Impact of proton therapy on antitumor immune response

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Abstract

Radiotherapy delivered using photons induces an immune response that leads to modulation of the tumor microenvironment. Clinical studies are ongoing to evaluate immune checkpoint inhibitors in association with photon radiotherapy. At present, there is no publication on the radio-induced immune response after proton therapy. Balb/c mice bearing subcutaneous CT26 colon tumors were irradiated by a single fraction of 16.4 Gy using a proton beam extracted from a TR24 cyclotron. RNA sequencing analysis was assessed at 3 days post-treatment. Proton therapy immune response was monitored by flow cytometry using several panels (lymphoid, myeloid cells, lymphoid cytokines) at 7 and 14 days post-irradiation. RNA-Seq functional profiling identified a large number of GO categories linked to “immune system” and “interferon signaling”. Immunomonitoring evaluation showed induced tumor infiltration by immune cells. This is the first study showing the effect of proton therapy on immune response. These interesting results provide a sound basis to assess the efficacy of a combination of proton therapy and immune checkpoint inhibitors.

Introduction

Radiation therapy is used in more than 50% of cancer patients. Initially, radiotherapy (RT) was used for its direct effects on cancer cell survival. In addition to its cytotoxic action on cancer cells, it is now well established that irradiation also induces an immune response [1]. This can be immune-activating and/or immunosuppressive [2]. For the past ten years, the association of radiotherapy and immunotherapy in preclinical, and more recently in clinical settings, has been in full development and seems promising for many indications. In order to improve the effectiveness of these combinations, it is necessary to optimize the modalities of radiotherapy administration, such as the dose [3], the fractionation scheme [4] or the dose rate [5]. Almost all the data in the literature regarding radio-induced immune response was obtained with irradiation based on photons.

It is becoming essential to evaluate another feature of radiotherapy, namely the impact of the type of particles used. Recently, Spina *et al.*, evaluated the effects of carbon-ion therapy on immune modulation. They highlighted an interesting induction of pro-inflammatory cytokines [6].

Proton therapy (PT) plays an important role in clinical radiotherapy with growing facilities and indications [7]. It has particularly interesting ballistic advantages defined by the Bragg peak, beyond which the dose delivered is almost nil, enabling the total avoidance of surrounding organs at risk. Protons have an increased Relative Biological Effectiveness (RBE) compared to photons, and they cause a different type of damage to DNA than do photons [8]. The effect of proton on the immune response currently remains completely unexplored.

In this study, we aimed to evaluate the radio-induced immune response with a 16.4 Gy single fraction of proton. We evaluated this response by transcriptomic analysis in order to describe the immune molecular

pathways modulated by proton therapy, and by analysis of the tumor microenvironment, by immunomonitoring of intratumoral infiltrated immune cells.

Results

Effect of 16.4 Gy with proton therapy on CT26 tumor growth. A single dose of 16.4 Gy proton therapy was delivered to the CT26 tumors of immunocompetent BALB/c mice. The dose of 16.4 Gy delivered with proton therapy induced enhanced CT26 tumor control compared to the non-irradiated (NI) control tumors (Figure 1). We highlighted a tumor growth delay for the PT group. For mice treated by protontherapy, the maximum tumor volume limit of 1500 mm^3 was reached from 54 days, while 4/10 mice had still complete response after 100 days.

Proton therapy activated transcriptomic antitumor immune response pathways. Gene transcript analysis was compared between 16.4 Gy and NI CT26 tumors 3 days post-irradiation to examine immune response after proton therapy. RNA-Seq profiling identified 68 genes differentially expressed with s-value < 0.005 and a Fold Change ≥ 2 (Table S1).

Enrichment analysis was then performed on the gene set, focussing on pathways linked to “immune response” and “interferon signaling”. The enriched GO and REACTOME categories listed in Table 1 represent all the pathways identified that included “immune activation” and “interferon signaling”.

