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Orignal Article

Synchrotron microbeam radiotherapy evokes a different early tumor immunomodulatory response to conventional radiotherapy in EMT6.5 mammary tumors

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ABSTRACT

Background: Synchrotron microbeam radiation therapy (MRT) is a new, evolving form of radiotherapy that has potential for clinical application. Several studies have shown in preclinical models that synchrotron MRT achieves equivalent tumor control to conventional radiotherapy (CRT) but with significantly reduced normal tissue damage.

Methods: To explore differences between these two modalities, we assessed the immune cell infiltrate into EMT6.5 mammary tumors after CRT and MRT.

Results: CRT induced marked increases in tumor-associated macrophages and neutrophils while there were no increases in these populations following MRT. In contrast, there were higher numbers of T cells in the MRT treated tumors. There were also increased levels of CCL2 by immunohistochemistry in tumors subjected to CRT, but not to MRT. Conversely, we found that MRT induced higher levels of pro-inflammatory genes in tumors than CRT.

Conclusion: Our data are the first to demonstrate substantial differences in macrophage, neutrophil and T cell numbers in tumors following MRT versus CRT, providing support for the concept that MRT evokes a different immunomodulatory response in tumors compared to CRT.

Radiotherapy (RT) has both local and systemic immunomodulatory effects [1,2]. Within the normally immunosuppressive tumor microenvironment, RT can either promote a more immunogenic environment or a more immunosuppressive environment [2,3]. Several mechanisms triggered by RT have been identified [4,5], with increasing evidence that it can enhance the efficacy of checkpoint inhibitors targeting PD-1 [6]. However, little is known regarding the impact of microbeam radiation therapy on the tumor-immune response.

Synchrotron microbeam radiation therapy (MRT) uses synchrotron-generated X-rays that are spatially fractionated to produce intense, planar microbeams [7–9]. While MRT configurations vary, typical 'in-beam' or peak doses are between 100 and 500 Gy and beams are 25–50 μm wide with 200–400 μm center-to-center spacing. Synchrotron MRT has shown equivalent tumor control to conventional radiotherapy (CRT) in a range of animal models, with a major benefit of significantly reduced damage to adjacent normal tissues compared to CRT [7,8,10–12].

The cellular mechanisms by which MRT achieves toxicity throughout the whole tumor rather than just within the 'in-beam' areas are currently unknown, however in normal zebrafish fins, different inflammatory and immune profiles were observed following MRT compared to broader minibeams (200–800 μm width) [13]. We showed in normal mouse skin that leukocytic infiltration and inflammatory responses are lower following MRT compared to CRT [12]. We and others have also identified differences in immune-related gene expression profiles and pathways between MRT and CRT in tumor models [9,14]. These observations raise the intriguing possibility that MRT may provide a novel approach for manipulating the immunomodulatory effects of radiation.

While CRT can induce anti-tumor immune responses, irradiated tumors also recruit large numbers of myeloid cells that are capable of contributing to tumor regrowth [2]. Increased tumor-associated

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macrophage (TAM) numbers correlate with the degree of malignancy and clinical outcome in many types of solid cancers [15,16]. TAMs are highly plastic cells that display a continuum of phenotypes, the extremes of which are the M1-like macrophages that exhibit a cytotoxic phenotype, and the alternatively activated M2-like macrophages that are anti-inflammatory, angiogenic and promote tissue remodeling [17]. The local tumor microenvironment can influence macrophages to become alternatively activated/tumor-promoting TAMs that contribute important functions required for tumor growth and progression [15].

Neutrophils are another major myeloid cell type found in tumors and associated with tumor progression [18–20]. Elevated tumor-associated neutrophil (TAN) numbers are associated with poor prognosis in a number of cancer types [21–23]. It is evident now that crosstalk exists between TAMs/macrophages and TANs/neutrophils in tumors [19,24].

