AWARD NUMBER: W81XWH-17-1-0032

TITLE: Dual Benefit of TGFB Inhibition on Tumor Control in the Context of Radiotherapy for Breast Cancer Brain Metastases

PRINCIPAL INVESTIGATOR: Mary Helen Barcellos-Hoff

CONTRACTING ORGANIZATION: University of California, San Francisco

REPORT DATE: April 2020

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
April 2020	Final	02/01/2017 - 12/31/2019

4. TITLE AND SUBTITLE

Dual Benefit of TGFB Inhibition on Tumor Control in the Context of Radiotherapy for Breast Cancer Brain Metastases

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0032

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Mary Helen Barcellos-Hoff

email:Mary.Barcellos-Hoff@ucsf.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Regents of University of California
San Francisco, CA 94143

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project evaluates whether transforming growth factor beta (TGFβ) inhibition during radiation therapy (RT) to breast cancer brain metastases (BCBM) provides greater therapeutic benefit than RT alone using a robust proof-of-concept therapeutic protocol in combination with innovative functional imaging. Successful demonstration that TGFβ inhibition increases durable RT response that may augment immunotherapy in preclinical BCBM models would provide a strong rationale for trials of clinically viable drugs that block TGFβ signaling with gamma-knife stereotactic radiosurgery (GKSRS) for women with metastatic disease. We incorporate molecular imaging of active TGFβ to assess target levels, drug delivery, therapeutic response via tumor metabolism, and identify potential immune – mediated responses that will enable rapid clinical translation of combined RT and TGFβ inhibitory drug regimens.

15. SUBJECT TERMS-

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU		

1. **INTRODUCTION:** This project evaluates whether transforming growth factor beta (TGFβ) inhibition during radiation therapy (RT) to breast cancer brain metastases (BCBM) provides greater therapeutic benefit than RT alone using a robust proof-of-concept therapeutic protocol in combination with innovative functional imaging. Successful demonstration that TGFβ inhibition increases durable RT response that may augment immunotherapy in preclinical BCBM models would provide a strong rationale for trials of clinically viable drugs that block TGFβ signaling with gamma-knife stereotactic radiosurgery for women with metastatic disease. We incorporate molecular imaging of active TGFβ to assess target levels, drug delivery, therapeutic response via tumor metabolism, and identify potential immune – mediated responses that will enable rapid clinical translation of combined RT and TGFβ inhibitory drug regimens.

2. **KEYWORDS:** breast cancer brain metastases, transforming growth factor beta (TGFβ), immunotherapy, radiation therapy (RT), gamma-knife stereotactic radiosurgery (GKSRS), molecular imaging, positron emission computed tomography

3. **ACCOMPLISHMENTS:**
   a. **What were the major goals of the project?**

	Proposed Timeline (Months)	**Revised Timeline (Months)**	**% Complete to Date**
Specific Aim 1: Ascertain the benefit of TGFβ inhibition in preclinical immunocompetent BCBM models using targeted radiation in a small animal radiation research platform that emulates GKSRS targeted delivery and determine whether this endorses response to immunotherapy.			
Major Task 1: Evaluate TRI-Modal Therapy			
Subtask 1: Establish brain tumor metastasis models • Establish and characterize the brain metastasis models	1-3	1-6	100
Subtask 2: Establish 2 cohorts of 80 mice • Image mice using bioluminescence and ascertain tumor burden • Randomize to treatment arms • Design single fraction treatment plan for each mouse • Irradiate and monitor mice • Transfer mice for functional imaging studies • Complete imaging-based tumor response and immune modulation	3-6	6-24	80

assessments in all cohorts and collect tissue at morbidity			
Major Task 2: Correlation of biological processes with outcome			
Subtask 1: Preparation of tissues for immunoscore and tumor analysis (e.g. embedding, sectioning)	6-7	12-24	90
Subtask 2: Analyze splenic immune repertoire and circulating cells by FACS	4-6	12-24	50
Major Task 3: Replicate experiment using 2 fractionated radiation protocols			
Subtask 4: • Establish second brain metastasis model • Use optimized protocol in second brain metastasis model	12-15		50

Specific Aim 2: Characterize functional imaging methods to assess drug distribution, tumor burden and immunological response to RT			
Major Task 1: Collect imaging information as a function of time post treatment for experiment 1			
Subtask 1: Synthesize PET radiolabeled drug and optimize yields of immune-probing imaging agents	1-3	1-9	100
Subtask 2: Correlate imaging and biological responses at 7 days post treatment • Complete imaging-based tumor response and immune modulation assessments in all cohorts and collect tissue at morbidity	4-12	12-24	60
Milestone #1: *Prepare manuscript on RT responses mediated by TGF β*	12-15	24-30	100
Subtask 3: Evaluate immunological responses mediated by RT • Assess systemic and localized processes that associate with decreased tumor burden following various arms of therapy • Re-evaluate experimental design and optimize protocol	12-15	12-18	80

• Subtask 4: Assess best evidence and predictors for biological efficacy of combination.	18-24	24-30	50
Milestone #2: *Prepare manuscript on use of the TRI-MODAL therapy in pre-clinical studies*	18-24	24-36	50

b. **What was accomplished under these goals?** For this reporting period describe:
   i. Major activities

Specific Aim 1: Ascertain the benefit of TGFβ inhibition in preclinical immunocompetent BCBM models using targeted radiation in a small animal radiation research platform that emulates GKSRS targeted delivery and determine whether this endorses response to immunotherapy.

- Generate and characterize two syngeneic mouse models of triple negative breast cancer (TNBC) brain metastasis.
- Image-guided radiotherapy (IGRT) of murine BCBM using the small animal radiation research platform (SARRP).
- Tumor microenvironment (TME) and immune system characterization as a function of radiotherapy and in combination with TGFβ blockade (1D11).
- Prepare the manuscript on RT responses mediated by TGFβ

   ii. Specific objectives

Assessing the benefit of TGFβ inhibition for BCM in the context of IGRT.
4T1-BrA intracranial tumor model: During year 1 of funding we established the intracranial 4T1-BrA tumor model of breast cancer brain metastases. As our primary objective was to evaluate response to treatment, we needed to control for location and time to tumor. We initially intended to use 4T1 murine breast cancer cells that were selected for brain-metastasis capacity in vivo (30). We obtained these cells from 2 different laboratories, but cells were positive for mycoplasma in both cases. Hence, we generated a pre-clinical model of breast brain metastasis using 4T1 cells that constitutively express luciferase and are labeled with mCherry fluorescent protein by inoculating these cells directly in the brain of female Balb/C mice, dissociating the brain and selecting for mCherry, expansion in vitro and re-injection of recovered cells into the brain. We designated these cells 4T1-BrA, representing brain-adapted rather than spontaneous metastases. Notably, the 4T1-BrA form highly immune cell-infiltrated tumors in the brain parenchyma, similar to parental 4T1 grown as subcutaneous tumors. CD3+ and CD8+ T-cells were present in the tumor mass as well as surrounding the tumor, suggesting that these tumors, like those grown as primary tumors, could be considered immunologically inflamed. The major myeloid cells in 4T1-BrA are myeloid, including F4/80^{+} macrophages and CD11b/Ly6G^{+} granulocytic myeloid derived suppressor cells (G-MDSC). Both tumor cells and immune cells expressed the checkpoint molecule, PD-L1.

4T1-BrA characterization. 4T1-BrA implanted into the brain of syngeneic female immunocompetent Balb/C mice were monitored for tumor growth by bioluminescent imaging (BLI). Upon morbidity, mice (n=3) were euthanized and brains collected for histology and characterization. The mouse brains were digested, and tumor cells were sorted based on

mCherry fluorescence. The resulting breast cancer brain-adapted (BrA) cells were expanded as a new cell line (4T1-BrA) and used in the following experiments.

Brains were collected and formalin-fixed paraffin-embedded (FFPE) and stained with hematoxylin-eosin to characterize the morphology and pattern of tumor growth. TME and immune system were profiled using immunofluorescence or Opal multiplexed immunofluorescent staining and Vectra multispectral microscopy. TGFβ activity was assessed using immunofluorescence.

4T1-BrA cells were injected into the brain and tumor growth was confirmed by BLI. Mice were randomized to control or treated arms based on CT based tumor volume. The small animal radiation research platform (SARRP) was used to deliver dose to the CT-defined tumor volume based on Muriplan software using a 5x5 collimator. We compared 3 radiation protocols: 1x14Gy, 3x8Gy and 5x6Gy. Tumor growth was monitored by BLI and Kaplan-Meier survival curves were generated. All 3 RT schedules controlled 4T1-BrA tumors and significantly ($p<0.0001$) increasing median survival to 27-31 days compared to 15 days for the sham-irradiated mice. This study established the baseline for subsequent studies in combination with TGFβ neutralizing antibody, 1D11 (10 mg/kg, i.p. 3 times a week, beginning 1 day before radiation therapy).

PET imaging of TGFβ activity: In collaboration with Dr. Ben Franc, we evaluated In order to evaluate the feasibility of immuno-PET imaging of active TGFβ with ^{89}Zr-DFO-fresolimumab we performed μPET/CT studies of ^{89}Zr-DFO-fresolimumab in mice bearing 4T1-BrA flank tumors. Target specificity was confirmed by lack of PET signal of either ^{89}Zr-DFO-isotype or ^{89}Zr-DFO-PEG indicating that ^{89}Zr-DFO-fresolimumab detects active TGFβ.

To evaluate radiation-induced activation, 4T1-BrM cells were injected subcutaneously on each flank. One tumor in each mouse was treated with 15 Gy, the mice were imaged and then the tumors harvested. Tumor sections were immunostained for active TGFβ and phosphorylated SMAD2/3 indicative of signaling and quantified using image analysis. Irradiated tumors showed higher active TGFβ ($p<0.05$) and phosphorylated Smad 2/3 ($p<0.05$) compared to controls, indicative of TGFβ activation. *Ex vivo* biodistribution studies were also conducted in order to quantify and correlate the μPET/CT images. ^{89}Zr-DFO-fresolimumab uptake in irradiated tumors was significantly higher than that of untreated tumors. After the *in vivo* studies, tumors were sliced and counted for radioactivity. The slices from irradiated tumors showed significantly ($p<0.0002$) higher ^{89}Zr compared to non-treated tumors.

Response to radiotherapy and TGFβ inhibition: In year 1, we next analyzed that combined treatment of RT and 1D11 provided benefit to fractionated RT (5 x 6 Gy) in the 4T1-BrA intracranial tumor model. Addition of 1D11 increased median survival by 58% compared to fractionated RT alone (31 days for RT vs 49 days for RT+1D11). Approximately 50% of mice of both RT (n=7) and RT+1D11 (n=9) survived more than 50 days without BLI evidence of intracranial regrowth. Combination of RT+1D11 significantly reduced the amount of infiltrating MDSCand increased CD3+ T-cell infiltrate, suggesting a shift towards anti-tumor immunity.

In year 2, we repeated the experiments using a sub-optimal radiation dose of 10 Gy to assess whether the addition of TGFβ blockade provides long term benefit. Consistent with the results obtained in the fractionated protocol, double-treated mice bearing 4T1-BrA intracranial tumors showed significant reduction of tumor burden as measured by BLI.

Kaplan-Meier survival plots demonstrate that the double treatment was superior to RT alone as demonstrated by an increase in median survival (Figure 1A). BLI graphs showed a decrease in tumor growth from a detectable signal to a complete regression in the RT alone and double treated group. Median survival was 17 days for control treated mice, 19 days for mice treated with 1D11, 33 days for mice treated with RT, and 41 days for double treated mice. However, 5/13 (38%) mice in the double treated group survived greater than 50 days whereas 2/12 (17%) irradiated mice did

so. Since 4T1 are highly metastatic to lung, we imaged whole lungs ex vivo by BLI to assess metastases. We found that RT alone decreased metastasis in lungs, which was decreased even more in the double-treated mice, which exhibited less lung burden.

The long-term survival suggest that tumor cure was achieved. To test for the contribution of the immune system, survivors were re-challenged with subcutaneous tumor injection of 4T1BrM. Such tumors were rejected in 2/2 from RT and 3/4 mice treated with RT+1D11, indicative of immune memory. No tumors recurred in the brain during the extended observation.