Table 1. List of GO and REACTOME categories obtained after enrichment on gProfiler2 and linked to “immune system” and “interferon signaling” after a dose of 16.4 Gy proton therapy on CT26 tumors with an s-value < 0.005 and a FC ≥ 2.

id	term_id	term_name	intersection_size	p_value
1	GO:0045087	innate immune response	20	4.5e-10
2	GO:0002376	immune system process	31	3.3e-09
3	GO:0002252	immune effector process	19	4.3e-09
4	GO:0006955	immune response	25	5.0e-09
5	GO:0034097	response to cytokine	18	2.7e-07
6	GO:0071345	cellular response to cytokine stimulus	15	2.5e-05
7	GO:0035456	response to interferon-beta	6	5.6e-05
8	GO:0035457	cellular response to interferon-alpha	4	1.7e-04
9	GO:0035458	cellular response to interferon-beta	5	7.5e-04
10	GO:0002682	regulation of immune system process	17	8.3e-04
11	GO:0035455	response to interferon-alpha	4	3.4e-03
12	GO:0032728	positive regulation of interferon-beta production	4	5.0e-03
13	GO:0001816	cytokine production	12	5.0e-03
14	GO:0001817	regulation of cytokine production	11	1.2e-02
15	GO:0002218	activation of innate immune response	4	1.2e-02
16	GO:0032608	interferon-beta production	4	2.9e-02
17	GO:0045088	regulation of innate immune response	6	2.9e-02
18	GO:0045089	positive regulation of innate immune response	5	3.3e-02

g:Profiler (biit.cs.ut.ee/gprofiler)

Next, we extracted all genes involved in the “immune response” pathway obtained with the enrichment (p value = 7.7e-05). This pathway was covered by 25 differentially expressed genes, which are presented in a heatmap below (Figure 2). All the genes observed presented up-regulation after 16.4 Gy proton therapy compared to NI controls. Among these genes, we identified several genes of *Ifit* (Interferon-induced protein with tetratricopeptid repeats) and *Ifi* (Interferon inducible protein) families, which are involved in Interferon alpha and beta signaling. Many induced genes are involved in the type I interferon pathway (*Oasl 1*, *Irf7,* etc).

Proton therapy induced intra-tumor immune cell infiltration. Proton therapy induced infiltration of two types of immune cells: antitumor and pro-tumor immune cells (Figure 3). Seven days after PT, significant infiltration of $CD8^+$ T cells, $CD4^+$ T cells and TAM1 was observed. For these cells, infiltration tended to be maintained 14 days after PT, albeit without reaching statistical significance. We did not observe radio-induced infiltration of NK cells in these conditions. $CD8^+$ T cells presented a cytotoxic activity phenotype, with co-expression of Granzyme B in more than 80% of them. Concerning immunosuppressive cells, we observed significant tumor infiltration of Treg, but no significant variation in MDCS and TAM2 infiltration, 7 and 14 days after PT (Figure 3). All significant infiltrations were transient, with loss of significance of these infiltrates compared to controls at D14.

Discussion

Proton therapy is an attractive therapeutic approach. It has both physical advantages, limiting the dose to surrounding organs at risk, and biological advantages, due to high linear energy transfer with different types of DNA damage induced by the densely ionizing radiation [9]. Several teams have described the potential effects of protons on the antitumor immune response, illustrating the importance of this issue, but also the difficulty of implementing experiments to demonstrate it [10-12]. Devices amenable to carrying out such studies *in vivo* are rare, and offer limited access for research studies. For example, in France, only few machines of this type are available, and each can be used for only a few slots per week.

This present study highlights that proton therapy enables the activation of several pathways implicated in the immune response. Activation of innate and specific immune responses was previously described to be induced by type I interferon (IFN-I), with activation of antigen recognition and cytolytic activity [13,14]. The IFN-I pathway was largely induced with a single dose of 16.4 Gy proton therapy in our study (up-regulation of *Ifi* and *Ifit* genes). These results suggest that the proton-induced mechanisms could be comparable to those induced by conventional photon radiotherapy treatment. Cytosolic DNA induced activation of the IFN-I pathway, mediated by cyclic GMP-AMP (cGAMP) synthase (cGAS) and its partner STING (stimulator of interferon genes) [15]. One of the activation mechanisms of the IFN-I pathway is the cGAS/STING pathway [16].

After conventional RT, Vanpouille-Box et al. demonstrated that photon RT induced cytosolic DNA accumulation, which activates the cGAS/STING pathway resulting in activation of IFN-I [3]. They also reported that doses greater than 10 Gy led to Trex1 expression, which degrades cDNA, thus avoiding IFN-I

activation. Proton therapy could also induce the release of DNA fragments into the cytoplasm, which enables IFN-I activation. With a similar dose delivered by photon (16 Gy), Vanpouille-Box et al. described an increase in Trex1 expression as well as a decrease in the amount of cDNA and activation of cGAS/STING in TSA cells [3]. With our gene transcript analysis we observed an increasing trend of *Trex1* expression after a 16.4 Gy proton therapy, however this induction was non-significant with an s-value = 0.07 (data not shown). It would be interesting to also evaluate the effect of protons on amount of cDNA.