Since little is known about how radiotherapy affects neutrophil numbers and function, we decided that it would be of value to assess MRT- and CRT-induced changes in both TAMs and TANs in mammary tumors. Based on the expression profiling analysis completed in our earlier study [9], we hypothesized that TAM and TAN recruitment to tumors following MRT would be different to that seen after CRT, and that MRT would result in fewer TAMs and TANs in the irradiated tumor. As a consequence, this would alter the extent of infiltration of lineages of the adaptive immune system, including T cells, given the known immunosuppressive capacity of myeloid derived cells in a cancer setting [25]. To explore this, we measured the extent of myeloid cell and T cell infiltration into CRT versus MRT irradiated tumors using doses shown previously to give equivalent tumor control [8,11]. Unlike previous reports for CRT [26,27], MRT did not induce a myeloid cell response within tumors. These data provide the first direct evidence that MRT has different tumor immunomodulatory properties to CRT and opens a new avenue for investigating the use of immunotherapy in combination with microbeam radiotherapy.

Materials and methods

Cell culture

EMT6.5 mouse mammary tumor cells tagged with mCherry fluorescent protein (called EMT6.5 hereafter) were derived from EMT6 [28] and were propagated in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L D-glucose and 25 mM HEPES, 10% fetal bovine serum (FBS), and 1% penicillin and streptomycin (Life Technologies).

Tumor cell inoculation and tumor growth

Female Balb/c mice (6–8 weeks, Walter and Eliza Hall Institute, Melbourne) were inoculated with 1×10^5 EMT6.5 cells into the leg, and irradiated with MRT or CRT 14 days later when tumors were approximately 10 mm in diameter. Tumors were excised at 48hr later for analysis.

Irradiation with MRT or CRT

Tumor-bearing mice were irradiated with MRT or CRT on the Imaging and Medical Beam Line (IMBL) at the Australian Synchrotron as described previously [9]. Details are given in Supplementary Data. Tumors were excised at 48hr following irradiation and analyzed by flow cytometry, immunohistochemistry and quantitative real-time PCR (qRT-PCR). All procedures involving mice were conducted in accordance with the National Health and Medical Research Council of Australia guidelines and approved by the University of Melbourne Animal Ethics Committee (Ethics ID: 1011910).

Flow cytometry analysis

Analysis of the myeloid composition of the EMT6.5 tumors was completed as described previously [19] and detailed in Supplementary Table 1.

Immunohistochemistry

BrdUrd (50 mg/kg) was injected i.p. into tumor-bearing mice 4 h before they were culled. The tumors were fixed overnight with 10% formalin and embedded in paraffin. Tumor sections (4–5 μm) from mice in each treatment group were immunostained with antibodies for BrdU (1:100), F4/80 (1:400), CD3 (1:100) or CCL2 (1:2000) using ABC or Envision kits coupled with DAB chromogen or HRP-conjugated secondary antibody (Supplementary Table 2). The co-staining of F4/80 and BrdU sections was achieved using Vector Blue and DAB chromogens (Supplementary Table 2).

Measurement of CCL2 or CD3 staining area

Tumor sections (one section from each tumor) were stained using anti-CCL2 or anti-CD3 antibody. Details are provided in Supplementary Methods.

RNA isolation, cDNA synthesis and quantitative real-time PCR

Tumor RNA (7 mice/group) was extracted using Trizol (Invitrogen) and RNeasy mini kits (Invitrogen) as described previously [29] and in Supplementary Methods.

Statistical analysis

A one-way ANOVA or ANOVA on ranks with Student–Newman–Keuls' test or Dunn's correction was applied for multiple-group comparisons. *P* values less than 0.05 were considered statistically significant (SigmaStat 3.5) [9].

Results

MRT treated tumors contain fewer TAMs and TANs than CRT treated tumors at 48 h after irradiation. Using the tumor treatment and flow cytometry protocols outlined previously [9] (Fig. 1A), we measured the numbers of the different subpopulations of myeloid cells in the tumors following MRT or CRT given at doses shown to be equivalent using cell based assays [11]. MRT did not increase TAM or TAN numbers in tumors, in contrast to CRT that caused significant increases in total TAMs ($F4/80^+$), $Ly6C^{hi}$ and $Ly6C^{lo}$ TAMs and TANs (Fig. 1B–E).

The increased number of TAMs seen after CRT is not due to increased local proliferation of these myeloid lineages. Since TAMs have been implicated in reducing the efficacy of radiotherapy and in promoting tumor recurrence [30], we asked if their increase following CRT was a result of local proliferation or increased recruitment. Tumor sections were double-stained with antibodies against $F4/80^+$ and BrdU to find evidence of *in situ* proliferation after RT. However, there were no BrdU-positive $F4/80^+$ TAMs (Fig. 2A–E), indicating that neither CRT nor MRT induces proliferation of TAMs within these tumors.

