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Proton flash therapy and immune infiltration: evaluation in an orthotopic glioma rat model

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Abstract

FLASH radiotherapy (FLASH-RT) is a promising radiation technique that uses ultra-high doses of radiation to increase the therapeutic window of the treatment. FLASH-RT has been observed to provide normal tissue sparing at high dose rates and similar tumor control than conventional radiotherapy, yet the biological processes governing these radiobiological effects are still unknown. In this study, we sought to investigate the potential immune response generated by FLASH-RT in a high dose of proton therapy in an orthotopic glioma rat model. Thus, we cranially irradiated rats with a single high dose (25 Gy) using FLASH dose rate proton irradiation (257 ± 2 Gy/s) or conventional dose rate proton irradiation (4 ± 0.02 Gy/s).

We first assessed the protective FLASH effect that resulted in our setup through behavioral studies in naïve rats. This was followed by a comprehensive analysis of immune cells in blood, healthy tissue of the brain and tumor microenvironment by flow cytometry. Proton FLASH-RT spared memory impairment produced by conventional high-dose proton therapy and induced a similar tumor infiltrating lymphocyte (TIL) recruitment. Additionally, a general neuroinflammation that was similar in both dose rates was observed. Overall, this study demonstrated a FLASH proton therapy offers a neuro-protective effect even at high doses while is able of mounting an effective lymphoid immune response in the tumor.

Introduction

An estimated 2.9M patients will be diagnosed with cancer in the EU-28 in 2025, of which about 50% will receive external beam radiotherapy [1]. FLASH radiotherapy (RT), involving the ultra-fast delivery of radiation at dose rates much higher than routine clinical practice and with potentially fewer

toxicities, targets this large patient population. There is a huge potential for FLASH-RT in cancers for which RT is one of the key elements, yet still burdened by a high rate of acute and late side effects such as adult and pediatric brain tumors, unresectable sarcomas or locally advanced head and neck squamous carcinoma (HNSCC) [2]. While the protective effect of high dose rates was first observed in the 50s in cells and mouse models [3], the FLASH effect was rediscovered in 2014 at Institut Curie [4]. A drastic reduction of pulmonary fibrosis in mice irradiated with FLASH (>40 Gy/s) electrons (e-) compared to same dose standard-rate (0.05 Gy/s) photons was reported. These findings have been replicated in other animal studies and organs [3] and, in particular, in murine brains [5]. No cognitive deficits were observed after FLASH irradiation in contrast to conventional irradiations, both delivered in one fraction. Despite the reduction in toxicity, FLASH-RT has demonstrated to maintain the same tumor control level than standard irradiations [3], suggesting that high dose rates could enhance the therapeutic window in radiotherapy. The magnitude of the observed benefit suggests that the FLASH effect could be reproduced in human patients and encourage its test in clinical trials. Indeed, the first clinical patient was safely treated in terms of skin tolerance with FLASH e- in 2019 [6]: a compassionate treatment of a CD30+ T-cell cutaneous lymphoma. Currently, there is an ongoing clinical trial (<https://clinicaltrials.gov/ct2/show/NCT04592887>) for the treatment of bone metastasis with proton FLASH-RT.

The FLASH effect has been observed after exposure of biological tissues to electrons, X-rays (at large synchrotrons) and protons [7]. Most of the experimental evidence was gathered using low energy electrons, which limits the application to deep-seated tumors. While the use of very high energy electrons (VHEE) could overcome this limitation, the technology is not yet ready [8], [9]. In the case of protons, proton therapy is already used in clinics, thus it could be a direct way to proceed to clinical trials of deep-seated tumors. Orsay proton therapy center (ICPO) was the first one to propose a dedicated setup of proton FLASH irradiation for pre-clinical research [10]. FLASH effect has been demonstrated in gastrointestinal irradiations with the same type of beam than the one at the ICPO [11], [12].