We also assessed the downstream signaling of active TGFβ in the paraffin fixed tissue of mice treated with the suboptimal radiation dose of 10 Gy. Therefore, we performed immunofluorescence staining of phosphorylated SMAD2 and extracellular matrix protein tenascin C (TNC). SMAD2 is phosphorylated after TGFβ is bound to its membrane receptors and therefore a direct downstream target of active TGFβ. We can show that the percentage of pSMAD2 positive cells is increased after RT and can be decreased by addition of 1D11 (Figure 1B). The expression of TNC is directly positively influenced by TGFβ. Confirming the pSMAD2 results we could show and RT dependent increase that was impeded by TGFβ inhibition (Figure 1C &D).

Notably, long term survival and demonstrable anti-tumor immunity demonstrates that blocking TGFβ is itself immunomodulatory and precludes the need for addition of an IO agent.

Immune monitoring: In the first year, we used flow cytometry and immunostaining to evaluate immune cells of 4T1Br-A tumors. Radiation increased the amount of infiltrating MDSC whereas RT+1D11 significantly reduced the infiltrate. Radiation also increased tumor infiltrating CD3+ T-cells. The combination of decreased MDSC and increased CD3+ T cells suggests a shift towards anti-tumor immunity.

In the second year, we used mass cytometry by time of flight (mass cytometry; CyTOF) to enable single cell resolution of up to 40 parameters in millions of cells. CyTOF combines flow cytometry with elemental mass spectrometry by using isotopes of different atomic weights to report antibody binding on single cells, rather than using fluorescence, which has been done for decades. Fluorochrome reporters can be used to identify about 15 targets, whereas CyTOF triples the content, and is more quantitative, in which 100,000 or more such events are compiled for each specimen.

Preliminary CyTOF analysis was conducted in collaboration with Dr. M. Spitzer (UCSF) using spleen samples collected five days post RT. Double-treated mice exhibited an increase in effector T cell populations, as shown by low CD27 T cells. Moreover, programmed cell death ligand 1 (PD-L1) positive macrophages, which are known to suppress cytotoxic T lymphocyte function against tumor cells, were increased in the mice treated with RT, but this population was diminished in mice treated with RT in combination with 1D11. The scaffold maps show that there was a decrease in PDL1+ population in the combined RT + 1D11 group, whereas there was an increase in PD1 compared to RT alone.

TS/1-BrA intracranial tumor model

We generated a second model of brain-adapted murine breast cancer cell line by inoculating TSA murine breast cancer cells into the brain of syngeneic Balb/C mice. Initially, we found that the erratic behavior of the TS/A-BrA cells did not show expected bioluminescence 1 week after induction. We next initiated an experiment to establish the baseline tumor growth with the second brain metastasis model, TS/A-BrA. Unexpectedly most mice injected with 1x104 and 1x105 cells did not exhibit bioluminescence and/or tumor growth, which is usually detected at 1 week post inoculation. However, the mice injected with 1x106 had a median survival of 27 days, with all mice dying of tumor burden. The lack of BLI suggests that either the cells have lost the reporter, that the cells were not viable and thus did not establish i.c. tumors, and/or other technical difficulties.

In vitro luciferase assays demonstrated that in fact TSA-BrA cells had lost significant expression of luciferase.

This made the approximate measurement of tumor sizes impossible. Therefore, we selected the transfected cells with Puromycin and confirmed successful selection of those cells using a Veritas luminometer. Compared to TS/A-BrA cells without luciferase we could detect a low signal in mixed population containing luciferase expressing and non-expressing TS/A-BrA cells. After selection (S) the total signal of those cells increased and was significantly different to non-selected and non-expressing cells and closer to the signal found in luciferase expressing SB28 cells (Figure 2A, B). After the successful selection we initiated an experiment to determine latency of tumor growth with the second brain metastasis model, TS/A-BrA. TS/A cells or TSA-BrA cells were injected into the brain of Balb/C 6-7 weeks old female mice with three groups (3-5 mice per group) $1x10^4$, $1x10^5$, and $1x10^6$ cells (Figure 2C, D). Tumor growth was monitored with BLI and Kaplan-Meier survival curves were generated. Murine brains from different groups were collected for FFPE.

Prepare manuscript on RT responses mediated by TGFβ

Combined results from three different brain cancer models demonstrating PET imaging and specific detection of TGFβ activity by radiolabeled fresolimumab and assessed response to single fraction and fractionated radiation +/- TGFβ inhibition.

“Positron Emission Tomography Imaging of Functional TGFβ Activity and Benefit of TGFβ Inhibition in Irradiated Murine Brain Tumors” was submitted to the *Journal of Radiation Oncology, Biology and Physics* (Attachment C).

i. other achievements. (Include a discussion of stated goals not met)

We successfully generated 2 syngeneic mouse models of brain adapted breast cancer cells. Both models show invasive growth and tumor nests comparable to the histology of human breast cancer brain metastases. Median survival for mice harboring intracranial 4T1-BrA have a similar latency and a median survival of 21 days but TS/A seems to be less aggressive than 4T1BrA, as are the orthotopic parental cells.

Completion of the objectives for the third year were delayed by three major events. The IVIS bioluminescence machine was down for 24 weeks due to technical problems that resulted in loss of experimental time. Dr. Borrero-Garcia, the postdoctoral fellow who led this project since August 2018, accepted a permanent research position at biotech company and left in August 2019. A new postdoctoral fellow, Dr. Oliver Reiners, joined the lab in June, 2019 and was trained by Dr. Borrero-Garcia and lab manager Mr. William Chou. He received a doctorate in Cancer Biology at the University of Duisburg-Essen, Germany, in 2019 for his thesis research in esophageal cancer and extracellular matrix changes induced by radiotherapy.

The award was a collaborative research award with Dr. Benjamin Franc (#BC160513P1), who did not request a NCE and completed his final report in 2019. In addition, he moved to Stanford University in December 2018.

c. **What opportunities for training and professional development has the project provided?**

The project has provided the postdoctoral fellow Dr. Borrero-Garcia the opportunity to share his work at the BOP retreat at UCSF. In addition, Dr. Borrero-Garcia trained Dr. Reiners, who is new to the field. He presented the research at the 2019 annual meeting of the Society for NeuroOncology.

d. **How were the results disseminated to communities of interest?**

This research has been presented at institutional and national meetings by the postdoctoral fellows.

Functional imaging platform to monitor progression and response to therapy in a pre-clinical model of BCBM. Alba Gonzalez-Junca, Denis Beckford-Vera, Niecholle Roco, Tony Hyunh, Dave Korenchan, Robert Flavell, Henry F VanBrocklin, Benjamin Franc, Mary Helen Barcellos-Hoff. POSTER UCSF Radiology Imaging Scientific Retreat 2018

TGFβ inhibition sensitizes breast cancer brain metastasis tumors to radiation treatment. Alba Gonzalez-Junca, Luis D. Borrero-Garcia, Denis Beckford Vera, Henry Van Brocklin, Benjamin Franc and Mary Helen Barcellos-Hoff. UCSF Breast Oncology Program Scientific Retreat 2019. POSTER

TGFβ activation by radiation opposes immune rejection of intracranial GL261 Alba Gonzalez-Junca, Denis Beckford Vera, Henry Van Brocklin, Benjamin Franc, Renate Parry and Mary Helen Barcellos-Hoff Society of Neuological Oncology (SNO) (November 2018)- *This presentation reported on the use of imaging agent developed by partnering PI Dr. Benjamin Franc (#BC160513P1) and his team.* POSTER

TGFβ inhibition sensitizes breast cancer brain metastasis tumors to radiation treatment. Alba Gonzalez-Junca, Luis D. Borrero-Garcia, Denis Beckford Vera, Henry Van Brocklin, Benjamin Franc and Mary Helen Barcellos-Hoff. UCSF Breast Oncology Retreat (BOP Retreat – February 2019) –PLATFORM PRESENTATION Luis D. Borrero-Garcia

Radiation primes glioblastoma for response to TGFβ neutralizing antibodies Oliver Reiners[1], Luis D. Borrero-Garcia[1], Alba Gonzales-Junca[1], Hideho Okada[2], Benjamin L. Franc[3], Henry Van Brocklin[3], Denis Vera-Beckford[3], and Mary Helen Barcellos-Hoff[1] – Society for NeuroOncology (SNO) (November 2019) - Oliver Reiners POSTER

-

e. **What do you plan to do during the next reporting period to accomplish the goals?**

N/A

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

   a. **What was the impact on the development of the principal discipline(s) of the project?**

   The development of the ^{89}Zr-DFO-fresolimumab is potentially relevant to human studies of response to therapy and can be readily moved to preliminary assessment in humans.

   The demonstrable benefit of TGFβ inhibition in the context of RT for breast cancer brain metastases is clinically important.

   Notably, long term survival and demonstrable anti-tumor immunity in mice bearing intracranial 4T1-BrA tumors demonstrates that blocking TGFβ is itself immunomodulatory in the context of RT, which precludes the need for addition of an IO agent as originally proposed.

   b. **What was the impact on other disciplines?**

Nuclear imaging of TGFβ activity has a wide range of potential applications in other disease states. Here, we showed that TGFβ activity can be detected non-invasively in breast brain metastasis. In parallel studies, we also documented a imaging of glioblastoma models, and a similar immune mediated rejection in one model.

In addition, based on the studies we conducted, there is potential to develop a theranostic approach in which a tumor imaged with 89Zr-Fresolimomab, could be irradiated to induce further TGFβ activation, and then treated by administering Fresolimomab radiolabeled with a therapeutic isotope to deliver radiation specifically to the tumor. This might conceivably be useful to decrease adverse effects like decline in cognitive function from external beam radiation therapy for breast cancer brain metastases.

c. **What was the impact on technology transfer?**

Nothing to Report.

d. **What was the impact on society beyond science and technology?**

Nothing to Report.

5. **CHANGES/PROBLEMS:**
   a. Technical problems
      i. Malfunction of IVIS needed for mouse bioluminescence imaging
   b. Personnel changes
      i. Dr. Luis Borrero-Garcia left for an industry position in July, 2019
         1. Dr. Borrero-Garcia assisted in training new postdoc
      ii. Dr. Oliver Reiners joined June, 2019
         1. Dr. Borrero-Garcia, who received a doctorate in Cancer Biology at the University of Puerto Rico in 2018 for his thesis research in breast cancer.
         2. Dr. Borrero-Garcia completed training in September and has learned the brain metastasis model, radiation protocol and laboratory procedures

6. **PRODUCTS:** (PLEASE ALSO SEE APPENDIX A)

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

f. What individuals have worked on the project? 2019-2020

Name	Role	Person Months Worked	Contribution	Funding Support
Mary Helen Barcellos-Hoff, Ph.D	PI	1.2	Designed expts and analyzed experimental data	

Luis Borrero-Garcia, Ph.D.	Postdoctoral Fellow	5	Execution of experiments and analysis of results	
Oliver Reiners, Ph.D.	Postdoctoral Fellow	3	Execution of experiments and analysis of results	
William Chou	Specialist	1.8	Assistance on in vivo experiments and technical support	
Trevor Jones	Assoc Specialist	6	Assistance on in vivo experiments and technical support	

g. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

CHANGES IN OTHER SUPPORT:

PI: BARCELLOS-HOFF, MARY HELEN

Role: P.I.

Completed:

Varian Medical Systems 02/02/2019 - 02/01/2020 1.2 calendar
CyTOF Analytics of Systemic Immune Responses to Radiation Therapy $131,755
Role: P.I.
We propose to use mass cytometry by time of flight (mass cytometry; CyTOF) for state of the art single cell analysis to document the systemic immune response to RT in cancer patients to enable single cell resolution of up to 40 parameters in millions of cells.

UCSF Resource Allocation Pilot 04/1/19-3/31/20 0 Calendar
Role: MPI (Contact PI) $50,000
Functional Analysis to Stratify HNSCC Patients for PARP Inhibition
Goal: To functionally evaluate TGFβ competency and response to radiation and PARPi in head and neck squamous cell carcinoma (HNSCC).

Initiated:

R01CA239235 04/01/2019-03/31/2024 1.80 calendar
NIH/NCI $303,482
Role: PI (Contact PI)
Definition of immune infiltrate phenotype and DNA damage response deficits across diverse murine mammary carcinomas
Goal: We proposed a comprehensive description of a heterogeneous murine mammary carcinoma model to bridge the gap in which the broad spectrum of human breast cancer is insufficiently represented within the limited diversity of models.

h. What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Dr. Franc completed collaborative project in 2018 and submitted the final report for GRANT NUMBER W81XWH-17-1-0033.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. DO NOT RENUMBER PAGES IN THE APPENDICES.