CD3 positive T cells are increased after MRT. Since TAMs and TANs suppress T cell responses [31], we measured the number of CD3 positive T cells present in the irradiated tumors. Scored both by number and area of positivity, T cells were increased in MRT

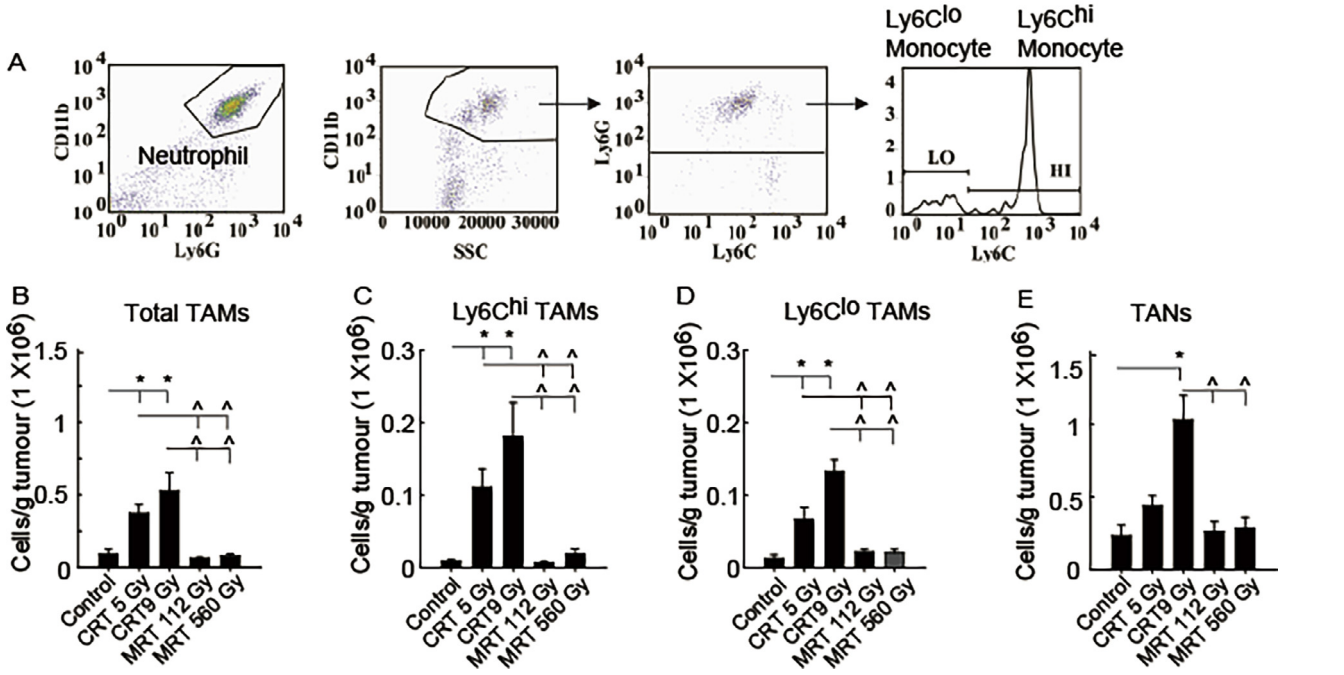

Fig. 1. TAM and TAN numbers are increased only after CRT. (A) Flow cytometry gating strategy. Representative gating strategy for flow cytometric analysis of myeloid subpopulations in blood. The same gating strategy was used for analysis of tumors. (B–E) EMT6.5 tumor-bearing mice (n = 7 mice/group) were irradiated with MRT or CRT and tumors were excised at 48hr following irradiation and analyzed for myeloid cell content. Numbers of (B) Total TAMs, (C) Ly6C^{hi} TAMs, (D) Ly6C^{lo} TAMs and (E) TANs were measured by flow cytometry. A one way analysis of variance on ranks was applied for multiple-group comparison. Data are expressed as cells/gram of tumor tissue (Mean ± SEM). CRT and MRT compared to the control group: $^{*}p < 0.05$. CRT compared to MRT groups: $^{\wedge}p < 0.05$.

treated tumors, despite no change in the number of myeloid cells (Fig. 2F–L). The numbers of CD3+ cells within the tumor is low, but consistent with a previous report of the EMT6 tumor showing an "immune-excluded" phenotype [32].