The underlying radiobiological basis of the FLASH effect remains to be conclusively demonstrated. Current theories on the protective effect of FLASH-RT include transient oxygen depletion resulting from radiolytic oxygen consumption, differential activation of metabolic and detoxification pathways in response to reactive oxygen and nitrogen species between normal and tumor cells, or radical-radical recombination [7]. In addition, an immune hypothesis has also been proposed, namely, FLASH-RT impacts differentially on circulating immune cells, tumor immune microenvironment, cytokine production and inflammatory responses [13]. However, there is currently a lack of substantial biological data to support this hypothesis, and presently only a few evaluations have been performed.

A recent study pointed towards a differential expression of inflammation-regulating cytokines associated with immune response as influencing factors of reduced toxicities after FLASH irradiation [13]. Kim et al. [14] reported increased infiltration of S100A8 neutrophils and CD8 α T cells in subcutaneously implanted Lewis lung carcinoma tumors following electron FLASH irradiation compared with tumors that received standard treatment. However, the use of a subcutaneous model can modify the immune activation and bias the results. Indeed, in an orthotopic model of murine ovarian cancer, Eggold et al. [15] observed only an increase of intratumoral CD4 T cells in the animals that received electron FLASH-RT 96 h before as compared with those receiving conventional RT. No significant differences were observed in the other immune cell types as a function of irradiation mode neither at 96 h nor 17 days after irradiation. Finally, significant reduced number of distant metastases was observed in carbon-FLASH irradiated animals bearing subcutaneous murine sarcoma (LM8) compared with those receiving conventional carbon irradiations [16], indicating a higher

capacity of carbon FLASH-RT to induce abscopal effects than standard irradiations and suggest a differential interaction of FLASH-RT with the immune system.

The lack of biological data motivated this study, in which we have evaluated for the first time the possible impact of proton FLASH-RT versus conventional proton therapy on circulating immune cells and tumor infiltration in an orthotopic tumor model of rat glioblastoma.

Materials and methods

Irradiations and dosimetry

Orsay proton therapy center (ICPO) uses an “universal” nozzle-equipped gantry supplied by a Proteus 235 isochronous cyclotron (IBA, Belgium) capable of delivering both pencil beam scanning and double scattering treatment modalities. The dose rate was set using the maximum nominal cyclotron current of 500 nA and 226.899 MeV energy, and the pencil beam scanning irradiation was optimized to reduce the dead time between each pencil beam position. An accelerated narrow monoenergetic proton pencil beam (in our case with a spatial spread of the pencil beam of 4.0 ± 0.3 mm in air at isocenter) was scanned over a 15×15 mm² square field (a uniform broad field was therefore generated by the superposition of a nine equally-spaced and weighted proton pencil beams). With this specific set-up, the pencil beam scanning, performed with two scanning magnets, was therefore optimised to satisfy the requirements for an irradiation duration shorter than 100 ms and mean dose rates greater than 100 Gy/s. Reference dosimetry was performed with the Advanced Markus chamber (PTW, Germany) and applying corrections for temperature, pressure, polarity effects, and recombination. The chamber was previously cross-calibrated against a Semiflex cylindrical ionization chamber calibrated under reference conditions in a ⁶⁰Co beam at the French national metrology institute (CEA-LNHB), following the methods described in [10].

To precisely align and deliver proton radiation to the rats’ brains, we used an in-house 3D-printed rat immobilizer allowing anesthesia of the animals on a transportable bed (as also described previously [17], [18]). Positioning and immobilization were ensured acquiring kV X-ray images and monitoring the animal during irradiation with surface cameras. Using this setup, all groups received unilateral transmission irradiations using the plateau area of a mono energy 226 MeV proton beam. The original scanned beam was modified into a 12×12 mm² (at 90%) collimated scanned beam at the irradiation point using a brass collimator (and 7 cm airgap), with a flatness of $\pm 5\%$ at maximum dose level. In all cases, the dose prescription was 25 Gy at 1 cm depth in the brain or in the tumor. One single dose was chosen to disentangle the specific effects of ultra-high dose rates from temporal fractionation or dose. A dose of 25 Gy was used since it has been shown to be the therapeutic dose in the glioma model (RG2) that we are using [19]. Proton irradiation of rats was delivered using standard (4 ± 0.02 Gy/s) versus FLASH (257 ± 2 Gy/s) dose rates.

