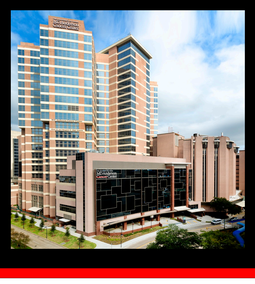

Neuroinflammatory Response in Human Derived Cerebral Organoids to Proton FLASH and Conventional Radiotherapy

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Introduction

FLASH radiotherapy (FLASH-RT) refers to the delivery of radiation at ultra-high dose rates. Preliminary data has suggested that there is a reduction in radiation-induced toxicity and preservation of surrounding normal tissue when radiation is delivered at FLASH dose rates compared to conventional dose rates; however, the mechanisms behind this phenomenon are unknown. Using human-induced pluripotent stem cell (iPSC) derived cerebral organoids (COs) as a 3D *in vitro* model of normal human brain tissue, we investigated the effects of proton FLASH and conventional dose-rate RT on microglia, the innate immune cells of the central nervous system, as chronic neuroinflammation following RT including microglial activation has been correlated with normal tissue damage.

Methods

COs were used to determine the number of activated microglia following exposure to conventional or FLASH-RT. Samples to assess microglia development within the CO model at 1 and 2 months of maturation were also prepared. After six months of maturation, COs were irradiated to 2, 5, and 9Gy at the MD Anderson Proton Therapy Center with proton FLASH and conventional dose rates. One month following irradiation, COs were fixed, sectioned, and immuno-stained with markers for activated microglia, Iba1 and CD68. Slides were imaged on a Vectra Polaris and analyzed using the QuPath software package. The percentage of activated microglia cells was defined as cells with positive expression of both CD68 and Iba1 divided by the number of all Iba1 positive cells.

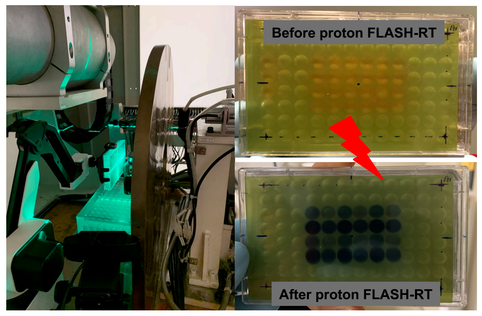

Figure 1. The proton FLASH beamline setup at the MD Anderson Proton Therapy Center including the robotic arm and laser positioning system for the experimental procedure (**left**). A GAFChromic EBT-XD dosimetric film is attached to the bottom of the 96-well plate to measure the absorbed dose and display positional accuracy (**right**).

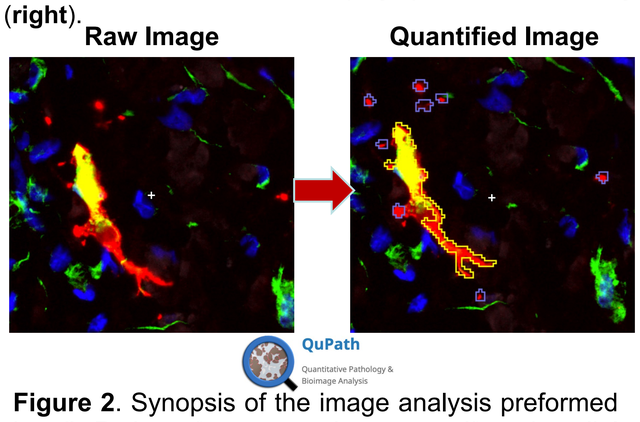

Figure 2. Synopsis of the image analysis preformed by QuPath software used to quantify microglial presence and activation. Cells expressing Iba1 (microglial protein marker) are detected and outlined in blue. The dual detection of CD68 (lysosomal protein marker) and Iba1 are outlined in yellow indicating an activated microglia cell. The total number of active and inactive cells are then computed. Data is exported for further analysis and plotting.