The increase in TAMs after CRT may be due to increased CCL2-mediated recruitment. The chemokine CCL2 has an established role in the recruitment of macrophages to tumors and is known to be induced by CRT [33]. High CCL2 correlates with increased TAMs and poor outcome in breast cancer patients [34]. We therefore undertook an immunohistochemical analysis of CCL2 and its localization with respect to TAMs in EMT6.5 tumors following RT. CCL2 increased in CRT-treated tumors compared to either MRT-treated or non-irradiated tumors (Fig. 3A–F). This was consistent with the pattern of TAM localization in the tumors (Fig. 3G–K) with higher numbers of TAMs within CRT-treated tumors (Fig. 3H,I), compared to non-irradiated tumors, where TAMs accumulated along the tumor periphery (Fig. 3G). MRT also resulted in some infiltration of TAMs toward the tumor center (Fig. 3J,K), but this was markedly less than after CRT (Fig. 3H,I). When analyzed on an individual tumor basis, the extent of the positive area of CCL2 staining correlated with the extent of infiltration of the different myeloid populations into the tumor, most evident for TAMs and TANs, (Supp. Fig. 1). These data are consistent with the hypothesis that CRT may increase CCL2 mediated recruitment of TAMs, while MRT does not. However, CCL2 is not the only monocyte/-macrophage chemoattractant, and other factors, such as CSF-1 and CCL5, may also be involved in the increased TAM numbers seen after CRT.

MRT induces a higher level of pro-inflammatory genes than CRT. The induction of a trophic and immunosuppressive environment is a major cause of tumor recurrence after conventional radiotherapy and either stimulating a cytotoxic/anti-tumor response or inhibiting immunosuppressive factors can improve the efficacy of this treatment [27]. We therefore measured the expression of genes associated with trophic/immunosuppressive and cytotoxic functions in tumors [17] (Fig. 4). *CD11c* and *Tnfα* are associated with cytotoxicity and anti-tumor functions [35], and their expression was higher after MRT compared to CRT (Fig. 4A,C). There was a trend for elevated levels of *Ifnγ* after MRT as well (Fig. 4B). In contrast, there was little difference in genes associated with immunosuppressive and trophic functions apart from an increase in *Arg1* expression after MRT (Fig. 4F). *Arg1* is typically associated with pro-tumor functions [36]. Overall these data support the concept that MRT induces a more cytotoxic, anti-tumor microenvironment, which may explain the equivalent tumor control when compared to CRT, despite the fact that the majority of the tumor receives only valley dose radiation that is comparable to the levels given during CRT. Our demonstration of elevated numbers of intra-tumoral T cells after MRT (Fig. 2) further support this hypothesis. Further investigation of gene expression and protein levels in peak and valley microenvironments of MRT-treated tumors is required to better understand the mechanisms behind these observations.

Discussion

The results from this study confirm our hypothesis that the immunomodulatory effects of MRT are different to those following CRT. The major finding is the marked decrease in TAMs and TANs that infiltrate the tumor after MRT compared to CRT at doses that we have shown previously to be equivalent [8,11]. Consistent with the well-known immunosuppressive properties of these myeloid cells [31], we find enhancement of T cell infiltration following MRT, where TAM and TAN numbers are low. The intra-tumoral cytokine profile is also substantially different between MRT and CRT, although further research is required for a fuller understanding of the pathways involved in regulating TAM and TAN recruitment to the irradiated tumor. The elevation of CCL2 in the CRT