For the irradiation, the animals were anesthetized with no oxygen added in the anesthesia (isoflurane, 2.5% in air) for 10 minutes. Prior to the experiments, some film dosimetry experimental campaigns were carried out as a cross check to verify the irradiation conditions. Moreover, radiochromic films were placed on the rats’ skin for quality assurance of the irradiation.

Animals’ follow up

In the case of normal rats the animals were followed for 3 months. Behavioral evaluations were performed at 3 months after irradiation. The tumor bearing animals were irradiated 14 days after implantations and sacrificed 8 days after irradiations for flow cytometry analysis. In the case of non-irradiated animals, they were sacrificed 14-17 days after implantation. The clinical status of the

animals was checked five times per week. All animal experiments were conducted in accordance with the animal welfare and ethical guidelines of our institution. They were approved by French Ministry of Research (permit n° 2019122418442057).

Assessment of FLASH effect in behavioral tests

Rats that beard no tumor were housed in groups of two per cage in a temperature- and humidity-controlled colony room and maintained on a 12:12-hours light/dark cycle with *ad libitum* access to water and food. All behavioral tests were performed during the day by the same experimenter. The experiments were performed at approximately the same hour each day for each animal to avoid disrupting the sleep cycle.

The open field test (OF) was adopted as a basal assessment to measure locomotor and exploratory activity and general anxiety. Each rat was placed in an open arena (1 m x 1 m) and allowed to freely explore. Each animal was placed in the OF arena three times per day (for 5, 3, and 3 min) with an interval of 3 hours between each trial. The total distance travelled, the time spent to rearing and the time spent in the center were recorded. Anxiety was inversely correlated with the time the rat spent in the center of the arena.

Wheel activity test was also used to assess spontaneous physical activity [20]. The wheel was housed in a standard clear polycarbonate cage and with *ad libitum* access to water and food. Each animal was placed individually in the cage containing a wheel and was free to access it during a 48-hours period. Sensors record the use of the wheel (number of wheel turns, duration, and periods of use).

The memory capacity of the animals was assessed with the object recognition task (ORT), which evaluates the ability to recognize a novel object in a known environment. Each rat was allowed to familiarize itself with two identical objects in the OF arena for 5 min. Three hours later, the rat was placed in the OF arena for 5 min with one novel object and the same familiar object. The time spent exploring each object and the total distance travelled were measured and used to calculate the discrimination ratio with the following formula: (time exploring the novel object – time spent exploring the familiar object) / time spent exploring both objects

Statistical analyses were performed using the software JASP with a threshold of 0.05. Fisher's exact test and Student's t test were also used. In the case in which the result was non-significant, a second statistical analysis was performed, the Bayesian analysis. This analysis was used to confirm the probability of the alternative hypothesis.

Tumor Inoculation

The RG2-[D74] (ATCC® CRL-2433™) glioma cell line transfected with the luciferase gene was used (RG2-luc). 5000 RG2-luc cells were resuspended in 5 µL DMEM and then injected intracranially into 6-weeks old male wild-type rats (strain F344, Janvier Labs) using a Hamilton syringe through a burr hole in the right caudate nucleus (2.5 mm anterior to the ear bars, i.e., at the bregma site, 4.7 mm lateral to the midline and at a depth of 5.5 mm from the skull).

Tumor follow up

The presence of a tumor was confirmed either by magnetic resonance imaging (MRI), by Bioluminescence imaging (BLI) or both before irradiation.

Concerning MRI, for each imaging session, a catheter was inserted into the tail vein for contrast agent administration. A 7-Tesla preclinical magnet (Bruker Avance Horizontal 7-T Bruker, Inc., Billerica, MA, United States) equipped with a 35 mm-diameter “bird-cage” antenna was employed. The employed T2 sequence is described in our previous work [17]. The average volume one day before irradiation was 32.5 mm³ (See figure S2 in supplementary materials).