Results

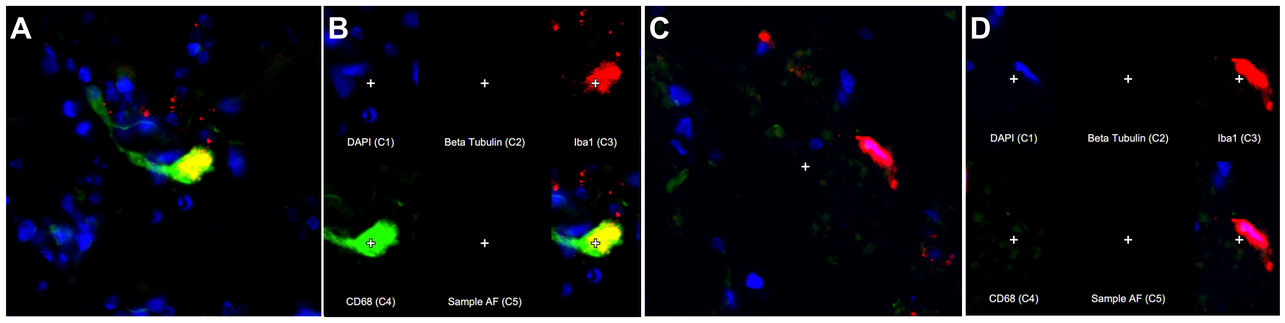

Figure 3. Cellular Characterization of COs. Color combined immunofluorescence images of frozen cross sections of COs labeled for microglial markers. Activated microglia cells **(a-b)** display dual detection of CD68 (green; lysosomal marker) and Iba1 (red, microglia marker) expression. Inactive microglia cells **(c-d)** are characterized by cellular detections Iba1 but not CD68. All nuclei are labeled with DAPI (blue).

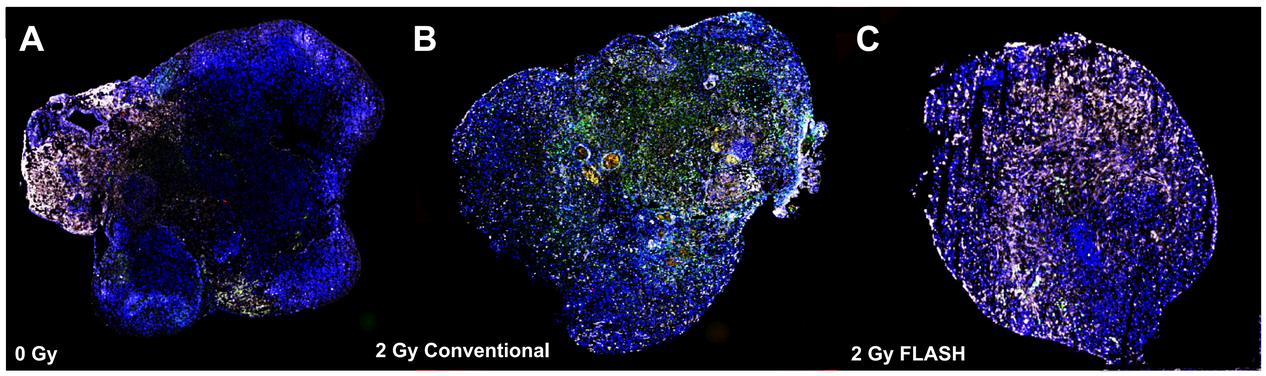

Figure 4. Effect of Irradiation on COs. Representative images of unirradiated (A), conventional (B), and FLASH irradiated (C) COs. Cross section COs are immunolabeled for Ki-67 (pink), Iba1 (red), CD68 (green), and DAPI (blue).

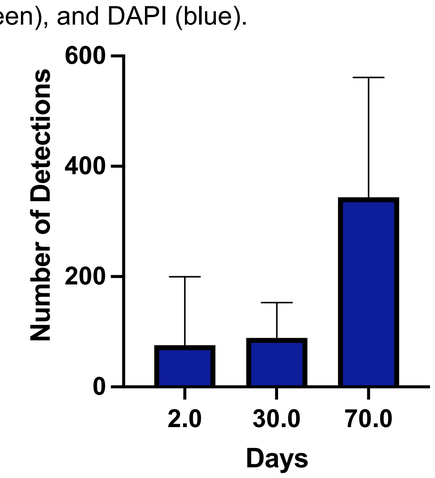

Figure 5. Microglial maturation increased with time in control COs. The detected microglia cells were immunolabeled for Iba1 (macrophage specific protein) expression. Error bars represent standard deviation (n=3).

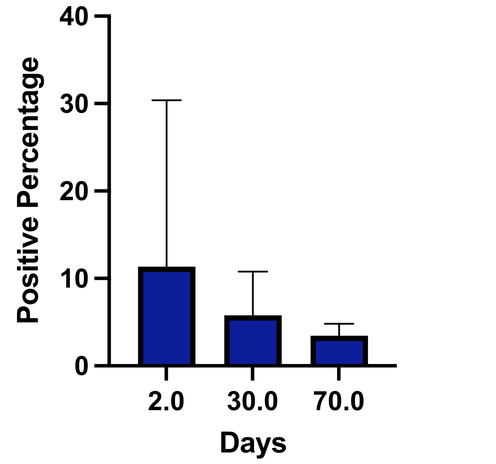

Figure 6. Control COs display a reduced quantity of activated microglia cells with time. Quantified number of activated microglia cells defined as Iba1 positive cells expressing CD68 (lysosomal protein). Error bars represent standard deviation (n=3).