Moreover, a single fraction of 16.4 Gy proton therapy in our cancer model induced a significant antitumor response, with tumor infiltration of different types of immune cells, such as $CD8^+$ T cells, which express granzyme B, translating cytotoxic activity. This is related to the induction of expression of certain chemokines observed in RNA-Seq (such as *Cxcl11*), which contribute to the recruitment of effector T cells in the tumor [17]. $CD8^+$ T cell activation is one of the mechanisms involved in the induction of the abscopal effect, which is a rare systemic effect first described more than 60 years ago after conventional radiotherapy [18,19]. Brenneman *et al.*, recently described the first case report of an abscopal effect after proton therapy in a patient with sarcoma [20]. The incidence of this abscopal effect is increased when photon RT is combined with immunotherapy [21]. The association of proton therapy with immunotherapy could therefore yield a beneficial effect both locally and outside of the radiation field in metastatic patients.

Combinations of immunotherapy with conventional radiotherapy are increasingly being evaluated in preclinical and clinical situations [22,23]. Some studies have attempted to optimize the combination conditions to achieve a radio-induced immune response, leading to an *in situ* vaccine, which may be amplified with immunotherapy by inhibiting tumor microenvironment immunosuppression [24].

Yet, no published preclinical study has described the effectiveness of combining proton therapy with immunotherapy. We searched for the query string “proton” and “immunotherapy” in the ClinicalTrials.gov database in December 2020, and identified only 4 verified trials worldwide evaluating the safety and/or efficacy of a combination of PT and immunotherapy (NCT03765190; NCT03818776; NCT03267836 and NCT03764787) that are currently ongoing or not yet recruiting. They are all early-stage studies (phase I or II) with small sample sizes (maximum 30 patients). All these trials are investigating proton therapy with anti-PD-1 or anti-PD-L1 safety and efficacy for metastatic cancers (neoplasm or head and neck), non-small cell lung cancer, head and neck cancer or meningioma.

In our experiments, we showed that there was tumor infiltration by Tregs, which have an immunosuppressive effect. These interesting findings could encourage the evaluation of an association of proton therapy with for example an anti-CTLA4 or an anti-CCR4, which, by targeting Tregs, induces reactivation of $CD8^+$ T cells against tumor cells [25]. We previously demonstrated with conventional RT treatment that inducing expression of immunotherapy targets by RT could have an impact on the efficacy of the combination of RT with a specific immunotherapy [4].

There is a keen interest within the scientific community in developing and evaluating the combination of proton therapy with immunotherapy. Several research teams have expressed an interest, on the assumption that proton therapy could activate the immune response and increase immunotherapy efficacy [10,26-28]. However, to date, no study has demonstrated the concept biologically.

For the first time, we demonstrate here that proton therapy can activate the immune response and can “heat up” the tumor by infiltration of antitumor immune cells. Our results pave the way for future studies that could evaluate the effect of the proton therapy dose delivered, and its possible fractionation scheme, the effects of variations in RBE, and the best combination of proton therapy and immunotherapy. It therefore seems essential to evaluate the effect of proton therapy on the expression of specific targets, in order to guide clinicians in the choice of immunotherapy to be combined with proton therapy in future clinical trials.

A single fraction of 16.4 Gy proton therapy induced an interesting induction of immune response biological pathways and immunostimulatory antitumoral effects. This study reveals the possible potential of combining proton therapy with immunotherapy in order to enhance tumor control and survival.

Methods

Cell culture and animals. The murine colon carcinoma cell line CT26 was purchased from American Type Culture Collection and cultured in RPMI 1640 (Dutscher, France) supplemented with 10% fetal bovine serum (PAN Biotech GmbH, Aidenbach, Germany) at 37°C in a humidified atmosphere containing 5% CO_2 and 95% humidity.