BLI was carried out with an IVIS spectrum (Perker Elmer, Houten, The Netherlands). For the BLI procedure, the rats were injected intraperitoneally with a concentration of 150 mg/kg (P/N 122799) of D-luciferin (Perkin Elmer) in 500 µL. The peak of luminescence was reached 25 min after injection. The presence of a tumor was confirmed when the bioluminescent signal overcame the background level. Thus, only the rats expressing a BLI signal significantly higher than that of the background on the day of the irradiation were included in the study. Based on the BLI signal, the rats were randomized into groups, assuring that each group had a similar BLI average signal. Magnetic resonance imaging (MRI) was also performed the day before irradiations, to confirm the tumor presence and evaluate tumor size.

Analysis of blood immune cell populations

At 24 hours and 7 days after irradiation, blood was collected in EDTA (ethylene diamine tetra-acetic acid) tubes from the tail vein. Blood was collected from animals in the flow cytometry analysis group (n = 8 for the non-irradiated group, n= 10 for the pFLASH irradiated group and n = 7 for conventionally irradiated group). Red blood cells were lysed using Red Blood Cell Lysis Solution (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, cells were stained with antibodies in buffer containing PBS and 3% of Fetal Bovine Serum (FBS) and a viability stain diluted 1/1000 (FVS780, BD Biosciences, RRID: AB_2869673) at 4°C for 30 min. The cells were stained with antibodies detailed in table S1 in supplemental materials. After the wash, cells were resuspended in PBS and were analysed using a multiparameter flow cytometer (Fortessa LSR, BD Bioscience, USA) and analysed using FlowJo v10.6.

Data are given as means ± standard error of the mean. Statistical analyses were performed by Brown-Forsythe and Welch ANOVA with multiple comparisons performed by Unpaired t with welch's correction. These statistical analyses were performed on GraphPad Prism 9 (GraphPad Software, CA, United States).

Analysis of tumor and brain immune cell populations

Tumors and the contralateral hemisphere of the rat brain were dissected and washed in 4°C Dulbecco's Phosphate Buffered Saline (D-PBS, Gibco). Brain samples were carefully removed of meninges and visible blood vessels. Tissues were weighed and incubated for 30 min at 37°C in digestion solution in agitation (D-PBS containing 1 mg/mL Collagenase D (Roche), 0.1 mg/mL DNase I (Sigma) and 3% fetal calf serum (FCS)). Tissues were then mechanically disrupted with a 2 mL-syringe piston on top of 100 µm filters to obtain a single cell suspension in FACS buffer (DPBS with 0.5% bovine serum albumin (BSA) and 2 mM ethylene-diamine-tetra-acetic acid (EDTA)).

Cells were centrifuged at 320g for 7 min, resuspended in 6mL of 30% Isotonic Percoll Solution (IPS) and centrifuged for 20 minutes at 500g with no break at 20°C. Myelin and debris were disposed of by carefully removing 5 mL of supernatant. Cells were then washed twice in 10mL ice cold DPBS and

resuspended in FACS buffer with purified anti-CD32 (FcγRII) as a blocking agent. Cells were incubated for 15 minutes at 4°C and then immunolabelled for FACS analysis with the appropriate fluorescence-coupled antibodies (see table S2 in supplemental materials) and a viability stain diluted 1/1000 (FVS780, BD Biosciences, RRID: AB_2869673) for 30 minutes at 4°C. Counting beads were added to the sample before acquisition (CountBright™ Plus Absolute Counting Beads, Thermofisher). Cell profiles were recorded using a multiparameter flow cytometer (Fortessa LSR, BD Bioscience) and analysed using FlowJo v10.6.

Data are given as means \pm standard error of the mean. Statistical analyses were performed by Brown-Forsythe and Welch ANOVA with multiple comparisons performed by Unpaired t with welch's correction. These statistical analyses were performed on GraphPad Prism 9 (GraphPad Software, CA, United States).

Results

1. Observation of FLASH effect in rats after high dose proton radiation

Similar mean distance travelled, and mean time spent rearing was observed between the 3 groups in the open-field (OF) test 3 months after the irradiation (See Figure 1A-B), suggesting that locomotor and exploratory activity were not altered in any of the irradiation setups. In contrast, the OF test revealed that both irradiated groups presented higher anxiety levels than the non-irradiated controls, as measured by the time spent in the center of the arena, with no significant difference observed between proton Flash therapy (pFLASH) and conventional proton therapy (CPT) groups (see Figure 1C). The wheel activity test yielded no significant difference in terms of spontaneous physical activity of the rats between the three groups (see Figure 1D). A 3 x 15 ANOVA (groups x 3-hour periods) analysing the number of wheel turns was performed, resulting in no main effect of hours and no group x hours interaction. Therefore, no tested conditions affected the spontaneous activity of the rats.