APPENDIX A: RELATED ABSTRACTS

Functional imaging platform to monitor progression and response to therapy in a pre-clinical model of BCBM. Alba Gonzalez-Junca, Denis Beckford-Vera, Niecholle Roco, Tony Hyunh, Dave Korenchan, Robert Flavell, Henry F VanBrocklin, Benjamin Franc, Mary Helen Barcellos-Hoff. POSTER UCSF Radiology Imaging Scientific Retreat 2018

INTRODUCTION: Radiotherapy (RT) is administered to brain metastases using a high single dose (radio-surgery) or with whole-brain radiation, according to the number of metastatic foci at the time of discovery. We have previously shown that radiation induces activation of TGFβ, a cytokine whose activity is tightly regulated in normal tissue but deregulated in the tumor microenvironment. Our prior work shows that TGFβ signaling compromises the efficacy of RT by both endorsing an effective DNA damage response, as well as through the suppression of immune response in different cancer models. Our ultimate goal is to develop a non-invasive functional imaging to monitor TGFβ activity to determine whether activity in irradiated brain metastases correlates with outcome. METHODS: For these studies, we luciferase expressing, brain-adapted 4T1 cells (BrA4T1), a model of triple-negative breast cancer, and stereotactic injection into syngeneic mouse brains. The Small Animal Radiation Research Platform (SARRP) at Mt Zion was used to perform image-guided dose delivery (1x14Gy, 3x8Gy, 5x6Gy)to intracranial tumors. A multi-modal functional imaging approach was used to monitor tumor growth and response to different radiotherapy protocols. RESULTS: The growth of tumors was measured using bioluminescence. RT treatment planning was based on computerized tomography (CT) using the SARRP. The characteristics of BrA4T1 tumors were imaged using diffusion weight imaging magnetic resonance imaging. We determined metabolic changes within the tumor using FDG-PET-CT and hyper-polarized 13-C pyruvate. Fresolimumab, a humanized monoclonal antibody that recognizes the active form of TGFβ, was labeled with ^{89}Zr to monitor the activity of this cytokine in situ. CONCLUSIONS: Pre-clinical use of multi-modal functional imaging platform will stimulate the development of agents to assess functional characteristics of cancers that may predict response to treatment with RT as well as combinations with immunotherapy.

TGFβ inhibition sensitizes breast cancer brain metastasis tumors to radiation treatment. Alba Gonzalez-Junca, Luis D. Borrero-Garcia, Denis Beckford Vera, Henry Van Brocklin, Benjamin Franc and Mary Helen Barcellos-Hoff. POSTER UCSF Breast Oncology Program Scientific Retreat 2019.

Radiation therapy (RT) is commonly used as a primary treatment for both glioblastoma and solid tumor metastatic brain cancers. Recent evidence point to the critical role of the brain tumor microenvironment (TME) as a barrier for successful therapy. We have shown that radiation induced transforming growth factor beta (TGFβ) activity in the irradiated TME has opposes the benefits of radiation by two mechanisms. First, TGFβ prevents radiation-induced cell killing by regulating the DNA-damage response to promote DNA repair, conferring radio- resistance to the tumor cells. Second, TGFβ is immunosuppressive through its important regulation of both the innate and adaptive immune system. Here we studied whether the inhibition of TGFβ in the context of RT could provide durable benefits, by reprograming the glioblastoma (GBM) TME.

To test this, we used a pre-clinical syngeneic orthotopic model, GL261,and state of the art image-guided RT delivered with the small animal radiation research platform to focally target the tumor regionwith either single high dose (10 Gy) or 5 fractions of 6 Gy.. The combination of RT with a pan-isoform TGFβ-neutralizing antibody (1D11) provided a significant survival benefit leading to more than 80% mice with complete tumor regression with either radiation schedule. This was associated with fewer $CD11b^{+}/Gr1^{+}$ myeloid-derived suppressive cells (MDSC), and which expressed less PD-L1, accompanied by increased T-cell tumor infiltration and activity. MDSC, which are prominent in GBM, can limit anti-tumor immunity and contribute to a permissive TME. To investigate the role of TGFβ in the regulation of this myeloid lineage, we established primary cultures from human monocytes chronically exposed to granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin 6 (IL-6), which promotes the generation of HLA-$DR^{low}/CD14^{+}/CD11b^{+}/CD33^{+}$ MDSC. The addition of TGFβ significantly increased the percentage of MDSC, at the expense of macrophages and antigen-presenting dendritic cells, and amplified their immune-suppressive capacity. When cells were cultured in the presence of a small molecule TGFβRI inhibitor (LY2109761), MDSC generation was significantly reduced and antigen-presentation was increased. Our results suggest that the blockade of radiation-induced TGFβ promotes a more favorable anti-tumor response by regulating the myeloid composition, thus improving anti-tumor immunity.

TGFβ activation by radiation opposes immune rejection of intracranial GL261 Alba Gonzalez-Junca, Denis Beckford Vera, Henry Van Brocklin, Benjamin Franc, Renate Parry and Mary Helen Barcellos-Hoff POSTER Society of Neuological Oncology (SNO) (November 2018)

Enabling anti-tumor immunity in brain is a challenge due to its unique microenvironment that includes tissue-specific extracellular matrix, immune cells and vasculature, and because many glioblastoma patients require rapid treatment, usually surgery followed by radiation therapy, that may oppose immunotherapy. Here we hypothesize that transforming growth factor β (TGFβ) is at the root of the profoundly immunosuppressive tumor microenvironment, and is perpetuated by standard of care, radiation therapy. We first localized TGFβ activation in situ using GC1008, a humanized pan-isoform TGFβ neutralizing antibody, radiolabeled with ^{89}Zr for PET-CT imaging. The antibody localized to a murine intracranial tumor compared to the injury-control brain injected with PBS. Paired comparisons of dual flank tumors in which one was irradiated (15 Gy) showed that radiation significantly increased ^{89}Zr-GC1008 uptake ($p<0.0002$). This was confirmed by immunostaining with an antibody that detects active TGFβ and nuclear pSMAD, indicative of signaling. Administration of TGFβ pan-isoform neutralizing antibody, 1D11 (25 mg/kg), to mice bearing irradiated intracranial tumors reduced immunostaining for active TGFβ and p-SMAD and blocked induction of a critical TGFβ target, tenascin-C, compared to treatment with isotype control antibody. These data support radiation-induction of TGFβ activation. Mice bearing i.c. GL261 (n=10-12) treated with 1D11 compared to isotype IgG had similar (17d vs 16d) median survival, which was doubled by tumor irradiation (10 Gy). Combined treatment with 1D11 and radiation led to durable control (Kaplan-Meier, $p>0.0009$), in which mice that showed complete regression by bioluminescence imaging for >45 days rejected a flank GL261 re-challenge. TGFβ inhibition with tumors treated with 5 daily 6 Gy fractions also eradicated most intracranial GL261. The undetectable brain bioluminescence and successful re-challenge suggest that TGFβ inhibition in the context of radiation can release the immunosuppressive microenvironment, elicit anti-tumor immunity and enable immune memory.

TGFβ inhibition sensitizes breast cancer brain metastasis tumors to radiation treatment. Alba Gonzalez-Junca, Luis D. Borrero-Garcia, Denis Beckford Vera, Henry Van Brocklin, Benjamin Franc and Mary Helen Barcellos-Hoff. PLATFORM PRESENTATION --Luis D. Borrero-Garcia UCSF Breast Oncology Retreat (BOP) Retreat – February 2019)

Breast cancer brain metastases (BCBM) are associated with poor prognosis and limited therapeutic options. Here we focus on approaches to improve response to radiation therapy (RT) by testing whether inhibition of transforming growth factor beta (TGFβ) improves response of brain adapted (BrA) intracranial (i.c.) murine breast cancer. The rationale is that previous studies showed that RT activates TGFβ, which affects the composition of the tumor microenvironment and enhances the ability of tumor cells to survive DNA damage. We first imaged TGFβ activity in situ using fresolimumab (GC1008), the humanized 1D11 TGFβ neutralizing antibody, radiolabeled with 89Zr for PET-CT imaging (89Zr-

fresolimumab). Mice harboring irradiated (15 Gy) 4T1-BrA flank tumors displayed significantly increased 89Zr uptake compared to contralateral un-irradiated tumors. We collected irradiated and non-irradiated tumors for dual active TGFβ and phospho-SMAD2 immunofluorescence staining. TGFβ intensity of each tumor correlated with the 89Zr uptake, which supports the specificity of 89Zr- fresolimumab to detect TGFβ activity in vivo. Next, we tested if TGFβ inhibition using monoclonal antibody 1D11 improves radiation response of 4T1-BrA i.c. tumor. Tumor growth was quantified by bioluminescence (BLI) using IVIS-Xenogen. Image-guided radiation therapy of a single dose of 10 Gy (sRT) or 5 daily fractions of 6 Gy (fRT) was delivered using a small animal radiation research platform. TGFβ neutralizing monoclonal antibody, 1D11, or irrelevant monoclonal, 13C4, were administered i.p. and mice were monitored by BLI and physical symptoms. Double treatment with 1D11 and RT led to increased median survival using sRT (41 vs 33 days) or fRT (49 vs 31 days)compared to RT alone. sRT eliminated 2/12 tumors, which double-treatment increased (5/13) whereas fRT eliminated 4/9 tumors, similar to that in double-treated mice (3/8). Mice that showed complete rejection of i.c. tumor were re-challenged with subcutaneous injections of the same cells. Re-challenge showed that only mice that were sRT double-treated rejected 4T1-BrA flank tumors. Thus, effective intracranial control of BCBM was achieved by RT and TGFβ inhibition, and effective intracranial tumor control can elicit immune memory indicated by subsequent rejection of tumor re-challenge following sRT indicates that regime matters.

Radiation primes glioblastoma for response to TGFβ neutralizing antibodies Oliver Reiners1, Luis D. Borrero-Garcia1, Alba Gonzales-Junca1, Hideho Okada2, Benjamin L. Franc3, Henry Van Brocklin3, Denis Vera-Beckford3, and Mary Helen Barcellos-Hoff1 – POSTER Society for NeuroOncology (SNO) (November 2019)

SB28 is a new luciferase expressing murine glioblastoma (GBM) cell line whose morphology, growth pattern, mutational burden immune infiltrate more closely resembles human GBM and is unresponsive to immunotherapy (OncoImmunology, 7:12, e1501137). Radiation therapy (RT) has the potential to improve response to combinations by multiple means: cell kill that may activate an immune response, effects on vascular may improve drug access, induction of DNA damage that can be augmented by targeted or chemotherapy, and modulation of the tumor microenvironment. High transforming growth factor β (TGFβ) activity in most GBM, which increases with radiation, likely opposes effective therapy by endorsing an effective DNA damage response and being immunosuppressive. Here evaluated the response of SB28 to radiation in combination with PD-L1 checkpoint blockade or neutralizing TGFβ. Establishment of intracranial tumors was monitored by bioluminescence. We determined that SB28 endogenously activate TGFβ as evaluated by biomarkers. We used radiolabeled antibodies and PET-CT to demonstrate access to intracranial tumors, indicative of a compromised blood-brain barrier. TGFβ neutralizing antibody, 1D11, had little effect on tumor growth compared to mice receiving IgG control antibody (median survival control: 19 days vs 1D11: 20.5 days). A single dose of radiation of 10 Gy delivered to the tumor site increased median survival to 21 day, which was increased to 31 days in mice treated with 1D11. In a second experiment in which the radiation dose was 6 Gy, 1D11 provided no benefit. In contrast, median survival of mice treated with PD-L1 neutralizing antibodies 3 days after being irradiated with 6 Gy demonstrated increased median survival (Control: 18 day; anti-PD-L1 20.5; RT: 25 days; RT + anti- PD-L1: 28 days). Double treatment resulted in 1/7 long term survival and loss of bioluminescence indicative of tumor elimination. Thus, radiation can prime SB28 GBM for response to biological agents.