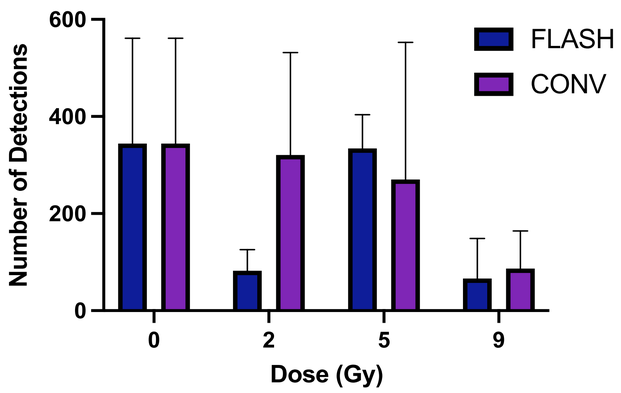

Figure 7. Microglial expression following exposure to conventional or FLASH-RT. The number of microglia cells irradiated with convention therapy display a downward trend. FLASH-RT COs have a higher quantity of microglia cells at 5Gy and a decreased quantity at 2Gy and 9Gy. Error bars represent standard deviation (n=3).

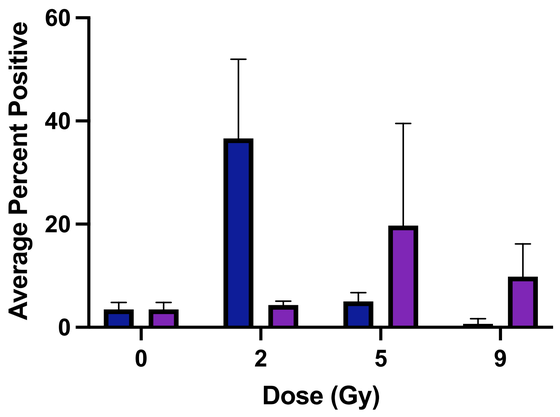

Figure 8. Activated microglial expression following exposure to conventional or FLASH-RT. A significant increase in activated microglial cells is present at 2Gy (P=0.002) Error bars represent standard deviation (n=3).

Discussion

The original report on the development of the stem cell-derived CO model described a brain tissue model solely comprised of cells from the ectodermal stem cell lineage indicating there would be no cells or tissues present from the other lineages. However, recent work discovered that mesodermal-derived microglia develop within the CO model. We performed compositional analysis of COs to reveal a population of cells positive for the microglia marker Iba1 and active microglia cells positive for both CD68 and Iba1 markers indicating the potential of the CO model for *in vitro* neuroinflammatory studies.

The CO model was used to evaluate the effects of conventional and FLASH radiation on microglial cells (Figure 4).The average number of microglia cells detected at 1, 2, and 7 months of CO maturation was found to increase (76, 89, 344) (Figure 5) while the percentage of activated microglia detected decreased (11, 6, 3%)(Figure 6). A significant increase in the percentage of activated microglia cells was detected at 2Gy FLASH (37%, P=0.002) followed by a decrease at 5Gy and 9Gy (5, 1%) (Figure 8). For COs irradiated at conventional dose rates, the percentage of activated microglia at 2Gy (4%) was approximately the same as the control but was higher at 5Gy and 9Gy (19, 10%) (Figure 8). The number of microglial detections in FLASH-RT decreased at 2Gy (82) and 9Gy (66) but increased at 5Gy (334) (Figure 7). In conventional radiation, a reduction in the number of microglial cells is seen with doses (344, 320, 270, 87) (Figure 7).

Conclusions

Our results further confirm the presence of microglia within the CO model. Our findings begin to characterize the long-term maturation and radio response of this unique cell within the CO model. Irradiated COs display dose-rate dependent differential effects in neuro-inflammation markers where FLASH appears to result in reduced microglial activation for higher doses compared to conventional radiotherapy.

Further Study

Our initial findings motivate further investigation into the neuro-inflammatory response of normal brain tissue at FLASH dose-rates compared to conventional dose rates including determining the mechanism behind the differential effects. Incorporating vasculature within the CO model is being actively investigated in order to further develop and improve the model's ability to recapitulate *in vivo* settings.

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