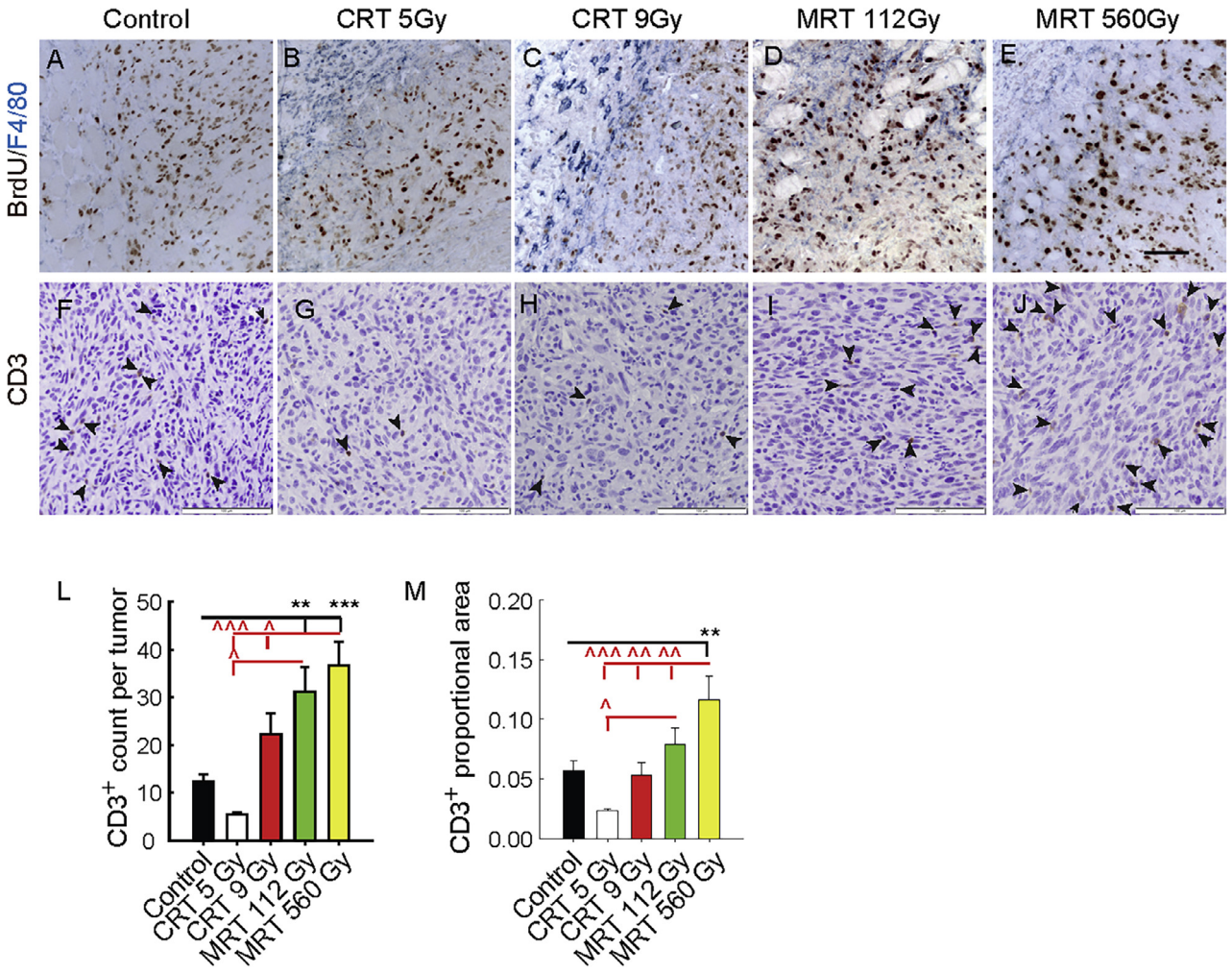

Fig. 2. Immunohistochemical analysis of TAMs and T cells in irradiated tumors. Mice bearing EMT6.5 tumors were injected ip with BrdUrd (50 mg/kg) (n = 7 mice/group) 4 h before they were culled at 48 h following irradiation. Paraffin-embedded tumor sections from control and radiation groups were co-stained for BrdU (brown) and F4/80 (blue). Other sections were immunostained with anti-CD3 to indicate T cell infiltration. (A,F) Control, (B,G) CRT (5 Gy), (C,H) CRT (9 Gy), (D,I) MRT (112 Gy) and (E,J) MRT (560 Gy). Black arrowheads point to CCL2 positive cells. Scale bar, 50 μm for BrdU and F4/80 staining and 100 μm for CD3. Quantitation of the CD3 staining by cell number (K) and area of staining (L).