APPENDIX B: FIGURES CITED IN TEXT

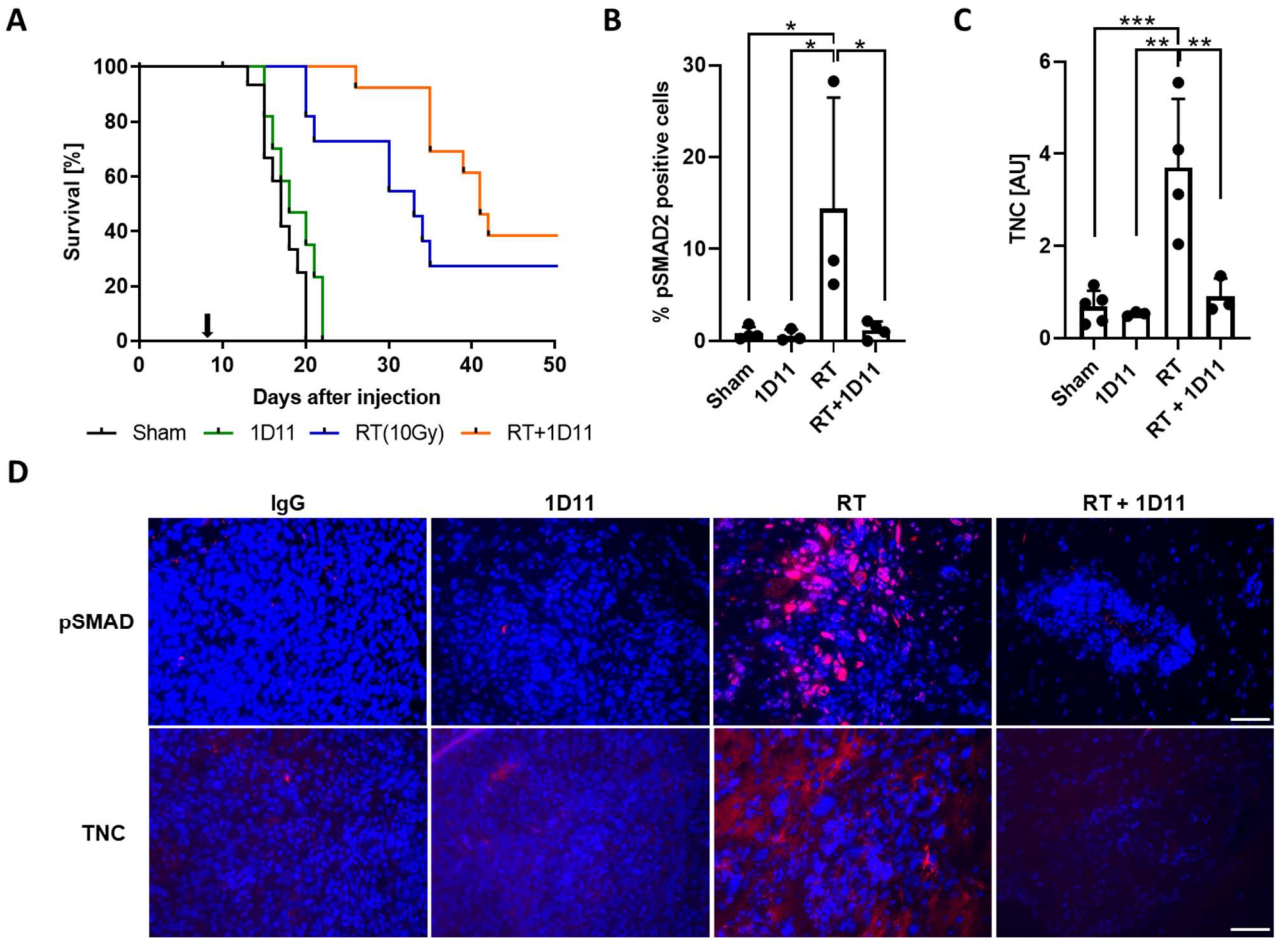


Figure 1: Molecular effects on 4t1-BrA tumors treated with TGFβ inhibition. (A) Survival of Balb/C mice were intracranially injected with 30*103 4T1-BrA cells and treated with TGFβ inhibition (1D11, n=11), 10 Gy (RT, day 9 as indicated by arrow, n=12), double treatment (RT+1D11, n=13) or IgG (Sham, n=15). **(B)** Percentage of pSMAD positive cells from tumors harvested 5 days after radiotherapy. **(C)** Quantification of TNC immunofluorescence in tumors harvested 5 days after radiotherapy**. (D)** Example pictures of immunofluorescence localization for pSMAD and TNC. The scale bar indicates 100 µm. Results in (B) and (C) were analyzed using One-way ANOVA with Turkey's multiple comparison test (*p≤0.05, **p≤0.01, *** p≤0.001), means for each analyzed animal are shown.

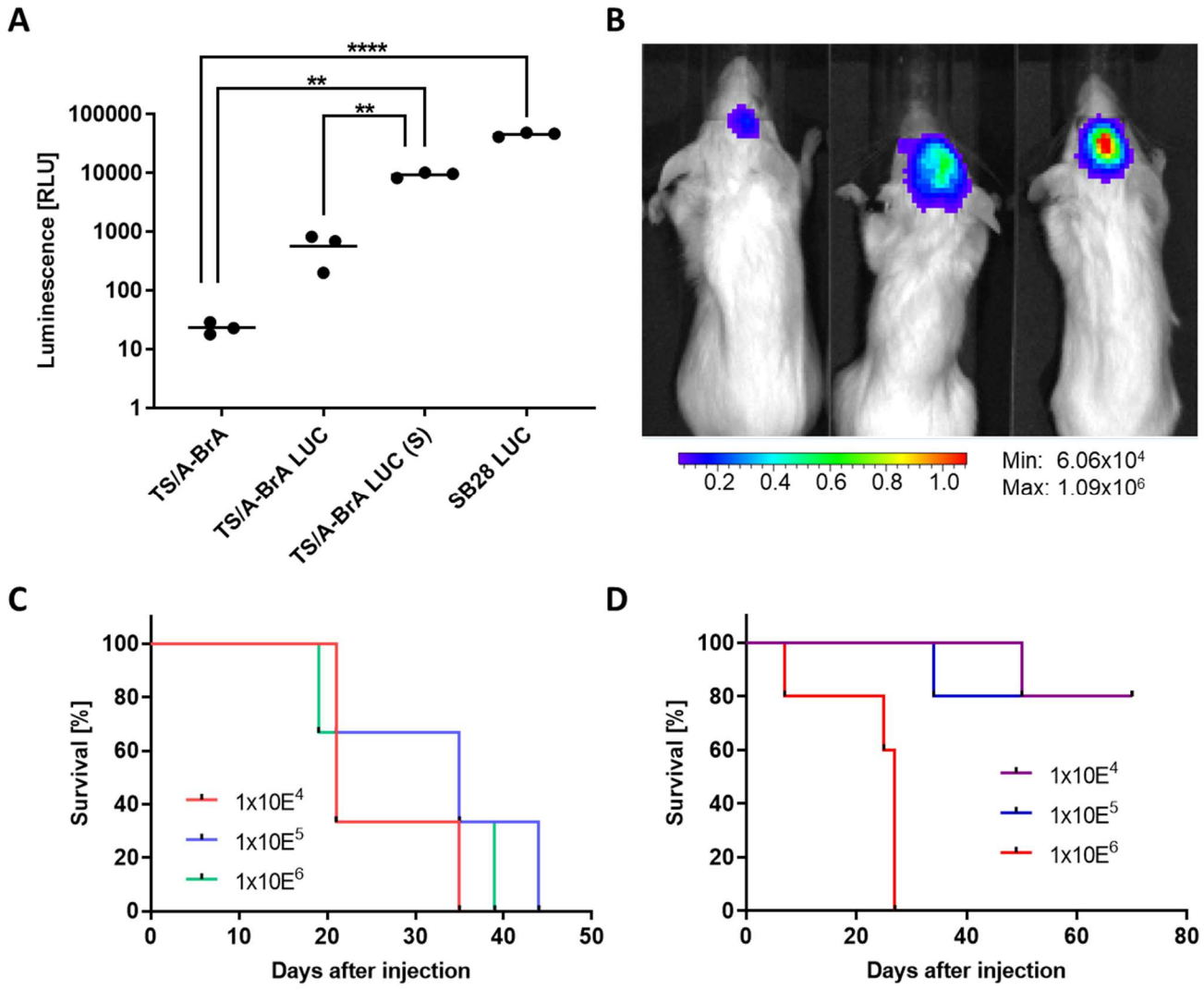


Figure 2: TS/A and TS/A-BrA tumor models. (A) TS/A-BrA cells were transfected with luciferase (LUC) and selected with Puromycin (S). Cells were stimulated with luciferin, luminescence measured and compared with transfected SB28 cells. Results were analyzed using One-way ANOVA with Turkey's multiple comparison test (**p≤0.01, **** p≤0.0001). **(B)** Representative bioluminescence pictures of mice intracranially injected with $1x10^6$ TS/A-BrA cells and imaged 25 days after injection. Minim and maximum of the intensity scale is given**.(C)** Survival of mice intra injected with $1x10^4$, $1x10^5$ or $1x10^6$ TS/A cells (n=3, per group). **(D)** Survival of mice intra injected with $1x10^4$, $1x10^5$ or $1x10^6$ TS/A-BrA cells (n=5, per group).

APPENDIX C: MANUSCRIPT SUBMITTED to *International Journal of Radiation Oncology, Biology and Physics*

International Journal of Radiation Oncology • Biology • Physics

Positron Emission Tomography Imaging of Functional TGFβ Activity and Benefit of TGFβ Inhibition in Irradiated Murine Brain Tumors

--Manuscript Draft--

Manuscript Number:	
Article Type:	Full Length Article
Section/Category:	Biology Contribution
Corresponding Author:	Mary Helen Barcellos-Hoff, Ph.D. University of California San Francisco San Francisco, CA UNITED STATES
First Author:	Alba Gonzalez-Junca, PhD
Order of Authors:	Alba Gonzalez-Junca, PhD
	Oliver Reiners, PhD
	Luis D. Borrero-Garcia, PhD
	Denis Beckford-Vera, PhD
	Ann A. Lazar, PhD
	William Chou
	Steve Braunstein, MD, PhD
	Henry VanBrocklin, PhD
	Benjamin L. Franc, MD
	Mary Helen Barcellos-Hoff, Ph.D.
Abstract:	Introduction: Assessing the promiscuous activation of transforming growth factor β (TGFβ) in cancer patients in response to radiation therapy is potentially targetable because it promotes cell survival by endorsing DNA damage repair. Methods & Materials: We used positron emission tomography (PET) to image 89Zr -fresolimumab, a humanized TGFβ neutralizing monoclonal antibody, in murine tumor models in which TGFβ activity has an important role: Glioblastoma (GL261 and SB28) and breast cancer-derived brain metastases (brain-adapted 4T1 breast cancer (4T1-BrA). TGFβ pathway activation was assayed by immunofluorescence. Mice bearing intracranial tumors were treated with neutralizing monoclonal antibody 1D11 and/or radiation (10 Gy) and survival was evaluated using Kaplan Meier. Results: 89Zr -fresolimumab PET-CT imaging of murine tumors detected TGFβ activation in multiple tumor models using engineered, physiological and radiation-induced activation, which was confirmed by immunostaining of biological markers of TGFβ pathway activation. GL261 glioblastoma tumors had more PET signal compared to similar sized SB28 glioblastoma tumors, whereas widespread PET signal of 4T1-BrA intracranial tumors is consistent with highly dispersed histological distribution. Despite evidence of TGFβ activation, survival of mice bearing intracranial tumors treated with 1D11 neutralizing antibody alone was similar from that of mice treated with control antibody. In contrast, 1D11 improved survival of mice when given in combination with local radiation. The extent of survival benefit of combination of RT+1D11 in brain tumor models associated with the levels of TGFβ activity detected by PET. Conclusions: This study demonstrates that 89Zr -fresolimumab PET imaging detects TGFβ activity and confirmed radiation-induced activation in tumors. Functional imaging indicated a range of TGFβ activity in murine tumors, and TGFβ blockade provided survival benefit in the context of radiation treatment, which supports the evidence that radiation-induced TGFβ activity opposes therapeutic response.

Cover Letter

UCSF Helen Diller Family Comprehensive Cancer Center

Mary Helen Barcellos-Hoff, Ph.D.
Vice Chair, Research
Professor
Dept. of Radiation Oncology

1600 Divisadero Street
Suite H1031
San Francisco, CA 94143-1708
Tel: 415/476-8091
Fax: 415/353-9883
EMAIL: mary.barcellos-hoff@ucsf.edu

May 26, 2020

Dear Dr. Zietman,

I am pleased to submit on the behalf of my co-authors our manuscript entitled "Positron Emission Tomography Imaging of Functional TGFβ Activity and Benefit of TGFβ Inhibition in Irradiated Murine Brain Tumors". All authors have read and approved the content of the manuscript, which is neither published nor submitted elsewhere.

