissues (31). FTY-720 has been shown to have direct anti-tumor effects (32,33). We initially confirmed that treatment with low doses of FTY-720 had no effect on tumor growth whilst retaining the capacity to reduce both circulating and tumor-infiltrating T-cell populations (Supplemental Fig. 6A and B). We then determined the effect of blocking T-cell infiltration into the tumor either prior to implantation or prior to treatment (experimental schema outlined in Fig. 6A). Our data demonstrates that T-cells resident in the tumor at the time of treatment are capable of mediating therapeutic responses following combined therapy with ~40% of mice that received combined RT/αPD-1 mAb therapy undergoing curative responses when FTY-720 treatment was initiated on the day prior to therapy (6 days post tumor implantation) (Fig. 6B and C). However, infiltration of circulating T-cells into the tumor post therapy was required to achieve maximal responses (~80% of mice which did not receive FTY-720 achieved curative responses).

These data demonstrate that in a proportion of mice, tumor-resident T-cells have the capacity to mediate local tumor control and clearance. However, the response mediated by tumor resident T-cells appears insufficient and infiltrating T-
Fractionated RT modulates the local TCR repertoire cells from outside of tumor are also required for successful clearance of tumor in the majority of mice following combination RT/αPD-1 mAb therapy.

Discussion

In this study we demonstrate for the first time that low-dose daily fractionated RT leads to polyclonal T-cell infiltration and expansion at the site of treatment but that the generation of systemic anti-tumor immunity is suppressed through the PD-1/PD-L1 axis. Inhibition of this axis facilitated the generation of a systemic polyclonal T-cell response capable of mediating out-of-field (abscopal) effects following local RT. Our data demonstrate that both tumor-resident and infiltrating T-cells contribute to tumor control following combined therapy. Furthermore, immunosequencing of the CDR3 regions of TCRβ revealed that all of the dominant T-cell clones were present in both the irradiated and out-of-field tumors.

We have previously demonstrated that fractionated RT leads to local T-cell activation and production of IFNγ which drives adaptive resistance through the PD-1/PD-L1 axis (20). In this study we provide additional insight into this T-cell response and demonstrate that low-dose daily fractionated RT modulates the TCR repertoire leading to polyclonal expansion of pre-existing TIL populations within the irradiated tumor but not within the out-of-field non-irradiated tumor, co-incident with the emergence of immunological suppression. Here we show that RT leads to increases in PD-L1 expression on both tumor cells and CD11b+ Gr1+ cells only within the irradiated tumor. Given that PD-L1 expression in the tumor microenvironment appears to be a biomarker for local CD8+ T-cell activation this data suggests that the immune response is restricted to the site of RT. Complete regression of both the...
Fractionated RT modulates the local TCR repertoire

irradiated and out-of-field tumors following treatment with fractionated RT alone was a rare event across independent experiments and it remains unclear what local and systemic events underpin these responses. In contrast, concurrent treatment with RT and αPD-1 mAb overcomes this local immunosuppression facilitating systemic anti-tumor immunity capable of mediating the regression of distal, non-irradiated lesions which also exhibit increased expression of PD-L1 on tumor and CD11b+ Gr1+ cells. Sequential therapy where PD-1 blockade began 7 days after completion of the fractionated RT cycle was ineffective suggesting that whilst fractionated RT can lead to changes in the TCR repertoire in the irradiated tumor, exhaustion and subsequent atrophy of tumor-reactive TILs may occur rapidly after RT unless signaling through the PD-1/PD-L1 axis is blocked. Whilst regression of irradiated tumor has previously been shown to be CD8+ T-cell dependent (27) further mechanistic studies would be required to determine the relative contribution of the distinct T-cell clones at the irradiated and out-of-field tumor sites and confirm causality to response.