Interestingly, the object recognition task was performed differently by the three groups (Figure 1E). The control group showed a significantly positive discrimination ratio compared to 0 (one-sample t test: $t = 1.7$, $p = 0.075$, but the Bayesian one-sample t-test ($BF+0 = 1.73$), in favour of the alternative hypothesis, confirmed the significant result), as well as the pFLASH group (one-sample t test: $t = 2.75$, $p = 0.02$, confirmed by the Bayesian one-sample t-test ($BF+0 = 4.75$) in favour of the alternative hypothesis). The results confirmed that both control and pFLASH groups had a good memory of the familiar object. This was not the case for the conventionally irradiated rats, which did not recognize the familiar object significantly (ratio (one-sample t test: $t=0.44$, $p=0.34$, confirmed by the Bayesian one-sample t-test ($BF+0 = 0.52$) in favour of the null hypothesis). The result was significantly different between CPT and pFLASH group (independent sample t-test: $t = -2.39$, $p = 0.04$, confirming by the Bayesian independent sample t-test ($BF10 = 2.16$) in favour of the alternative hypothesis), corroborating that CPT irradiation disturbed the recognition memory compared to pFLASH dose rate condition. Overall, a FLASH protective effect in terms of memory was assessed in the dose rate of protons used in this study.

2. Circulating immune cells

In order to study the systemic impact of the brain irradiations, we assessed peripheral immune populations 24 hours and 7 days after the irradiations. The gating strategy to isolate the population can be found in FigS2.

No significant changes were observed 24 hours after irradiations in all populations analysed. At 7 days post-irradiation, CD4 T cells of both the CPT and pFLASH groups were decreased compared to the pFLASH analysed at 24 hours although not compared to the control group. A significant increase of B cell proportion was observed exclusively in the pFLASH group 7 days after irradiations compared to the control and the population observed at 24 hours after irradiation. Finally, a significant increase of His48^{high} CD43^{low} monocytes was observed both in the CPT and the pFLASH group 7 days after irradiation compared to the rest of the groups.

3. Inflammation of the brain parenchyma is also observed in pFLASH irradiation

The healthy tissue was analysed by flow cytometry 8 days post irradiation for several immune populations (see Figure 3, gating strategy in Figure S3). Proton irradiation provoked an infiltration of peripheral immune cells (CD45^{high}) in the brain parenchyma in both CPT and pFLASH groups, while non-irradiated controls showed negligible presence of immune infiltrates in steady state, as expected (Figure 3A). Brain resident macrophages, microglia (isolated as CD45^{low} CD11b/c⁺ cells), were severely affected by both irradiations irrespectively of the dose rate (See figure 3A) showing a 4-fold decrease in microglia density in the tissue (Figure 3A-B).

The infiltration of immune cells in the brain parenchyma was evidence of a general inflammation in the tissue, which was significant in terms of lymphoid cells in both groups but specially of CPT in the case of myeloid cells (dendritic cells, neutrophils and His48^{high} monocytes) (Figure 3H-K). This showed a tendency for a higher inflammation in the CPT dose rate group.

Additionally, we observed that irradiated microglia upregulated the peptide processing major histocompatibility protein complex II (MHC-II), as apparent by the expression of rat RT1B (Rano class II histocompatibility antigen, β -1 beta chain) (see Figure 3L-M).