Tumor grafting was performed as previously described [4]. Briefly, CT26 cells (5.10^5) were suspended in 100 µl of NaCl and injected subcutaneously in the right flank of immunocompetent 8-week BALB/c female mice (Charles River Laboratories, Saint-Germain-des-Monts, France). Tumor size was measured until tumor volume (TV) reached the limit point of 1500 mm^3. TV was calculated according to the equation $TV = (L \times W^2)/2$, where L and W are the length and width of the tumor, respectively. Ten days after injection, mice were randomized to treatment and control groups to obtain an equivalent average tumor volume in each group of 130 ± 20 mm^3 (n = 5-6 mice for RNA-Seq profiling analysis and n = 4-5 mice for immunomonitoring). Mice were euthanized as soon as the limit point was reached or at 100 days after treatment for the growth delay study; for RNA-Seq experiments, mice were sacrificed 3 days after irradiation; for immunomonitoring experiments mice were sacrificed 7 and 14 days after irradiation (Figure 4). As requested by the ethics committee and French regulations, the mice were sacrificed by cervical dislocation after general gaze anaesthesia (Isoflurane 2.5%)

All mouse procedures were performed with approved protocols in accordance with the legislation on the use of laboratory animals (directive 2010/63/EU), and with ethical rules for the care and use of animals for research from the small animal ethics committee (C2ea Grand Campus n°105 and C2ea Cremeas

n°35, C2ea Icomech n°38) and the French Ministries of Research and Agriculture (APAFIS#13961-2018022215413276 v2, APAFIS#22350-201910091738155 v2 and APAFIS#8235-201612161350414_v1). All procedures used follow ARRIVE guidelines to respect 3R recommendations. Thus, animals are randomized before treatment in order to have comparable tumor volume averages. The numbers of mice in each group allow statistical analysis. The control groups of untreated mice allow the analysis of the effect of the treatment.

Treatments. Ten days after injection of cancer cells, tumors were irradiated by a single dose of 16.4 Gy under anaesthesia (isoflurane 2.5% mixed with oxygen compact unit, Minerve, France) with a proton beam extracted from CYRCé platform (CYclotron pour la ReCherche et l'Enseignement) in the Institut Pluridisciplinaire Hubert Curien (Strasbourg, France) with an average energy beam of 25 MeV [29]. Using an in-house immobilization bed, tumors were irradiated directly in contact with the collimator with a dose rate of 3.1 Gy/min.

Flow cytometry. The modulation of the immune system by 16.4 Gy proton therapy was evaluated by flow cytometry at 7 and 14 days after treatment, as previously described [4]. Briefly, after dissection, tumors were dissociated using a mouse tumor dissociation kit (Miltenyi Biotech). To analyze myeloid cell infiltration, tumor cell suspension (10^6 cells) was stained in Flow Cytometry Staining Buffer (FSB, eBioscience) with specific antibodies according to manufacturer's recommendation (antibody details are presented in Table S2) for 15 minutes at room temperature in the dark, washed twice in FSB and analyzed by flow cytometry. To analyze lymphoid cell infiltration, tumor cell suspension was performed according to manufacturer's recommendation (Miltenyi Biotech). For lymphoid and myeloid cell infiltration assays, Viability Dye eFluor 780 was used to identify live cells. Flow cytometry acquisition was performed on a Cytoflex 13C cytometer (Beckman Coulter). CytExpert (Beckman Coulter) was used for analysis. For lymphoid and myeloid cell identification, and for lymphoid cell functionality, see the gating strategy presented in Figure S1.

RNA extraction, RNA sequencing (RNA-Seq) and Gene Set Enrichment Analysis. Single-end transcriptome reads were pseudo-aligned to the UCSC mm 10 reference genome and quantification of gene expressions was performed with the Kallisto algorithm (v 0.44.0) [30]. The program was run with default options. RNA-Seq profiling analysis was assessed with R software (R version 4.0.3). Differential analysis was performed with DESeq2 R package (version 1.30.0) [31] using log fold change shrinkage [32]. A gene was considered significantly differentially expressed when the corresponding s-value was < 0.005 and a log2 fold change ≥ 1. Gene set enrichment analyses were performed using gProfiler2 (v0.2.0) [33].

Statistical analysis. The results are expressed as mean ± SEM (standard error of the mean). Figures were designed using GraphPad Prism V8. Software (GraphPad Software, USA). Comparisons between groups were carried out using a non-parametric Mann-Whitney test. Statistical analyses were achieved using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). A p-value < 0.05 was considered statistically significant.