The frequency of T-cells in the tumor is well documented to predict outcome (34); however, the relative contribution of tumor resident versus infiltrating T-cells on therapeutic response following combination RT and αPD-1 mAb therapy are unclear. The analysis of the TCR repertoires in the blood and tumor demonstrate that fractionated RT leads to T-cell infiltration in the irradiated tumor but not the distal non-irradiated tumor site. However, whilst both tumor resident and infiltrating T-cells were required for complete responses in approximately 80 % of mice following combined therapy, a proportion of mice elicited complete tumor regressions mediated by resident T-cells alone. This is despite the radiosensitivity of lymphocytes and subsequent observed reduction in TIL number within 24 hours after a fractionated RT cycle. Furthermore, by day 7 post combination therapy both the irradiated and out-of-
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Field tumors had significantly greater numbers of CD8^+ T-cells after daily fractionated RT. Although it cannot be ruled out that T-cell numbers increased due to shrinkage of the tumor this data suggests that those T-cells which are activated following RT may be more radioresistant, potentially through modulation of anti-apoptotic proteins such as BCL-2, BCL-xl and Bim (35). However, maximal therapeutic responses required both resident and infiltrating T-cells. Preclinical studies demonstrate that naïve TILs can undergo activation and differentiation within the tumor microenvironment in the presence of sufficient antigen and co-stimulation provided by local APC (36,37). Given the immunogenic nature of RT, further studies are required to delineate the relative contribution of in situ priming by tumor resident APC versus classical priming in secondary lymphoid organs.

The generation of neo-antigens secondary to DNA-damage following ionizing radiation is hypothesized to contribute to tumor control through broadening of the TCR repertoire (4). Our data suggests that following fractionated RT only a small fraction of T-cells (<0.5%) are unique to the irradiated tumor, and therefore we speculate may be specific for a neo-antigen generated by RT. In addition, the high level of concordance in the high-abundance clones present in both the irradiated and out of field tumor suggest that the antigens may be shared across the 2 tumors. These data suggest that following low-dose fractionated therapy the T-cell response may be dominated by clones responding to pre-existing tumor antigens and we speculate that the response against potential neo-antigens generated as a consequence of RT-induced DNA-damage may be minimal. Interestingly, the addition of αPD-1 mAb to RT does not significantly alter the clonality or diversity of the T-cell repertoire above that of RT alone. Presumably this is because inhibition of signaling through PD-1 may principally operate to reinvigorate T-cells. However, NGS of TCRs does not enable
Fractionated RT modulates the local TCR repertoire identification of specific peptide antigens or define the lineage of the TCR expressing cell and further mechanistic studies would need to be undertaken to confirm the specificity of TCR clonotypes restricted to CD4$^+$ and CD8$^+$ T-cells. Moreover, targeting co-activatory immune checkpoints such as CTLA-4 have been shown to broaden the peripheral TCR repertoire (38,39). Therefore, combining RT and αCTLA-4 mAb may co-operate to further enhance repertoire diversity and potentially improve therapeutic outcome (22,25).