Here we used three syngeneic intracranial murine brain tumor models, two that represent glioblastoma (GBM) and a model of brain metastases. GBM generally exhibit high expression levels of the cytokine transforming growth factor β (TGFβ). A vast literature on TGFβ biology in cancer indicates that it is key to many aspects of tumor biology, from growth control to vascularity, extracellular matrix composition and immune infiltrate yet the context in which TGFβ activity is clinically actionable has yet to be established.

To ascertain the endogenous TGFβ activity in brain tumors, we radiolabeled the humanized form of a TGFβ neutralizing antibody, fresolimomab, with ^{89}Zr for positron emission tomography (PET) imaging and computerized tomography (CT). We demonstrate that PET imaging specifically detects both engineered and physiological active TGFβ and localizes to intracranial tumors. To further assess its biological consequences, we show that TGFβ neutralizing antibody increases mouse survival when tumors are treated with radiation. Thus, systemic administration of TGFβ inhibitors augments radiation response of intracranial brain tumors.

Together these studies to provide a means to non-invasively assess TGFβ activity and support clinical development of TGFβ inhibitors in glioblastoma and brain metastases.

Very Best Regards,

Mary Helen Barcellos-Hoff

Mary Helen Barcellos-Hoff, Ph.D.
Professor and Vice Chair, Department of Radiation Oncology

NCI CCC
A Comprehensive Cancer Center Designated by the National Cancer Institute

Positron Emission Tomography Imaging of Functional TGFβ Activity and Benefit of TGFβ Inhibition in Irradiated Murine Brain Tumors

Alba Gonzalez-Junca, Ph.D[1*]; Oliver Reiners, Ph.D[1*]; Luis D. Borrero-Garcia, Ph.D[1]; Denis Beckford-Vera, Ph.D[2]; Ann A. Lazar, Ph.D[4,5,6]; William Chou, BA[1]; Steve Braunstein, MD/PhD[1], Henry VanBrocklin, Ph.D[2], Benjamin L. Franc, MD[2,3]; Mary Helen Barcellos-Hoff, Ph.D[1,4*]

[1] Department of Radiation Oncology, School of Medicine, University of California San Francisco, San Francisco, CA, USA

[2] Department of Radiology and Biomedical Imaging, University of California San Francisco, San Francisco, CA, USA

[3] Current address: Department of Radiology, Stanford University, School of Medicine, Palo Alto, CA, USA

[4] Helen Diller Family Comprehensive Cancer Center, School of Medicine, University of California San Francisco, San Francisco, CA, USA

[5] Division of Oral Epidemiology, School of Dentistry, University of California San Francisco, San Francisco, CA, USA

[6] Division of Biostatistics, School of Medicine, University of California San Francisco, San Francisco, CA, USA

* Contributed equally

Running title: Functional Imaging of TGFβ Activity

Keywords: Glioblastoma, brain metastases, TGFβ, ionizing radiation, positron emission tomography, functional imaging

Financial Support: This research was supported by Varian Medical Systems research grant, National Institutes of Health R01NS109911 and U.S. Department of Defense Congressionally Directed Medical Research Award BC160513 to MHBH, and U.S. Department of Defense Congressionally Directed Medical Research Award BC160513P1 to BLF at UCSF.

Corresponding Author:

Mary Helen Barcellos-Hoff, Ph.D.
Department of Radiation Oncology
Helen Diller Family Comprehensive Cancer Center
School of Medicine
University of California, San Francisco
2340 Sutter St., San Francisco, CA 94143

(415) 476-8091

MaryHelen.Barcellos-Hoff@ucsf.edu

Statistical analysis:

Ann A. Lazar, Ph.D.

Division of Oral Epidemiology

School of Medicine

University of California. San Francisco

Ann.Lazar@ucsf.edu

Conflicts of Interest: The authors declare there are no conflicts of interest regarding these data.

Data sharing statement: Research data are stored in an institutional repository and will be shared upon request to the corresponding author.

Author Contribution or Task: AGJ, OR, LBG, and WC conducted experimental studies; AGJ and OR analyzed data and prepared the data for publication; AL analyzed data; DBV and HVB conducted radiochemistry and image analysis; SB, BF and MHBH designed study; AGJ, OR, BF and MHBH wrote manuscript; all authors revised and approved final manuscript.

Word count: 5292

References: 30

Figures: 4

Tables: 0

Supplementary Figures: 3

Supplementary Tables: 0

Acknowledgements: The authors wish to thank Mr. Trevor Jones and Ms. Hui Zhang for expert technical assistance. These studies were supported by awards to MHBH from Varian Medical Systems, Inc., NIH R01NS109911, and DOD-BCRP W81XWH-17-1-0032, and to BF from DOD-BCRP W81XWH-17-1-0033.

Positron Emission Tomography Imaging of Functional TGFβ Activity and Benefit of TGFβ Inhibition in Irradiated Murine Brain Tumors

Running title: Functional Imaging of TGFβ Activity

Keywords: Glioblastoma, brain metastases, TGFβ, ionizing radiation, positron emission tomography, functional imaging

Financial Support: XXX

Conflicts of Interest: The authors declare there are no conflicts of interest regarding these data.

Data Repository: Research data are stored in an institutional repository and will be shared upon request to the corresponding author.

Author Contribution or Task: XXX

Word count: 5292

References: 30

Figures: 4

Tables: 0

Supplementary Figures: 3

Supplementary Tables: 0

Acknowledgements:

ABSTRACT

Introduction: Assessing the promiscuous activation of transforming growth factor β (TGFβ) in cancer patients in response to radiation therapy is potentially targetable because it promotes cell survival by endorsing DNA damage repair.

Methods & Materials: We used positron emission tomography (PET) to image ^{89}Zr -fresolimumab, a humanized TGFβ neutralizing monoclonal antibody, in murine tumor models in which TGFβ activity has an important role: Glioblastoma (GL261 and SB28) and breast cancer-derived brain metastases (brain-adapted 4T1 breast cancer (4T1-BrA). TGFβ pathway activation was assayed by immunofluorescence. Mice bearing intracranial tumors were treated with neutralizing monoclonal antibody 1D11 and/or radiation (10 Gy) and survival was evaluated using Kaplan Meier.

Results: ^{89}Zr -fresolimumab PET-CT imaging of murine tumors detected TGFβ activation in multiple tumor models using engineered, physiological and radiation-induced activation, which was confirmed by immunostaining of biological markers of TGFβ pathway activation. GL261 glioblastoma tumors had more PET signal compared to similar sized SB28 glioblastoma tumors, whereas widespread PET signal of 4T1-BrA intracranial tumors is consistent with highly dispersed histological distribution. Despite evidence of TGFβ activation, survival of mice bearing intracranial tumors treated with 1D11 neutralizing antibody alone was similar from that of mice treated with control antibody. In contrast, 1D11 improved survival of mice when given in combination with local radiation. The extent of survival benefit of combination of RT+1D11 in brain tumor models associated with the levels of TGFβ activity detected by PET.

Conclusions: This study demonstrates that ^{89}Zr -fresolimumab PET imaging detects TGFβ activity and confirmed radiation-induced activation in tumors. Functional imaging indicated a range of TGFβ activity in murine tumors, and TGFβ blockade provided survival benefit in the context of radiation treatment, which supports the evidence that radiation-induced TGFβ activity opposes therapeutic response.

INTRODUCTION

Transforming growth factor β (TGFβ), with its 3 isoforms TGFβ 1, 2 and 3, is a prominent regulator of tumor microenvironment by influencing extracellular matrix (ECM) remodeling, angiogenesis and immunosuppression, and also regulates tumor cell motility and invasion (1). Elevated TGFβ levels in plasma correlate with poor outcome for breast, lung and hepatocellular carcinoma patients (reviewed in (2)). Of particular clinical significance is the role of TGFβ in glioblastoma (GBM) because increased TGFβ signaling is associated with poor prognosis (3). *TGFB1*, its receptors and its direct target, tenascin C mRNA levels are increased in recurrent GBM (4). Consistent with pronounced TGFβ signaling in GBM treated with standard of care radiation therapy, 11% of recurrent GBM harbor a *de novo* mutation that further increases TGFβ activity (5), suggestive of a strong selective pressure from TGFβ during tumor recurrence. Pharmaceutical inhibition of TGFβ signaling in murine and human cell lines prior to radiation increases radiation sensitivity, decreases cancer stem cell resistance, and reduces tumor growth in breast, brain and lung tumor murine models (6-8). Huber and colleagues showed that blocking TGFβ signaling increases response to radiation and temozolomide in preclinical brain tumors (9,10). Thus, the clinical motivation for targeting TGFβ inhibition in cancer is predicated on its multiple actions that promote malignant phenotypes, remodel the TME, and compromise therapeutic efficacy, which may contributes to failure of tumor control (reviewed in (11)).

TGFβ activation is controlled by its production as a latent complex which is bound to the latency associated peptide (LAP) (12). The latent TGFβ complex is secreted, often in association with binding proteins that sequester the complex in the ECM. An extracellular or pericellular process referred to as activation can be triggered by changes in pH, reactive oxygen species, mechanical stress, or enzymatic cleavage, and is required for functional TGFβ to bind to ubiquitous receptors. While TGFβ activation is essential to initiate signaling and subsequent pathway stimulation, it is difficult to monitor when and where functional TGFβ contributes to cancer biology and hence, obscures its potential as a therapeutic target.

Here we radiolabeled fresolimumab, a humanized form of TGFβ neutralizing monoclonal 1D11, with ^{89}Zr to evaluate positron emission tomography (PET) as a non-invasive means to assess TGFβ activity in murine tumors. We used genetically engineered tumor models to demonstrate that ^{89}Zr -fresolimumab PET specifically detects active TGFβ, which was validated by immunofluorescence staining of TGFβ targets, confirming TGFβ pathway activation. Despite functional imaging of TGFβ activity in three intracranial tumor models, neutralizing TGFβ antibody treatment had no effect on tumor control unless mice had received radiation therapy yet significantly improved survival in combination with radiotherapy in all 3 tumor models. Together these studies demonstrate the feasibility of functional monitoring of TGFβ activity and potential benefit of its targeting in combination with radiotherapy.

MATERIAL & METHODS

Cell lines

Cell lines were authenticated and routinely assayed to confirm that they were free of mycoplasma. C57Bl/6 murine GBM cell line GL261-luciferase (GL261) cell line was provided by Dr. Nalin Gupta and cultured in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum (FBS). C57Bl/6 murine GBM cell line SB28-luciferase cell line (SB28), generated using sleeping beauty transposon system (13), was obtained from Dr. Hideho Okada and cultured with Roswell Park Memorial Institute medium (RPMI 1640), supplemented with 10% FBS (HyClone), GlutaMax (Thermo Fisher) and supplemented with 1 mM Sodium Pyruvate and 1% MEM non-essential amino acids (MEM NEAA 100X, Thermo Fisher). Lewis lung carcinoma (LLC) cells and transfected LLC cells expressing the integrin αvβ8 (β8 LLC) were obtained from Stephen L. Nishimura and cultured in DMEM + 10% FBS and 1 mg/mL geneticin. Human 293 renal sarcoma cell line clone C19 transfected with TGFβ expression plasmid in which site-directed mutagenesis of two cysteine codons to serine codons results in constitutively active TGFβ and clone B9 overexpresses wild type latent TGFβ1 (14) and were cultured with DMEM and 5% FBS. BALB/c murine breast cancer cell line 4T1-luciferase-mCherry was obtained from Dr. David Lyden. To create a model of brain metastases, 4T1-luc-mCherry cells were inoculated intracranially into BALB/c female mice and

mCherry^{+} tumor cells were sorted from resulting tumors using Sony SH800 cell sorter. The brain-adapted cells were pooled and expanded to generate the 4T1-BrA cell line, which was authenticated and confirmed mycoplasma free. Cells were cultured in DMEM containing 10% FBS and expanded *in vitro* for 2 passages prior to viable freezing in aliquots for subsequent animal inoculation.