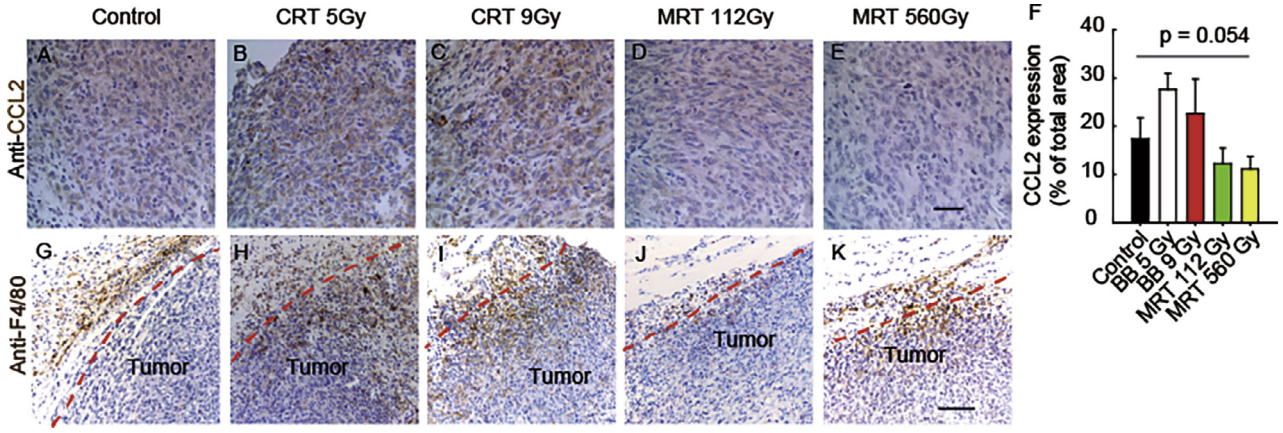

Fig. 3. Levels of CCL2 and localization of TAMs in irradiated tumors. Paraffin-embedded tumor sections from control and irradiated EMT6.5 tumors (n = 7 mice/group) were co-stained for CCL2 in (A) control, (B) CRT 5 Gy, (C) CRT 9 Gy, (D) MRT 112 Gy, or (E) MRT 560 Gy irradiated tumors. Scale bar, 10 μm. (F) Quantitation of CCL2 staining area in tumor sections. (G-K) Immunostaining for F4/80 positive TAMs in (G) control, (H) CRT 5 Gy, (I) CRT 9 Gy, (J) MRT 112 Gy, or (K) MRT 560 Gy irradiated tumors. Scale bar, 50 μm.