4. Intratumoral immune cell inflammation

Tumor weight at the end of the experiment showed a decrease in tumor size in irradiated tumors compared to the control tumors at the time of the irradiation (Figure S4), which validated a similar tumor regression capacity of both dose rates. A significant change of immune composition of the tumor was observed in the tumor microenvironment 8 days after conventional and pFLASH irradiation (Figure 4A). The details of the gating strategy and all p-values are available in Figure S5. Tumor infiltrating lymphocytes (TILs), characterized as CD3⁺ cells and containing CD4 and CD8 T cells (dark and light blue, respectively) represented $21.23 \pm 7.65\%$ of the tumoral hematopoietic cells in the control group, $49.27 \pm 5.05\%$ in the CPT group and $49.24 \pm 5.59\%$ in the FLASH group, with no significant difference observed between the two irradiated groups (Figure 4A). Quantification of the cell number normalized by tumor weight yielded a significantly higher infiltration of all infiltrating lymphoid populations in the irradiated groups (Figure 4B-H). Interestingly, regulatory T cells (Treg, characterized as CD25⁺ CD4⁺ T cells) increased very significantly after both CPT and pFLASH (see Figure 4E), and this was also the case for tissue resident memory cytotoxic T cells (CD8⁺ TRM T cells, characterized by the expression of CD103) (see Figure 4F). No significant difference was observed with respect to the radiation dose rate, although a trend for higher TIL infiltration was noticed in the CPT group. NK cells were also increased (characterized as CD161^{high} CD8⁺ cells) (Figure 4G), and more particularly B cells (characterized as B220^{high} Rt1b⁺) (Figure 4H). These results indicated an activated adaptative immune response to the tumor following both CPT and pFLASH.

In terms of tumor myeloid cells, CPT was particularly efficient in recruiting conventional type 1 dendritic cells (cDC1, characterized as CD11b/c⁺ CD103⁺ Rt1b⁺ cells), CD8⁺ macrophages (characterized as CD11b/c⁺ CD8⁺ Rt1b⁺ SSC-A^{high}, see Figure S6) and neutrophils (characterized as CD11b/c⁺ His48⁺ CD43⁺ SSC-A^{high}), while the group irradiated in FLASH dose did not increase these

populations significantly. Overall, CPT dose rate managed to increase immune cell density in the tumor microenvironment more efficiently, while FLASH dose rate only increased the density of TILs.

Discussion

FLASH therapy is a novel radiotherapeutic approach that uses ultra-high dose rates of radiation and has shown a significant reduction of toxicity as compared to conventional radiotherapy [4], [16], [21]–[23], while maintaining the same tumor control [6], [16], [23], [24]. To date, the biological mechanisms under FLASH-RT remain elusive. Several mechanisms have been proposed in the literature, namely the lower creation of oxygen-reactive species depending on tissue hypoxia level or a different immune activation through the systemic immune cells or inflammatory response in the tissue [7], [13], [14], [21], [23]. In this study, we chose to evaluate the potential differential impact of ultra-high dose rates on the immune landscape *in vivo* in a clinically relevant model of FLASH-RT. The choice of 25 Gy single fractionation was due to previous studies [19], [25] indicating that it is the therapeutic dose for this glioma model. Since this is a very high dose, we hypothesized that the putative sparing effect of FLASH-RT might be pertinent to this model. Moreover, there are indications in the literature that a minimum dose (7-9 Gy) is needed to observe the FLASH effect [24], [26]. Therefore, FLASH-RT might likely be translated to clinics using hypofractionation or single dose schemes, and not classical fractionation of 2 Gy/session. Indeed, the response of normal tissues to the combination of temporal fractionation remains to be explored in FLASH-RT. Thus far, only one study assessed tumor response in hypofractionation schemes [24] in glioma-bearing animals. The remarkable healthy tissue preservation in FLASH-RT might enable the use of more aggressive fractionation schemes than current ones in conventional RT, and particularly one single dose or hypofractionation scheme. To the best of our knowledge, this is the first data assessing the sparing effect of proton FLASH therapy in rats at very high doses and the cellular immune response generated after the treatment.