Tumor models and treatment

For intracranial tumors, a stereotaxic device was used to inject cells 1 mm anterior, 1.8 mm lateral and 3.5 mm beneath the skull surface of the bregma in the right brain hemisphere into the corpus striatum of 6–7 week old syngeneic mice anesthetized with ketamine/xylazine (90 mg/kg) and buprenorphine (0.5 mg/kg) whilst maintaining body temperature. SB28 ($5x10^4$) or GL261 (10^5) cells were injected into C57BL/6J mice and 4T1-BrA cells ($3x10^3$) were injected into BALB/cJ female mice. Intracranial tumor growth was measured by bioluminescence (BLI) imaged (Xenogen IVIS 100 Imaging System) every 5 days following intraperitoneal (i.p.) injection of 200 µl (15 mg/mL) luciferin under anesthesia using 2% isoflurane. Tumor burden was calculated based on bioluminescence flux (photons/sec) and was used to randomize mice to treatment groups: Sham IgG, Sham 1D11, radiation treatment (RT)+IgG, or RT+ID11. TGFβ neutralizing antibody 1D11 (25 mg/kg, i.p., 3x week, up to 4 weeks, Bioxcell, BP0057) or IgG control antibody (25 mg/kg, i.p., 3x week, up to 4 weeks, Bioxcell, BP0083) was administered 24 h before RT using individualized plans (Muriplan, XStrahl) based on arc beam computerized tomography (CT) using small animal radiation research platform (SARRP, XStrahl). Mice bearing intracranial tumors were monitored for neurological symptoms or weight loss (≥ 15% body weight and sacrificed in accord with the Institutional Animal Care and Use Committee guidelines at the institution.

For subcutaneous tumors, 4T1-BrA (10^5) were injected into the flanks of BALB/cJ female mice, human renal cell sarcoma B9 or C19 ($5x10^6$) cells were injected into flanks of BALB/c Foxn1/nu mice and LLC and β8 LLC ($2x10^6$) cells were injected into the flanks of C57BL/6J mice. In some studies, subcutaneous tumors were irradiated with 15 Gy using the SARRP under anesthesia using 2% isoflurane.

Preparation and validation of the ^{89}Zr-fresolimumab imaging probe

For radiolabeling, 20 mg of fresolimumab in 1 mL of 0.1 M sodium carbonate solution (pH 9) was mixed with 3-fold molar excess of DFO-p-SCN (Macrocyclics, Inc., Plano TX) previously dissolved in 20 µL of anhydrous DMSO. The reaction mixture was incubated for 45 min at 37 °C with gentle stirring. The DFO-fresolimumab conjugate was purified by size-exclusion chromatography using a PD-10 column. Fractions containing DFO-fresolimumab were pooled and concentrated via ultracentrifugation using an Amicon filter device (30 MWCO). DFO-fresolimumab conjugate was characterized by size-exclusion High Performance Liquid Chromatography (SE-HPLC) (Supplementary **Fig.** 1). Protein concentration was determined by Bradford spectrophotometric assay. The average number of chelates linked to an antibody molecule was determined by isotopic dilution assay, as previously described (15), using ^{89}Zr spiked with a known amount of $ZrCl_4$.

For radiolabeling, 10 µL (130 MBq/ 3.5 mCi) of ^{89}Zr-Oxolate (3D Imaging LLC, Little Rock AR) and 10 µL of 1M Na_2CO_3 were added to 20 µL of water, and allowed to stand for 3 min. Subsequently 200 µL of 1M ammonium acetate and 100 µg of DFO-fresolimumab (16 mg/mL in PBS), were added to the vial containing neutralized ^{89}Zr and the reaction mixture was incubated for 1h at room temperature. Radiolabeled conjugate was purified from unbound radiometal by size exclusion using a PD-10 desalting column with 0.9% NaCl as the eluent. Fractions (1 mL) were collected, and radioactivity was measured. Isolated radiolabeling yield was calculated from the ratio of activity in the fractions containing ^{89}Zr-DFO-fresolimumab (^{89}Zr-fresolimumab) to the initial activity used for the radiolabeling. Chemical and radiochemical purity was determined by SE-HPLC and iTLC.

To assay binding competition, a 96 well microtiter plate was coated with recombinant human TGFβ 3 (100 µL, 4 µg/mL in PBS) overnight at 4 °C. The following day, the solution in each well was discarded and wells were washed three times with 0.5% Tween in PBS. A solution of 1% nonfat milk (150 µL) was added to each well and the microtiter plate was blocked at room temperature for 1 h. Afterwards, the solution in each well was discarded. Solutions containing equal amount of ^{89}Zr-fresolimumab (100 µL, 20 MBq) and increasing amount of unmodified ("cold") fresolimumab (0.08 to 92 nM) were added to the

microtiter plate and incubated for 1 h at room temperature. The solution in each well was discarded and wells were washed three times with 0.5% Tween in PBS. Sodium hydroxide (NaOH) solution (1 M, 200 µL) was added to each well and incubated for 20 min. The NaOH solution in each well was pipetted into test tubes and the radioactivity was measured using a HIDEX automated gamma counter (Turku, Finland). Concentration values that caused 50% of inhibition (IC50) of ^{89}Zr-fresolimumab to recombinant human TGFβ 3 were estimated from the non-linear fitted curves using GraphPad Prism (GraphPad Software, Inc., San Diego, CA) (Supplementary **Fig.** 1).

***In vivo* PET/CT imaging**

Mice were injected i.p. with a dose of 140 – 150 µCi ^{89}Zr-fresolimumab or 200 µCi ^{18}F-flurodeoxyglucose (FDG). Following injection, mice were anesthetized with inhaled 2% isoflurane and imaged under isoflurane and body temperature maintenance using a pre-clinical PET/CT scanner (GNEXT PET/CT, SOFIE Inc., California, USA). In some experiments, mice harboring subcutaneous 4T1-BrA tumors were injected intravenously with ^{89}Zr -fresolimumab. Half of the mice were injected with unlabeled (cold) fresolimumab 24 h prior to radioactive-fresolimumab injection. 96 h after injection, mice were euthanized, and tumors were collected. Radioactive counts in harvested tissues was measured by the HIDEX automated gamma counter and the percentage of injected dose per gram (%ID/g) calculated (16).

Mice with subcutaneous tumors were imaged 96 h after ^{89}Zr-fresolimumab injection and mice with intracranial tumors were imaged after 24 h to 120 h after ^{89}Zr-fresolimumab injection. Mice with ^{18}F-FDG were imaged 30 min after injection. PET scans were 10 minutes each. Images were reconstructed using 3D-OSEM with an energy window of 350-650 keV. CT scans were 1 minute each with 720 projections acquired over 360 degrees. CT images were reconstructed using a modified Feldkamp method. Analysis of PET/CT was performed using AMIDE (Amide's a Medical Imaging Data Examiner). Volumes of interest were drawn and the uptake (Bq/mL) calculated. Image data were corrected for attenuation, decay and volume, and displayed as percentage of injected dose per mL (% ID/mL). Representative images are shown using the same display settings in AMIDE.

Immunostaining

Tumor bearing brains were collected at 5 days post radiation for TGFβ marker analysis. Harvested tissues were fixed in a 10% buffered formalin phosphate solution. After 24 h, brains were transferred into a 70% ethanol solution and embedded in paraffin (FFPE). Acidic antigen retrieval (Antigen Unmasking Solution, Citric Acid Based, Vector, H-3300) of 3-µm sections was followed by blocking with 0.5% casein (Spectrum, CA205). Subsequently the samples were incubated with primary antibody against TGFβ (Bioxcell, BP0057), pSMAD2 (Cell Signaling #3108) or TNC (Abcam, #AB108930) at 4 °C overnight. The next day slides were incubated for 1 h with fluorochrome-labeled secondary antibodies against rabbit (Alexa-fluor595, Life Technologies, #A21207 & Alexa-fluor488, Life Technologies, #A21206) or mouse (Alexa-fluor488, Life Technologies, #A21202) and stained with 4,6-diamidino-2-phenylindol (DAPI, Thermo Fisher, #D1306) for nuclear content. Sections were washed and mounted with Vectashield (Vector Labs, H1000) and stored at -25 °C in the dark. Slides were imaged with a Zeiss Axiovert epifluorescence microscope and representative pictures and fields of interest were further analyzed with in house developed macros and ImageJ (17). Fluorescence intensity of different markers relative to field or amount of positive stained nuclei were calculated and reported as arbitrary units (AU).

Statistical Method

In each experiment, mice were randomized to one of the four treatments. Descriptive statistics were used to summarize the data, for continuous variables, means and standard deviations (SD), and for categorical variables, frequencies and percent. N represents the number of animals per group. For continuous variables with matched groups, paired t-test was used, and for independent groups, analysis of variance was used with adjustment of alpha level for multiple testing using Bonferroni correction for post-hoc pairwise t-tests. Overall survival was calculated using Kaplan–Meier. Log-rank test was used to compare groups. Two-sided p-values less than 0.05 were considered statistically significant. SAS v. 9.4 was used to perform the analyses.

RESULTS

^{89}Zr-fresolimumab specifically detects active TGFβ in engineered tumor models

Active TGFβ is the ligand for ubiquitous receptors and promotes tumor progression, immune evasion and migration. First, we labeled fresolimumab, the humanized version of murine pan-isoform TGFβ neutralizing antibody 1D11, with ^{89}Zr using DFO conjugation (Supplementary **Fig.** 1A). ^{89}Zr-fresolimumab yield, radiochemical purity and biological activity were determined after conjugation and radiolabeling (Supplementary **Fig.** 1 B - D).

To determine if PET imaging of ^{89}Zr-fresolimumab can effectively discriminate between active and latent TGFβ, we established tumors from human tumor cell line, B9, which is stably transfected with a construct to express wild-type latent TGFβ1, and isogenic C19 cell line, which expressed constitutively active TGFβ1 (14,18). Mice bearing contralateral flank tumors of each cell line were imaged by PET and computerized tomography (CT). PET imaging of ^{89}Zr -fresolimumab showed increased signal in the C19 tumors expressing the constitutively active form of TGFβ1, compared to the contralateral B9 tumors, which had minimal PET signal (**Fig.** 1A). Analysis of the percentage of injected dose (ID) present in tumors 96 h after injection confirmed a significantly greater uptake of ^{89}Zr -fresolimumab in C19 tumors (**Fig.** 1B). Activation of TGFβ results in signaling via receptor-mediated phosphorylation of SMAD2, which ultimately mediates expression of TGFβ gene targets (19). To validate differential TGFβ activation, we used tumor sections for immunofluorescence (IF) staining of active TGFβ and phosphorylated SMAD2 (pSMAD2). Consistent with constitutive TGFβ activation, immunodetection of TGFβ was 5-fold greater in C19 compared to B9 and the percentage of pSMAD2 positive cells increased from 2% to 15% ($p<0.05$) (**Fig.** 1C).

Next, we assessed the discrimination between physiological activation of TGFβ in Lewis lung cell carcinoma (LLC) flank tumors. A physiological mechanism of TGFβ activation at the cell surface involves integrins that bind RGD sequences in LAP from TGFβ 1 and 3 (20). Parental LLC tumors, which do not express the αvβ8 integrin, were compared to β8 LLC tumors that have been stably transfected to express

αvβ8 (21). Mice bearing contralateral LLC tumors and β8 LLC tumors were imaged using PET ^{89}Zr -fresolimumab (**Fig.** 1D). β8 LLC tumors exhibit a higher dose relative to the minimal uptake of the contralateral parental LLC tumor (**Fig.** 1E). Immunostaining of tumor sections confirmed significantly greater active TGFβ ($p<0.05$) as well as an increased frequency of pSMAD2 cells from 2% to 14% ($p<0.05$) (**Fig.** 1F).

^{89}Zr-fresolimumab detects radiation-induced TGFβ activity

Radiation elicits rapid TGFβ activation via a redox mechanism (22), which promotes sustained TGFβ activity (23). We next sought to determine if ^{89}Zr -fresolimumab PET imaging could detect increased TGFβ activity in irradiated tumors. Mice were injected subcutaneously with 4T1-BrA cells in both flanks to establish bilateral tumors. The right tumor was irradiated with 15 Gy using a small animal radiation research platform and mice were injected with ^{89}Zr -fresolimumab immediately thereafter. Mice were imaged with PET/CT 96 h later. Irradiated tumors displayed significantly increased signal relative to non-irradiated contralateral tumors (**Fig.** 2A). Radioactivity was subsequently quantified *ex vivo* by autoradiography or gamma counting. Autoradiograms of tumor sections confirmed that the irradiated tumors were more radioactive than the contralateral non-irradiated tumors (**Fig.** 2B). Consistent with autoradiography, irradiated tumors contained more radioactive isotope compared to contralateral tumors (**Fig.** 2C). The specificity of ^{89}Zr -fresolimumab was confirmed by injecting 4T1-BrA tumor bearing mice with cold fresolimumab prior to radioactive-fresolimumab. The activity per gram of tissue was lower in mice that were pre-treated with cold fresolimumab (blocked) compared to non-blocked (**Fig.** 2D).