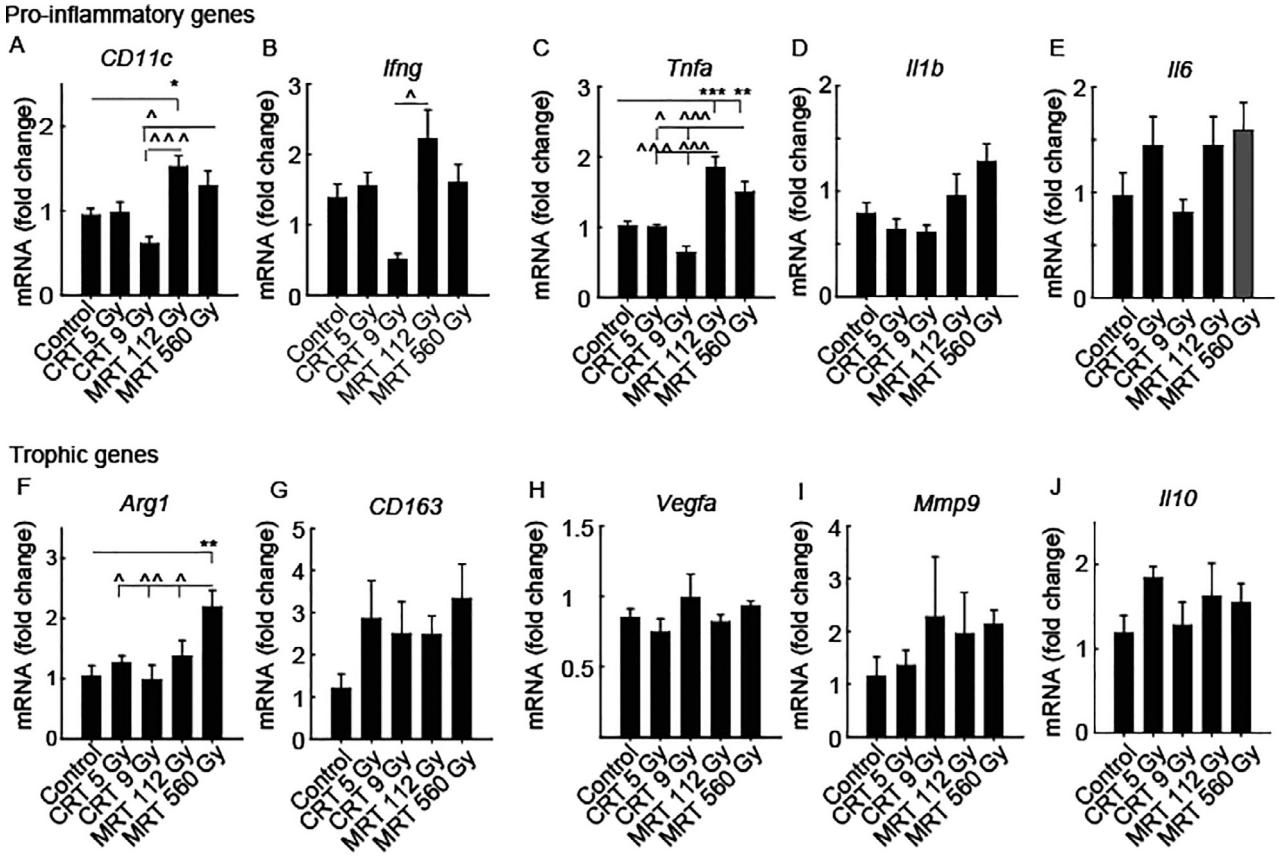

Fig. 4. Levels of pro-inflammatory and trophic genes after CRT and MRT. RNA was recovered from EMT6.5 tumors removed from mice 48hr after irradiation. Transcript levels of pro-inflammatory and trophic genes were measured by qRT-PCR in control, MRT and CRT irradiated tumors ($n = 7$ mice/group). A one-way ANOVA was applied for multiple-group comparison. Data are expressed as Mean ± SEM. CRT and MRT compared to the control group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. CRT compared to MRT groups: $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$.

treated tumors is consistent with its known ability to attract myeloid cells [34], but other chemoattractants such as CSF-1, G-CSF and CCL5 should also be assessed. Identifying these pathways and factors may reveal new ways of increasing the anti-tumor effects of radiation treatment, or minimizing recurrence post-radiation, for both CRT and MRT.

It is increasingly apparent that immune responses can affect the success of cytotoxic therapies [3]. TAMs are known to be important regulators of tumor growth and metastasis [15] and inhibition of macrophages/TAMs improves the effectiveness of radiotherapy [30]. Thus, our finding that MRT given at doses that can control tumor growth without recruiting TAMs/TANs is important. In contrast to our findings, Bouchet et al. reported that MRT irradiation of rats with glioma, while confirming an improved tumor response compared to CRT, resulted in an increase in macrophage infiltration into the tumor, as measured by transcript levels of the macrophage marker, CD68 [37]. However, total leukocyte infiltration as measured by nuclear morphology in H&E stained sections, was not altered. The reasons for the difference in macrophage levels after MRT between our study and that of Bouchet et al. may relate to the different tumor type under analysis or, probably more likely, to the method of scoring macrophages. Bouchet et al measured CD68 transcript levels in their tumors whereas we counted macrophage numbers by flow cytometry using the more lineage specific F4/80 protein marker.

The increase in TAMs following conventional radiotherapy is in line with previous studies [2]. M1 macrophages are regulated by IFNγ and bacterial products to induce the expression of proinflammatory genes including IL1β, TNFα, iNOS and IL-6. In contrast, M2 macrophages are generated in response to IL4 or IL13, stimulating the expression of anti-inflammatory and pro-tumorigenic genes such as IL10 and TGFβ [17].