Declarations

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Author contributions

C.Mi., H.B. and G.N. were involved in the conception and design of the study. H.B., V.M., C.Mu. and C.Mi. were involved in the tumor-graft production and volume tumor evaluation and animal housing. C.Mu., H.B. and M.R. were involved in proton therapy session preparation and implementation. E.L., V.M., F.G. and C.Mi. were involved in the immuno-monitoring analysis by flow cytometry. R.B. performed the RNA sequencing and A.N. and C.R. analyzed all RNA sequencing data. H.B. and C.Mi. supervised the project and were in charge of the statistical analysis of the study. H.B., C.Mi., A.N. and E.L. had access to the raw data and analyzed and interpreted the data. H.B., C.Mi., A.N. and E.L. were involved in writing the report, which was corrected and approved by all authors. All authors have read and agreed to the published version of the manuscript.

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Competing interests

The authors declare no conflict of interest.

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Figures

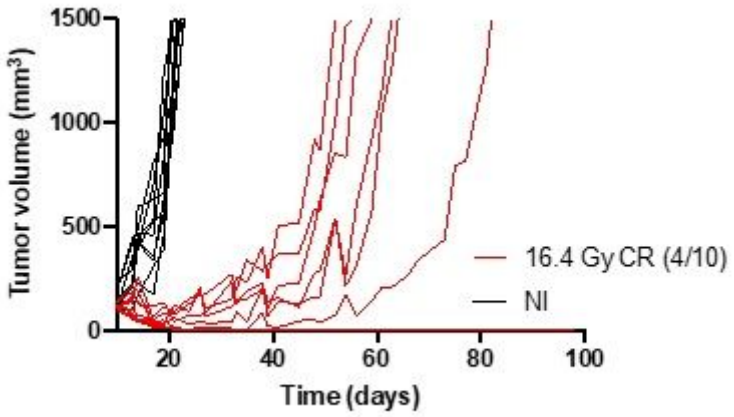

Figure 1

Effect of 16.4 Gy proton irradiation on CT26 tumors implanted on immunocompetent BALB/c mice (red) compared to un-irradiated (NI) tumors (black). n = 10 mice per group. CR: complete response.

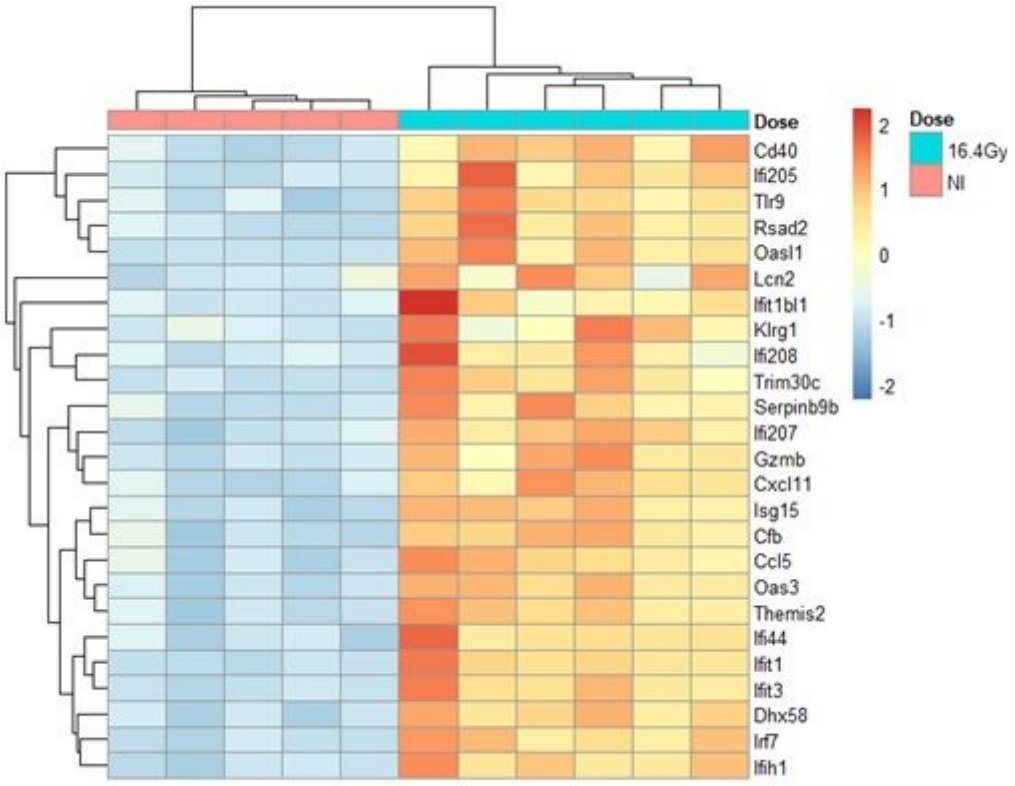

Figure 2

Heatmap representing “immune response” biological process, with 25 genes differentially expressed, at 3 days post-irradiation. Comparison between 16.4 Gy (green) and un-irradiated controls (NI, pink) of CT26 tumors. s-value < 0.005. Groups contained 5-6 mice.