Immunostaining of tumor sections indicated increased active TGFβ and pSMAD2 positive cells in irradiated compared to non-irradiated tumors (**Fig.** 2E). The frequency of pSMAD2 positive cells per field significantly increased ($p<0.02$) from 15% to 22% after radiation (**Fig.** 2F). Tenascin C (TNC) is an ECM protein whose expression is induced by TGFβ (24). Irradiated tumors showed significantly increased TNC (**Fig.** 2G), which was quantified by image analysis (**Fig.** 2H). These markers confirm the expected biological consequences of RT-induced activation of TGFβ and its downstream signaling. These data

support the feasibility of functional imaging of active TGFβ by ^{89}Zr-fresolimumab PET in multiple contexts.

Imaging TGFβ activity in murine intracranial tumors

Brain tumors are highly aggressive and retrospective analysis of TGFβ activity suggests that it might be important in patient response but is difficult to ascertain prospectively. Due to the intracranial location and the rapid course of the disease, finding non-invasive means to detect TGFβ activity could be of great utility. Here, we used two orthotopic glioblastoma models (SB28 and GL261) and an intracranial breast cancer brain metastasis model (4T1-BrA). Mice were imaged with ^{18}F-FDG PET/CT and bioluminescence imaging (BLI) of a luciferase reporter was used to confirm intracranial SB28 (**Fig.** 3A), GL261 (**Fig.** 3B) and 4T1-BrA (**Fig.** 3C) tumors. Mice receiving sham surgery were injected intracranially with PBS and were used as control (Supplemental **Fig.** 2A). All mice were subsequently imaged with^{89}Zr-fresolimumab PET, which detected TGFβ activity in the tumor-bearing brains but not in control mice. Signal quantification in SB28 tumor 96 h after injection demonstrated significant increase in tumor-bearing brains compared to sham surgery (**Fig.** 3D). We noted that, despite comparable BLI and survival, GL261 tumors exhibited twice as much signal as SB28 tumors (**Fig.** 3E, Supplemental **Fig.** 2B).

Imaging invasive brain metastases can be challenging due to the simultaneous presence of multiple extra-cranial metastasis. To model this clinical presentation, we injected mice with intracranial and subcutaneous tumors and imaged with ^{89}Zr-fresolimumab PET/CT (**Fig.** 3F). Signal was detected in both brain and flank tumors indicating that functional monitoring of multiple sites is possible. These data show that functional imaging of active TGFβ in intracranial tumors with different activation levels as well as extracranial tumor locations is feasible.

1D11 neutralizing antibody inhibits TGFβ signaling in intracranial tumors

We next investigated the efficacy of TGFβ inhibition by murine monoclonal TGFβ neutralizing antibody (1D11) in the established intracranial tumor models. Animals bearing intracranial tumors were

distributed into treatment groups based on BLI 7 days after implantation. 1D11 or a control antibody was injected i.p. one day before RT, those assigned to the RT groups received focal brain radiation (10 Gy). To evaluate the 1D11 treatment efficacy, tumors were harvested 5 days later for immunostaining of pSMAD2 and the TGFβ downstream target TNC (Supplemental **Fig.** 2C). The frequency of pSMAD2 positive cells was low in all tumor models in control (sham-irradiated, treated with control antibody) mice and was unaffected by 1D11 treatment (**Fig.** 4 A-C). In contrast, the frequency of pSMAD2 positive cells significantly increased in all irradiated tumors, which was prevented by concomitant treatment with 1D11.

To confirm biological activity, we used IF to evaluate TNC deposition (Supplemental **Fig.** 2D). Consistent with TGFβ signaling, TNC was increased in irradiated tumors and treatment with 1D11 effectively decreased deposition of TNC after RT in SB28 (**Fig.** 4D), GL261 (**Fig.** 4E), and 4T1-BrA (**Fig.** 4F) tumors. Although the relative change in pSMAD2 was similar among the models, the amount of TNC deposition in the irradiated TME was much greater in GL261 compared to either SB28 or 4T1-BrA. Taken together, these results show that radiation induced TGFβ signaling, which can be effectively inhibited by concomitant treatment with the neutralizing antibody 1D11, as evidenced by changes in pathway activity and ECM remodeling.

TGFβ blockade in combination with RT increases survival in brain cancer models

Next, we investigated the therapeutic response to TGFβ inhibition in the 3 models. GL261 is a widely used mouse GBM cell line that originated from chemical carcinogen-induced tumors. In comparison to GL261, SB28 has a lower mutational load, lower immune infiltration, and higher invasion, which more closely recapitulate the features of human GBM (13). We established the 4T1-BrA cell line by injecting 4T1 triple negative breast cancer cells into the brain of syngeneic mice. After tumors were formed, we isolated the brain-adapted tumor cells using fluorescence sorting and generated and characterized this new 4T1 brain adapted cell line for further experiments. Histological analysis of tumors at termination demonstrated the highly invasive behaviors of both SB28 and 4T1-BrA tumors compared to localized

GL261 tumor (Supplemental **Fig.** 2E). Some mice with SB28 tumors were found to have traversed the corpus callosum to the contralateral hemisphere at termination.

Despite PET functional imaging demonstrating TGFβ activity in untreated tumors, administration of 1D11 had no effect on tumor growth as evidenced by BLI (Supplemental **Fig.** 3) nor significantly affected survival, with the exception of one long-term survival of a GL261 tumor bearing mouse (**Fig.** 4 G-I). For SB28 tumors, RT provided modest increase in median survival (21 days) compared to sham (19 days), whereas RT+1D11 combination treatment significantly increased median survival to 31 days (**Fig.** 4G). In mice bearing intracranial GL261 tumors, RT significantly improved survival from 17 to 30 days (**Fig.** 4H). The combination of RT and 1D11 treatment significantly prolonged survival (median survival not reached), in which 80% of mice exhibited long term tumor-free survival (Supplemental **Fig.** 3B). In 4T1-BrA tumors, RT increased median survival from 17 to 33 days and resulted in 3 out of 12 (25%) long term survivors (**Fig.** 4I). Treatment with 1D11+RT significantly increased survival to 41 days with 4 out of 13 (30%) long term tumor-free surviving mice.

DISCUSSION

Abundant expression of TGFβ in GBM is associated with poor prognosis and malignant features (3). ^{89}Zr-Fresolimumab PET imaging was previously used to localize recurrent high-grade gliomas (25,26). Here we determined that ^{89}Zr-fresolimumab functional imaging can specifically distinguish active TGFβ using tumor models in which TGFβ activation had been engineered, physiologically activated, or radiation-induced. Consistent with prior studies, ^{89}Zr-fresolimumab PET imaging demonstrated increased TGFβ activation after irradiation, which was confirmed by autoradiograms and molecular markers of pathway activation in tumors. These findings were confirmed in three intracranial tumor models, where we determined that despite the distinct characteristics of the models employed, each showed significant ^{89}Zr-fresolimumab signal in the tumor, including both intra-cranial and extra-cranial tumors. TGFβ functional imaging was supported by analysis of pathway activity markers tumors. TGFβ blockade using a pan-

isoform neutralizing antibody 1D11 promoted tumor control and survival advantage when combined with radiation. Mice bearing intracranial brain tumors that were administered 1D11 prior to single dose (10 Gy) irradiation to the tumor volume demonstrated increased overall survival for each model compared to radiation alone.

Therapeutic control is determined by the degree and type of DNA damage inflicted and the cellular capacity to repair the damage. Successful repair requires the ability to recognize DNA damage, assemble the repair machinery, and execute repair; abrogation of any of these components decreases cell survival. TGFβ endorses homologous recombination (HR) and non-homologous end-joining (NHEJ) that are required for an effective DNA damage response (6,7,27,28). TGFβ inhibition compromises the molecular response to DNA damage and results in radiosensitization in cell lines and increased control of irradiated subcutaneous tumors. Inhibition of TGFβ signaling leads to reduced ATM mediated phosphorylation of critical DNA damage transducers, abrogation of the cell cycle checkpoint and increased radiosensitivity (27,29). Consistent with impaired ATM activity, phosphorylation of histone H2AX (γ-H2AX), an important mediator of cellular recognition of DNA damage, are decreased in human and mouse brain, breast and lung cancer models treated with either TGFβ neutralizing antibodies or small molecule inhibitors of TGFβ signaling (6,7,11). Decreased DNA damage recognition and ineffective cell cycle arrest are accompanied by greater radiation sensitivity in most (35/43) breast, GBM, head and neck, and lung cancer cell lines. Irradiation of tumors in mice treated with preclinical pan-neutralizing TGFβ antibody 1D11s also exhibit fewer γ-H2AX foci, indicating defective DNA damage recognition that results increased tumor control (6,7,11).

Inhibition of TGFβ in combination with radiotherapy has been shown to promote tumor control through multiple mechanisms. Pharmaceutical TGFβ inhibition in primary human GBM explants compromises the molecular response to radiation damage (4) and loss of TGFβ signaling in human papilloma virus positive head and neck cancer creates DNA damage deficits (28). A working model is that abundant TGFβ in the TME promotes HR and NHEJ, the two most accurate and effective DNA repair

pathways and inhibits the error-prone, alternative end-joining repair, which uses microhomology sequences that frequently generates insertions, deletions or chromosome breaks. Conversely, cancer cells in which TGFβ signaling is inhibited, or intrinsically impaired, are deficient in both HR and NHEJ and resort to alternative end-joining, which increases their sensitivity to therapy-induced DNA damage.

Combining 1D11 with localized brain RT resulted in long-term tumor-free survival in 80% of intracranial GL261 bearing mice and about 30% of the mice bearing 4T1-BrA tumors. Interestingly, neutralizing TGFβ provided durable control in most GL261-bearing animals but did not do so in those with SB28 tumors. Neutralizing antibody 1D11 effectively inhibits TGFβ signaling as shown by decreased pSMAD2, a mediator of the signaling cascade downstream of its receptors, and TNC deposition, a well-established TGFβ-induced extracellular matrix target (30). Interestingly, TGFβ – dependent TNC deposition was much more prominent in irradiated GL261 compared to a more modest increase in SB28. GBM express abundant TNC compared to normal brain (31). Weaver and colleagues showed that TNC increased in recurrent human GBM correlating with a stiffer microenvironment, enhanced focal adhesion and a shift to more immunosuppressive phenotypes (32). Together these data provide a strong rationale to use TGFβ inhibitors to increase response to RT.

In summary, we demonstrate the specificity of functional PET imaging using ^{89}Zr-fresolimumab as a novel non-invasive molecular imaging tool to assess TGFβ activity and expand the current spectrum for functional PET imaging of intracranial tumors. The importance of assessing TGFβ activity in response to treatment with RT, resides on the therapeutic potential of TGFβ inhibition in combination with radiotherapy. The conundrum is that even in tumors in which TGFβ activity is evident by PET functional imaging and molecular markers, only in the context of radiation did TGFβ inhibition increase median survival, which included long time survivors in 2 of 3 brain tumor models. These preclinical studies support the concept TGFβ opposes therapeutic benefit of radiation therapy and provide further motivation to identify clinically exploitable mechanisms by which TGFβ mediates the tumor control in response to radiotherapy.