Macrophage phenotype can vary with the dose of conventional radiation. Klug et al reported that low-dose radiation induced an M1-like, pro-inflammatory/cytotoxic phenotype in TAMs. These macrophages secreted iNOS and were essential for activating anti-tumor T cells [38]. Thus it appears that higher doses of radiotherapy may increase M2-like TAM numbers, while low-dose radiotherapy induces anti-tumor M1-like macrophages. Further evidence comes from a study showing that radiation doses (>12 Gy) sufficient to induce the endonuclease Trex1 can prevent activation of the immune system by degradation of cytosolic DNA fragments that otherwise stimulate interferon-β and activation of CD8 T cells and an anti-tumor response [39]. It is therefore not surprising that due to the wide range of doses in an MRT field, there will be a fundamentally different immune response in tumor tissue. In the highly heterogeneous MRT dose distribution, it is possible that some parts of the tumor may receive a more optimal immunogenic dose than others, and that this sub-population of irradiated cells could be sufficient to 'prime' the host to target the remaining tumor cells. If this 'MRT-optimised host immune response to the tumor' hypothesis is correct, it might explain why tumor cells that receive minimal or 'valley dose' radiation are still eliminated following MRT [8]. Hence, understanding the different immunomodulatory responses at a cellular level within the tumor following MRT will take substantial effort. Suffice to say, the integrated 'whole-of-tumor' response is very different following MRT compared to that seen following CRT.

There is growing awareness of the critical role of neutrophils in cancer progression [23,40] and recent studies have highlighted their importance in radiotherapy, suggesting that specific targeting of neutrophils may improve radiation response in patients [41]. We have shown previously that CRT recruits more neutrophils into the skin than MRT [12]. However, to our knowledge, this is the first publication showing that MRT does not recruit TANs into mammary tumors as seen for CRT. Given that we and others have shown that neutrophils can promote metastasis in preclinical models [18,19], it will be important to further investigate radiation-induced changes in TAN numbers and phenotype. It appears possible that the 'neutrophil-low' response after MRT may be an advantage in the treatment of cancer.

The toxic side effects of RT can greatly limit the dose given to patients [42] and insufficient dosing can lead to tumor recurrence. In preclinical studies, MRT has been shown to control tumors as effectively as CRT, while showing far less normal tissue toxicity [8,12]. On the one hand, it is possible that the different immune response following MRT contributes to reduced normal tissue toxicity. On the other hand, this different response may also limit the anti-tumor response following MRT. However, our data indicate that MRT may induce a more cytotoxic/anti-tumor microenvironment than CRT by reducing the number of TAMs/TANs and increasing the number of CD3+ T cells in the tumor. After MRT, there is an increase in the CD11c marker, which is expressed on many immune cell lineages, including myeloid cells, but is often used as an indicator of dendritic cells. Since there are fewer TAMs/TANs in the tumor, the increased expression of CD11c could be attributed to more dendritic cells.

In this study, TAMs and TANs were monitored 48hr after radiation. An analysis of TAM and TAN numbers over an extended time course following radiation will provide further information on the long-term, persistent effects of MRT and CRT on immune cell recruitment. In addition, analysis of trophic/pro-tumor versus cytotoxic/anti-tumor factors in TAMs and TANs isolated from MRT or CRT treated tumors will shed light on how radiotherapy alters their phenotype and how, or if they are involved in tumor recurrence and progression after radiation. Since TAM subpopulations appear to have different phenotypes and functions [43,44], it will also be worthwhile assessing changes in the $Ly6C^{hi}$ and $Ly6C^{lo}$ TAMs with time after radiation since they may be differentially affected by CRT and MRT.

In conclusion, we have shown that the immunomodulatory responses to MRT within the tumor are different to those of CRT, and that MRT results in significantly lower intratumoral TAMs and TANs and a higher infiltration of T cells at doses that have been shown previously to be equivalent. The intratumoral pro-inflammatory profile is also substantially different following MRT compared to CRT. These data support our hypothesis that MRT elicits different immunomodulatory pathways to CRT, and that it may be possible to develop MRT radiotherapy protocols that exploit this modified anti-tumor immune response.

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Conflict of interest

The authors claim no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.radonc.2019.01.006.

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