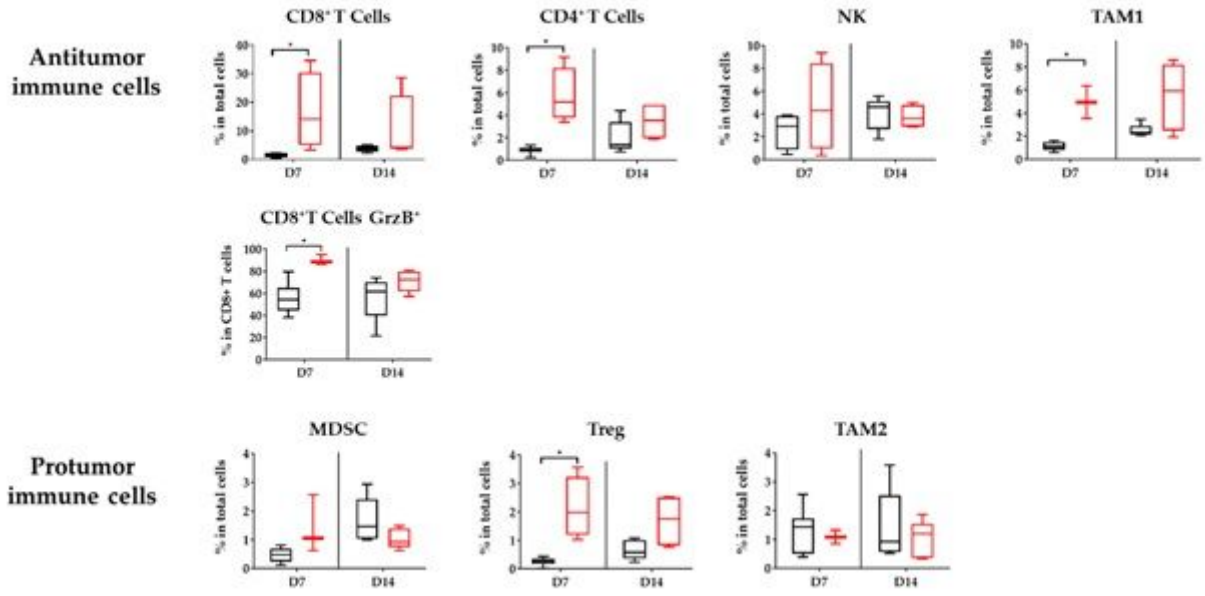

Figure 3

Modification of the tumor microenvironment induced by proton therapy: immunomonitoring of lymphoid and myeloid cells after PT. At 7 and 14 days after proton therapy, flow cytometry monitoring (FCM) was performed on dissociated tumors. Antitumor immune cell (CD8+ T cells, CD4+ T cells, Natural Killer (NK) cells, tumor associated macrophages (TAM) 1, CD8+ granzyme B+ (GrzB) and pro-tumor cell (myeloid derived suppressor cells (MDSC), Treg T cells and TAM2) infiltration was quantified. Black: un-irradiated control, Red: 16.4 Gy PT. All data are shown with box and whisker plots, with min to max values obtained from 4-6 independent samples per point. *p<0.05. The non-parametric Mann-Whitney test was used.

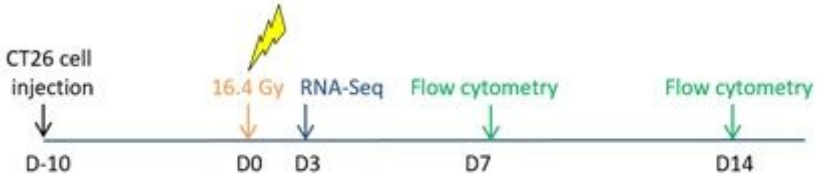

Figure 4

Time scale of the experiments. Ten days after injection of CT26 colon murine cancer cells into Balb/c mice, tumors were irradiated with a single dose of 16.4 Gy, with an un-irradiated control group. RNA-Seq profiling was performed 3 days post-irradiation and immunomonitoring was performed 7 and 14 days post-irradiation.

Supplementary Files

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