Recent advances in the delivery of RT such as stereotactic ablative radiation therapy (SBRT) permit high single-fraction doses to be administered to patients with minimal collateral damage to normal tissue. Preclinical evidence across a range of syngeneic models demonstrates that RT dose fractionation can influence systemic immune response and subsequent tumor control (23,27,40,41). A number of recent publications have demonstrated the enhancement of anti-tumor immune responses when mAbs targeting co-inhibitory/activatory receptors such as PD-1/PD-L1, CTLA-4 and CD137 are combined with high ablative single (e.g. 10 / 12 Gy (42), 12 Gy (19,24) or 20 Gy (25)) or hypofractionated RT-regimens (e.g. 3 x 8 Gy (21,23) or 5 x 6 Gy (22)). Moreover, increased TCR diversity in irradiated tumors was also described in a preclinical model following treatment with a single dose of 20 Gy RT (25). Despite our data in the CT26 model demonstrating that varying RT dose fractionation has little effect on therapeutic response when combined with blockade of the PD-1/PD-L1 pathway, the extent to which these different RT dose and fractionation approaches may differently modulate neo-antigen generation, TCR diversity and therapeutic response in the clinical setting remains unclear and requires further investigation. The impact of RT delivered as low-dose fractionated regimens, higher dose hypofractionated and single-dose ablative RT is not only likely to effect
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the amount, kinetics and type of tumor cell death it is also likely to be affected by the
intrinsic radiosensitivity and microenvironment of the tumor. Higher RT doses are
more likely to have very different effects on the tumor microenvironment and anti-
tumor immune response compared to the lower 2 Gy per day fractionated RT which
was used in this study and routinely given to a majority of cancer patients. The effect
of RT dose and fractionation has been shown in a recent study demonstrating that a
single 30 Gy dose of RT stimulated curative CD8⁺ T-cell dependent responses but
that this effect was abrogated when followed by 10 days of fractionated therapy (3 Gy
/day) (41). These data suggest that that repeated doses of RT to the tumor may be
detrimental to the anti-tumor immune response and this may have profound
implications for the current practice of irradiating tumor draining lymph nodes, which
needs further study.

A number of clinical trials are currently on-going to further delineate optimum
RT dose and fractionation schedules to improve immunologic response (43). Whilst
RT alone has been shown to induce abscopal effects in a limited number of patients
(44) reports of abscopal effects following concurrent RT and immunotherapy are
currently limited to case reports and results from clinical trials investigating RT plus
immune checkpoint blockade are eagerly awaited. In conclusion, the combination of
RT and immunotherapy holds great promise to improve cancer outcomes. Unlocking
this potential requires further investigation with well-designed clinical trials
investigating the effect of RT dose fractionation in different tumors to guide the next
phases of clinical development.

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References

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Figure Legends

Figure 1. Combination of fractionated RT and blockade of the PD-1/PD-L1 axis leads to improved survival and generation of tumor-specific memory immune responses. (A and B) CT26 (A) or 4434 (B) tumor bearing mice received fractionated RT delivered in 5 daily fractions of 2 Gy either in combination with either αPD-1/αPD-L1 mAb dosed at 10 mg/kg 3qw. Treatments started 7 days after tumor inoculation. Experimental groups contained at least 7 mice, except NT and RT groups which contained 14 mice. Data representative of 2 independent experiments. (C) Survival curve of long-term surviving (LTS) mice originally treated with RT and αPD-1 mAb/αPD-L1 mAb following rechallenge with 5x10^5 CT26 cells. Experimental groups contained at least 4 mice. (D) Frequency of IFNγ^+ CD8^+ T-cells isolated from either tumor naïve, or LTS mice originally treated with RT and αPD-L1 mAb following co-culture with either H2-Ld restricted peptides (AH1 (SPSYVYHQF); a defined CT26 tumor-associated antigen or β-galactosidase (TPHPARIGL); control peptide of prokaryotic origin) or 50 Gy irradiated CT26 cells for 5 days, followed by priming with 50 Gy irradiated CT26 cells. Experimental groups contained at least 4 mice, and the data shown are representative of at least 2 independent experiments. (E) CT26 tumor bearing mice received either 7 Gy or 3 daily fractions of 4 Gy alone or in combination with an αPD-1 mAb dosed at 10 mg/kg 3qw. The proportion of mice that experienced complete tumor resolution is indicated in each panel. C, **, P < 0.01, +, P < 0.05, Log-rank (Mantel-Cox) test. D, shows mean ± SEM. *, P<0.05, Mann-Whitney test.
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Figure 2. RT leads to local T-cell infiltration/expansion and broadening of the TCR repertoire but systemic responses are only observed when combined with αPD-1 mAb. (A) Schema for studies. Fractionated-dose RT (as 10 Gy in 5 daily fractions of 2 Gy) was delivered to tumor 1 with tumor 2 shielded from the ionising beam in combination with αPD-1 mAb dosed at 10 mg/kg 3qw for 1 week. (B), Individual tumor growth curves. The proportion of mice that experienced complete tumor resolution is indicated in each panel. Experimental groups contained at least 4 mice, and the data shown are representative of at least 3 independent experiments (for αPD-1 mAb only arms these data comprise a minimum of 5 mice and 2 independent experiments). (C-F) Cohorts of 5 mice per group were euthanized 7 days after the last dose of RT and TCR metrics determined. (C), T-cell infiltration/expansion. (D), the number of TCR molecules detected in post-therapy blood samples and the respective tumor sample divided by the total number of TCR molecules in the two samples. (E), TCR clonality. (F), number of unique TCRs identified in both tumors stratified by therapy. Asterisks with bars indicate pair-wise comparisons between the tumors within a therapy group **, P < 0.01, *, P<0.05 – paired t-test, and stand-alone asterisks denotes significance when compared to NT control **, P < 0.01, or *, P<0.05 level.