Firstly, we assessed a FLASH effect at the doses given in this study. pFLASH irradiations resulted in a significant memory sparing comparing to the conventional dose rate proton therapy, which significantly impaired object recognition. In our study, no significant lymphopenia was observed in any of the irradiated groups, apart from a slight decrease on CD4 T cells 7 days post-irradiation. Thus, we could not conclude on a protective effect of FLASH RT on peripheral immune cells as was suggested in the computational study by Jin et al. [27]. However, in the latter work the simulated irradiated area received 1.8–2 Gy per fraction over several weeks, which is the treatment plan that provokes the blood lymphopenia commonly seen in irradiated patients [28]. The memory and tissue sparing demonstrated by pFLASH might enable to use hypofractionation schemes safely and effectively instead of conventional schemes. We observed however an increase of circulating B cell proportion in pFLASH irradiated rats 7 days after the irradiation and a significant increase in His48^{high} CD43^{low} monocytes after both irradiations. His48 expression in rat monocytes is analogous to Ly6C on murine or CD14 in human monocytes, which mark “classical” or “inflammatory” monocytes involved in inflammatory cell-recruitment and wound healing [29], [30]. This might have indicated that a general inflammation occurred in both treatment groups.

Eight days after irradiation we observed a significantly higher T cell infiltration in the brain of the irradiated animals of both groups with respect to the controls, mostly of T cells and in the case of CPT, also neutrophils. Overall, immune cell infiltration was higher in the conventionally irradiated animals than in pFLASH. CD4 T cell infiltration has been suggested to play a role in neurodegeneration [31], although a certain level of lymphocyte infiltration within the brain parenchyma was reported to be necessary for rapid resolution of the inflammatory process and neuro-regeneration during the acute phase of neuroinflammation, probably by microglia suppression [32]. In our study, the number of microglia was significantly reduced eight days after irradiation

independently of the dose rate. Temporary microglia-depletion after irradiation has been suggested to prevent the development of cognitive deficits due to RT [33], [34]. It is also worth noting that the microglia depletion was observed at a shorter timepoint than most studies up to now [21], [35], and it could reflect a temporary depletion. Since FLASH-RT has shown to spare blood vessels in the lungs [4] and to not induce vasodilation of microvessels in the brain [36] compared to conventional radiotherapy at a similar high dose, it is possible that the reduced impact on brain function is explained by the sparing of vasculature by FLASH-RT. Early radiation vascular damage has been pointed as a direct cause of cognitive impairment, since it leads to ischemia and later to white matter necrosis [37], although likely not the sole cause, it is one of the major participants. Further studies at later time points would be needed to establish potential differences and the implications of the results between CPT and pFLASH in healthy brain tissue.

Both proton radiated groups showed a substantial infiltration of T cells in the tumor microenvironment (TME) with respect to non-irradiated controls which was similar in all subtypes analysed. No significant differences in T cells were observed between the two irradiated groups in agreement with Eggold et al. [15]. The higher CD8⁺ cell infiltration observed in the FLASH-irradiated animals in the work of Kim et al. [14] could be due to the subcutaneous model used in contrast to this work and Eggold's. However, only CPT significantly increased the number of dendritic cells, CD8⁺ macrophages and neutrophils suggesting a different inflammatory myeloid signaling cascade in the tissue. Indeed, intratumoral dendritic cells can provide *in situ* antigen processing and presentation to CD4 and also CD8 T cells [38] and MHC-II expressing macrophages have a pro-inflammatory phenotype (M1-like) in murine breast tumors that promotes tumor inhibition [39]. MHC-II coexpression with FOLR2 marks tumor-associated macrophages that are involved in T cell priming in human breast cancer [40]. On the other hand, the recent work of Tingannelli et al. [16] indicated a decrease in lung metastasis following FLASH with carbon ions further suggesting a different immune modulation in FLASH radiations that achieves abscopal effect and needs to be addressed in future works.

We chose RG2 as a glioma model since these cells form a highly invasive glioma with low immunogenicity compared to other rat glioma models like C6 and 9L [41], [42]. Therefore, the model allowed us to generate a proof-of-concept study to observe relative differences in the immune response generated by a FLASH irradiation compared to a conventional irradiation. However, it is important to note that the luciferase expressed by the tumor cells can be immunogenic and thus constrain tumor growth in some models, as it was shown in a model of breast cancer [43], although the contrary was observed in others, as it is the case in a model of ovarian cancer [44]. Although we have not directly evaluated the role of luciferase in this study, other study using RG2-luc at the same concentration showed a median survival time of 20 ± 2 days [45], similar to other studies using wildtype RG2 cell line with a similar concentration [46], [47]. Hence, the data suggest that luciferase in this specific model has no role in slowing down the tumor process.