Figure 3. Combination therapy leads to convergence of the TCR repertoires in both irradiated and out-of-field tumors. (A), scatterplots of clone abundance in tumor 2 versus tumor 1 following treatment. Points to the left of or below the dotted line were TCR clones detected in only tumor 2 or tumor 1, respectively. Quantitation of overlap metrics between the TCR repertoires of tumor 2 and tumor 1 are: (B), the number of shared TCR molecules identified in sequencing divided by the total
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number of TCR molecules in the two samples, (C), number of unique TCR clones that were found in both tumors, and (D), the number of clones with significantly greater frequency in tumor 2 than in tumor 1. (E), the top 25 clones in tumor 1 were tracked in pre-therapy blood, post-therapy blood, and in post-therapy tumor 2 samples; the plots quantify the T-cell fraction (T-cells per nucleated cell) for each clone in each sample. Each graph is representative of an individual animal. Asterisks with bars indicate pair-wise comparisons between the tumors within a therapy group: ***, P < 0.001, **, P < 0.01, *, P<0.05 – paired t-test. Experimental groups contained 5 mice.

**Figure 4. Fractionated RT leads to transient depletion of T cells and expansion of CD11b+ Gr1+ cells at the site of treatment.** (A) Schema for studies. Fractionated-dose RT (as 10 Gy in 5 daily fractions of 2 Gy) was delivered to tumor 1 with tumor 2 shielded from the ionising beam. (B) Fold change in CD8+ (left panel), CD4+ (middle panel) and CD11b+Gr1+ (right panel) cells compared to non-treated time-matched controls in the irradiated (1) and out-of-field (2) tumors. (C and D) Expression of PD-L1 on CT26 cells (C, gated as CD45- cells) and on CD45+CD11b+Gr1+ (D) 24 hours, 72 hours and 7 days post treatment with 5 fractions of 2 Gy RT. Fold changes were calculated by determining the frequency of cells as a population of CD45+ and then expressing these relative to their frequency in time/anatomical implant site-matched NT control mice. Dashed line represents the baseline for each tumor implanted in mice receiving NT. ***, P < 0.001, **, P < 0.01, *, P<0.05 (Mann-Whitney test).

Experimental groups contained 4-5 mice, and the data shown are representative of at least 2 independent experiments.
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**Figure 5.** Fractionated RT delivered concurrently with αPD-1 mAb leads to adaptive immunological changes in both irradiated and out-of-field tumors and systemic tumor control. (A) Schema for studies. Fractionated-dose RT (as 10 Gy in 5 daily fractions of 2 Gy) was delivered to tumor 1 with tumor 2 shielded from the ionising beam in combination with αPD-1 mAb dosed at 10 mg/kg 3qw for 1 week. (B), fold change in CD8$^+$ (left panel), CD4$^+$ (middle panel) and CD11b$^+$Gr1$^+$ (right panel) cells compared to non-treated time-matched controls in the irradiated (1) and shielded (2) tumors. Fold changes were calculated by determining the frequency of cells as a population of CD45$^+$ and then expressing these relative to their frequency in time/anatomical implant site-matched NT control mice. Dashed line represents the baseline for each tumor implanted in mice receiving NT. (C and D) Expression of PD-L1 on CT26 cells (C, gated as CD45$^-$ cells) and on CD45$^+$CD11b$^+$Gr1$^+$ (D) 24 hours, 72 hours and 7 days post treatment. ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$ (Mann-Whitney test). Experimental groups contained 3-5 mice, and the data shown are representative of at least 2 independent experiments.

**Figure 6.** Tumor resident and infiltrating T-cells contribute to the therapeutic response following fractionated RT and blockade and αPD-1 mAb therapy. (A), Schema for studies. CT26 tumor bearing mice received fractionated RT delivered in 5 daily fractions of 2 Gy in combination with αPD-1 mAb dosed at 10 mg/kg 3qw for 1 week. Cohorts of mice also received FTY-720 dosed at 25 µg/mouse for the first dose and 5 µg/mouse qd for up to 6 weeks. (B) Individual tumor growth curves. Pie charts represent the proportion of mice that experienced complete tumor resolution. (C), Kaplan Meier curves of therapy. Experimental groups contained at least 7 mice, and
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the data shown are representative of at least 2 independent experiments. n/s, P>0.05, Mann-Whitney test. ***, P < 0.001, Log-rank (Mantel-Cox) test.
Figure 1

A. Kaplan-Meier survival curves showing the effect of different treatments on tumor survival. NT, αPD-1 mAb 10 mg/kg, αPD-L1 mAb 10 mg/kg, 5x2Gy RT, RT + αPD-1 mAb, RT + αPD-L1 mAb.

B. Kaplan-Meier survival curves showing the effect of different treatments on tumor survival. NT, αPD-1 mAb, 5x2Gy RT, 5x2Gy RT + αPD-1 mAb.

C. Kaplan-Meier survival curves showing the effect of different treatments on tumor survival. NT, 5x2Gy RT + αPD-1 mAb, 5x2Gy RT + αPD-L1 mAb.

D. Flow cytometry analysis showing the percentage of CD8+ lymphocytes expressing IFN-γ. Naive + βGal, Naive + AH1, LTS + βGal, LTS + AH1, Naive + CT26, LTS + CT26. n/s indicates not significant.

E. Tumor growth curves showing the effect of different treatments on tumor volume. NT, 7Gy RT, 3x4Gy RT, 7Gy RT + αPD-L1 mAb, 3x4Gy RT + αPD-L1 mAb.
Figure 3
Figure 4

A.

s.c. implantation of tumor Day 1

4 Days

s.c. implantation of second tumor Day 4

Treatment with 5 fractions of 2 Gy RT

Survival

Tumor growth

Phenotyping of tumor microenvironment

B.

Fold change in tumor infiltrating CD8+ T cells

Tumor 1 (in IR field)

Tumor 2 (out of IR field)

Time post last fraction of RT

Fold change in tumor infiltrating CD4+ T cells

Tumor 1 (in IR field)

Tumor 2 (out of IR field)

Time post last fraction of RT

Fold change in tumor infiltrating CD11bGr1+ cells

Tumor 1 (in IR field)

Tumor 2 (out of IR field)

Time post last fraction of RT

C.

PD-L1 expression on CD45+ tumor cells (MFI)

Non Treated

5x2Gy RT

D.

PD-L1 expression on CD11bGr1+ cells (MFI)

Non Treated

5x2Gy RT
Figure 5
Figure 6
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Fractionated radiation therapy stimulates anti-tumor immunity mediated by both resident and infiltrating polyclonal T-cell populations when combined with PD1 blockade


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