In conclusion, we observed that both FLASH and conventional dose rate proton irradiation are capable of mounting an effective lymphoid immune response. However, conventional PT dose rate induced extensive brain damage, as shown in this work and previous ones with the same rat model [17], [45], [48] and is not possible to use in a clinical context in the case of large tumors such as GBM.

The specific radiobiological mechanisms of new radiotherapy techniques such as FLASH-RT are currently under rigorous study, and immune response generation is turning into a key point of observation. The strong immune changes in the brain's healthy tissue and the tumor microenvironment generated by proton FLASH need to be properly understood to provide an optimal clinical transfer. Additionally, the high lymphoid cell mobilization in the tumor reported in this study together with the reduction of neurotoxicity offered by proton FLASH might open the door to employ

this technique for T cell directed immunotherapies. Further studies should include the evaluation of the influence of hypofractionation schemes in FLASH-RT.

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Figure 1. Behavioral tests in naïve animals after cranial irradiation. (A) Locomotor, (B) exploratory activity and (C) anxiety assessments at 3 months post-irradiation measured in an Open Field (OF) test. (D) Spontaneous physical activity assessments measured in wheel activity. The grey area represents the dark periods (light off between 7 p.m. and 7 a.m.). (E) Memory assessment in the object recognition task (ORT) at 3 months post-irradiation. CPT irradiated rats did not score well in positive discrimination ratio contrary to pFLASH and controls rats. Control (n = 6), CPT (n = 6) and pFLASH (n = 6). The data are presented as the mean \pm SEM ; *p < 0.05. "CPT" and "pFLASH" refer to the groups receiving one fraction of 25 Gy of conventional proton therapy and one fraction of 25 Gy of proton therapy at ultra-high dose rate.

Figure 2. Impact of proton therapy dose rate in circulating immune populations. (A) Proportion of immune cells among CD45⁺ leucocytes: (A) CD4 T cells, (B) CD8 T cells, (C) B cells (D) NK cells, (E) Neutrophils, (F) CD43^{high} His48^{low} monocytes and (G) CD43^{low} His48^{high} monocytes. control (black), CPT-irradiated (green) and pFLASH (red) irradiated animals. Control (n = 9), CPT (n = 7), pFLASH (n = 9). The data are presented as mean \pm SD. p-values: *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001

Figure 3. Microglia and other immune cells in the brain parenchyma 8 days after proton irradiation. (A) Representative dot plots of total cells recovered from healthy tissue in terms of CD45 and CD11b/c expression. (B) Quantification of microglia per mg of tissue. (C) Quantification of T cells, (D) CD4 T cells, (E) CD8 T cells, (F) B cells, (G) NK cells, (H) cDC1, (I) neutrophils, (J) CD43^{hi} His48^{lo} monocytes and (K) His48^{hi} CD43^{lo} monocyte-macrophages. (L) Representative dot plots showing microglia in terms of expression of the activation marker Rt1b and side scatter (SSC-A). (M) Quantification of proportion of activated microglia. Control (black, n = 9), CPT (red, n = 7), pFLASH (green, n = 9). The data are presented as mean \pm SD. p-values: *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001

Figure 4. Flow cytometry analysis of immune cells in glioblastoma 8 days after irradiation. (A) Proportion of the identified immune populations, represented as percentage of the population among CD45⁺ cells, in RG2 glioblastoma. Immune cell composition was drastically altered following radiation. (B) Quantification of the cell density as recovered cells per mg tissue including all T cells, (C) CD4⁺ T cells, (D) CD8⁺ T cells, (E) Tregs, TRM T cells (F), NK cells (G), B cells (H), cDC1 (I), CD8⁺ macrophages (J), CD43⁺ His48^{neg} monocytes (K), neutrophils (L), His48⁺ monocytes-macrophages (mono-mac) (M), and CD49d^{neg} macrophages (N). Control (black, n = 10), CPT (red, n = 6), and pFLASH (green, n = 10). The data are presented as the mean \pm SD. p-values: *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001