References

1. Barcellos-Hoff MH, Newcomb EW, Zagzag D, Narayana A. Therapeutic targets in malignant glioblastoma microenvironment. Seminars in radiation oncology **2009** 19:163-70
2. Teicher BA. Transforming Growth Factor-β and the Immune Response to Malignant Disease. Clinical Cancer Research **2007**;13:6247-51
3. Bruna A, Darken RS, Rojo F, Ocaña A, Peñuelas S, Arias A, *et al.* High TGFbeta-Smad activity confers poor prognosis in glioma patients and promotes cell proliferation depending on the methylation of the PDGF-B gene. Cancer Cell **2007**;11:147-60
4. Bayin NS, Ma L, Thomas C, Baitalmal R, Sure A, Fansiwala K, *et al.* Patient-Specific Screening Using High-Grade Glioma Explants to Determine Potential Radiosensitization by a TGF-beta Small Molecule Inhibitor. Neoplasia (New York, NY) **2016**;18:795-805
5. Wang J, Cazzato E, Ladewig E, Frattini V, Rosenbloom DI, Zairis S, *et al.* Clonal evolution of glioblastoma under therapy. Nature genetics **2016**;48:768-76
6. Bouquet SF, Pal A, Pilones KA, Demaria S, Hann B, Akhurst RJ, *et al.* Transforming growth factor b1 inhibition increases the radiosensitivity of breast cancer cells *in vitro* and promotes tumor control by radiation *in vivo*. Clin Cancer Res **2011**;17:6754-65
7. Hardee ME, Marciscano AE, Medina-Ramirez CM, Zagzag D, Narayana A, Lonning SM, *et al.* Resistance of glioblastoma-initiating cells to radiation mediated by the tumor microenvironment can be abolished by inhibiting transforming growth factor-beta. Cancer research **2012**;72:4119-29
8. Du S, Bouquet F, Lo C-H, Pellicciotta I, Bolourchi S, Parry R, *et al.* Attenuation of the DNA Damage Response by TGFβ Inhibitors Enhances Radiation Sensitivity of NSCLC Cells In Vitro and In Vivo Int J Radiat Oncol Biol Phys **2014**;91:91-9
9. Zhang M, Herion TW, Timke C, Han N, Hauser K, Weber KJ, *et al.* Trimodal Glioblastoma Treatment Consisting of Concurrent Radiotherapy, Temozolomide, and the Novel TGF-β Receptor I Kinase Inhibitor LY2109761. Neoplasia (New York, NY) **2011** 13:537-49

10. Zhang M, Kleber S, Rohrich M, Timke C, Han N, Tuettenberg J, *et al.* Blockade of TGF-beta signaling by the TGFbetaR-I kinase inhibitor LY2109761 enhances radiation response and prolongs survival in glioblastoma. Cancer research **2011**;71:7155-67

11. Du S, Barcellos-Hoff MH. Tumors as organs: biologically augmenting radiation therapy by inhibiting transforming growth factor beta activity in carcinomas. Seminars in radiation oncology **2013**;23:242-51

12. Lawrence DA. Identification and activation of latent transforming growth factor b. Meth Enzym **1991**;198:327-36

13. Genoud V, Marinari E, Nikolaev SI, Castle JC, Bukur V, Dietrich PY, *et al.* Responsiveness to anti-PD-1 and anti-CTLA-4 immune checkpoint blockade in SB28 and GL261 mouse glioma models. Oncoimmunology **2018**;7:e1501137

14. Arrick BA, Lopez AR, Elfman F, Ebner R, Damsky CH, Derynck R. Altered metabolic and adhesive properties and increased tumorigenesis associated with increased expression of transforming growth b1. J Cell Biol **1992**;118:715-26

15. Deri MA, Ponnala S, Kozlowski P, Burton-Pye BP, Cicek HT, Hu C, *et al.* p-SCN-Bn-HOPO: A Superior Bifunctional Chelator for (89)Zr ImmunoPET. Bioconjugate chemistry **2015**;26:2579-91

16. Beckford-Vera DR, Gonzalez-Junca A, Janneck JS, Huynh TL, Blecha JE, Seo Y, *et al.* PET/CT Imaging of Human TNFalpha Using [(89)Zr]Certolizumab Pegol in a Transgenic Preclinical Model of Rheumatoid Arthritis. Molecular imaging and biology : MIB : the official publication of the Academy of Molecular Imaging **2019**

17. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, *et al.* Fiji: an open-source platform for biological-image analysis. Nature methods **2012**;9:676-82

18. Barcellos-Hoff MH, Ehrhart EJ, Kalia M, Jirtle R, Flanders K, Tsang ML-S. Immunohistochemical detection of active TGF-b *in situ* using engineered tissue. The American journal of pathology **1995**;147:1228-37

19. Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, *et al.* TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. The EMBO journal **1997**;16:5353-62

20. Sheppard D. Integrin-mediated activation of latent transforming growth factor beta. Cancer Metastasis Rev **2005**;24:395-402

21. Takasaka N, Seed RI, Cormier A, Bondesson AJ, Lou J, Elattma A, *et al.* Integrin alphavbeta8-expressing tumor cells evade host immunity by regulating TGF-beta activation in immune cells. JCI Insight **2018**;3

22. Jobling MF, Mott JD, Finnegan MT, Jurukovski V, Erickson AC, Walian PJ, *et al.* Isoform-specific activation of latent transforming growth factor beta (LTGF-beta) by reactive oxygen species. Radiation research **2006**;166:839-48

23. Barcellos-Hoff MH, Derynck R, Tsang ML-S, Weatherbee JA. Transforming growth factor-b activation in irradiated murine mammary gland. J Clin Invest **1994**;93:892-9

24. Barcellos-Hoff MH. Radiation-induced transforming growth factor b and subsequent extracellular matrix reorganization in murine mammary gland. Cancer research **1993**;53:3880-6

25. Oude Munnink TH, Arjaans ME, Timmer-Bosscha H, Schroder CP, Hesselink JW, Vedelaar SR, *et al.* PET with the 89Zr-labeled transforming growth factor-beta antibody fresolimumab in tumor models. Journal of nuclear medicine : official publication, Society of Nuclear Medicine **2011**;52:2001-8

26. den Hollander MW, Bensch F, Glaudemans AW, Oude Munnink TH, Enting RH, den Dunnen WF, *et al.* TGF-beta Antibody Uptake in Recurrent High-Grade Glioma Imaged with 89Zr-Fresolimumab PET. Journal of nuclear medicine : official publication, Society of Nuclear Medicine **2015**;56:1310-4

27. Kirshner J, Jobling MF, Pajares MJ, Ravani SA, Glick AB, Lavin MJ, *et al.* Inhibition of transforming growth factor-beta1 signaling attenuates ataxia telangiectasia mutated activity in response to genotoxic stress. Cancer research **2006**;66:10861-9

28. Liu Q, Ma L, Jones T, Palomero L, Pujana MA, Martinez-Ruiz H, *et al.* Subjugation of TGFb Signaling by Human Papilloma Virus in Head and Neck Squamous Cell Carcinoma Shifts DNA Repair from Homologous Recombination to Alternative End Joining. Clin Cancer Res **2018**;24:6001-14

29. Ewan KB, Henshall-Powell RL, Ravani SA, Pajares MJ, Arteaga CL, Warters RL, *et al.* Transforming growth factor-b1 mediates cellular response to DNA damage in situ. Cancer research **2002**;62:5627-31

30. Hau P, Kunz-Schughart LA, Rümmele P, Arslan F, Dörfelt A, Koch H, *et al.* Tenascin-C protein is induced by transforming growth factor-beta1 but does not correlate with time to tumor progression in high-grade gliomas. Journal of neuro-oncology **2006**;77:1-7

31. Bourdon MA, Wikstrand CJ, Furthmayr H, Matthews TJ, Bigner DD. Human glioma-mesenchymal extracellular matrix antigen defined by monoclonal antibody. Cancer Res **1983**;43:2796-805

32. Miroshnikova YA, Mouw JK, Barnes JM, Pickup MW, Lakins JN, Kim Y, *et al.* Tissue mechanics promote IDH1-dependent HIF1alpha-tenascin C feedback to regulate glioblastoma aggression. Nat Cell Biol **2016**;18:1336-45

FIGUREs & LEGENDS

Fig. 1: PET imaging of ^{89}Zr-fresolimumab detects active TGFβ *in vivo*. (A) PET imaging of ^{89}Zr-fresolimumab in mice bearing latent TGFβ producing B9 tumors (indicated by orange arrow) and active TGFβ producing C19 cells (indicated by arrow in green). Representative PET/CT transverse sections (lower image) and coronal sections (right image) are shown. (B) Quantification of radioactivity measured in flank tumors (p=0.004, paired t-test). (C) Immunofluorescence staining of B9 (orange) and C19 (green) tumors for TGFβ (green), pSMAD2 (red), and nuclei (blue). Percentage of total TGFβ signal normalized to cell count and pSMAD2 positive cells were quantified (p=0.03 and p=0.03, respectively, t-test). (D) PET imaging of mice bearing subcutaneous LLC (indicated by orange arrow) β8 LLC (indicated by green arrow) tumors. (E) Quantification of radioactivity measured in flank tumors (p=0.004, paired t-test). (F) Immunofluorescence staining of LLC (orange) and β8 LLC (green tumors for TGFβ (green), pSMAD2 (red), and nuclei (blue). Percentage of total TGFβ signal normalized to cell count and pSMAD2 positive cells were quantified (p=0.02 and p=0.03, respectively, t-test). Scale bars indicate 100 μm in C and F.

Fig. 2: ^{89}Zr-fresolimumab detects radiation induced changes of active TGFβ levels *in vivo*. (A) Cartoon and ^{89}Zr-fresolimumab PET/CT imaging of bilateral tumor bearing mice in which the right flank tumor was irradiated with 15 Gy (red arrow) or untreated (sham, blue arrow). (B) Autoradiograms of sham and irradiated (RT) 4T1-BrA subcutaneous tumors. (C) Radioactivity of 4T1-BrA tumors measured *ex vivo* 72 h after ^{89}Zr-fresolimumab injection (p=0.0002, paired t-test). (D) The radioactivity of 4T1-BrA tumors either injected with unlabeled (cold) fresolimumab followed by labeled ^{89}Zr-fresolimumab (Blocked, n=6) or with ^{89}Zr-fresolimumab (Not Blocked, n=10), (p=0.04, t-test). (E) Immunofluorescence localization of active TGFβ (green) and the TGFβ signaling readout pSMAD2 (red) in 4T1-BrA subcutaneous tumors. DAPI stained nuclei are blue. (F) Quantification of pSMAD2 positive cells from 4T1-BrA subcutaneous tumors (p=0.012, paired t-test). (G) Quantitation of TNC IF intensity in 4T1-BrA subcutaneous tumors (p=0.008, t-test). (H) Distribution of TNC IF (red) in 4T1-BrA subcutaneous tumors. DAPI stained nuclei are blue. Scale bars indicate 100 μm in E and G.

Fig. 3: Detection of active TGFβ in brain tumors by ^{89}Zr-fresolimumab PET imaging. Mice bearing intracranial (A) SB28, (B) Gl261, and (C) 4T1-BrA tumors were imaged with ^{18}F-FDG PET (upper panel) and ^{89}Zr-fresolimumab PET (bottom panel). Coronal (left), sagittal (middle) and BLI (right) are shown. (D) Total intracranial radioactivity in mice bearing SB28 tumors or PBS injected controls 120 h after injection (p=0.006, t-test). (E) Total intracranial radioactivity in mice bearing SB28 tumors or GL261 tumors or PBS injected controls analyzed 48 h after injection (p=0.01, t-test). (F) Accumulated radioactivity of ^{89}Zr-fresolimumab quantified from PET for mice bearing SB28 or 4T1–BrA intracranial (i.c.) and subcutaneous (s.c.) tumors.

Fig. 4: TGFβ neutralizing antibody 1D11 effectively decreases intracranial TGFβ downstream signaling after and increases response to RT The percentage of pSMAD2 positive cells for SB28 (A, p=0.005), GL261 (B, p=0.005) and 4T1-BrA (C, p=0.02) was quantitated (3 to 5 animals per group). Immunofluorescence of TNC was quantified for SB28 (D, p=0.001), GL261 (E, p=0.0001) and 4T1-BrA (F, p=0.0006) (3 to 5 animals per group). (G) Kaplan-Meier survival analysis of mice bearing intracranial SB28 tumors treated with 1D11 (n=6, green), RT (n=9, orange), combination of both (n=10, blue) or non-treatment (n=9, black). (H) Mice bearing intracranial GL261 tumors treated with 1D11 (n=5), RT (n=10), combination of both (n=10) or non-treatment (n=4). (I) Kaplan-Meier survival analysis of mice bearing intracranial 4T1-BrA tumors treated with 1D11 (n=9), RT (n=12), combination of both (n=13) or sham-treated (n=15). RT at day 9 indicated by arrow. One-way ANOVA was used in A-F and Kaplan-Meier analysis using the log-rank test in G-I (*P≤0.05, **P≤0.01, ****P≤0.0001).

Figure 1

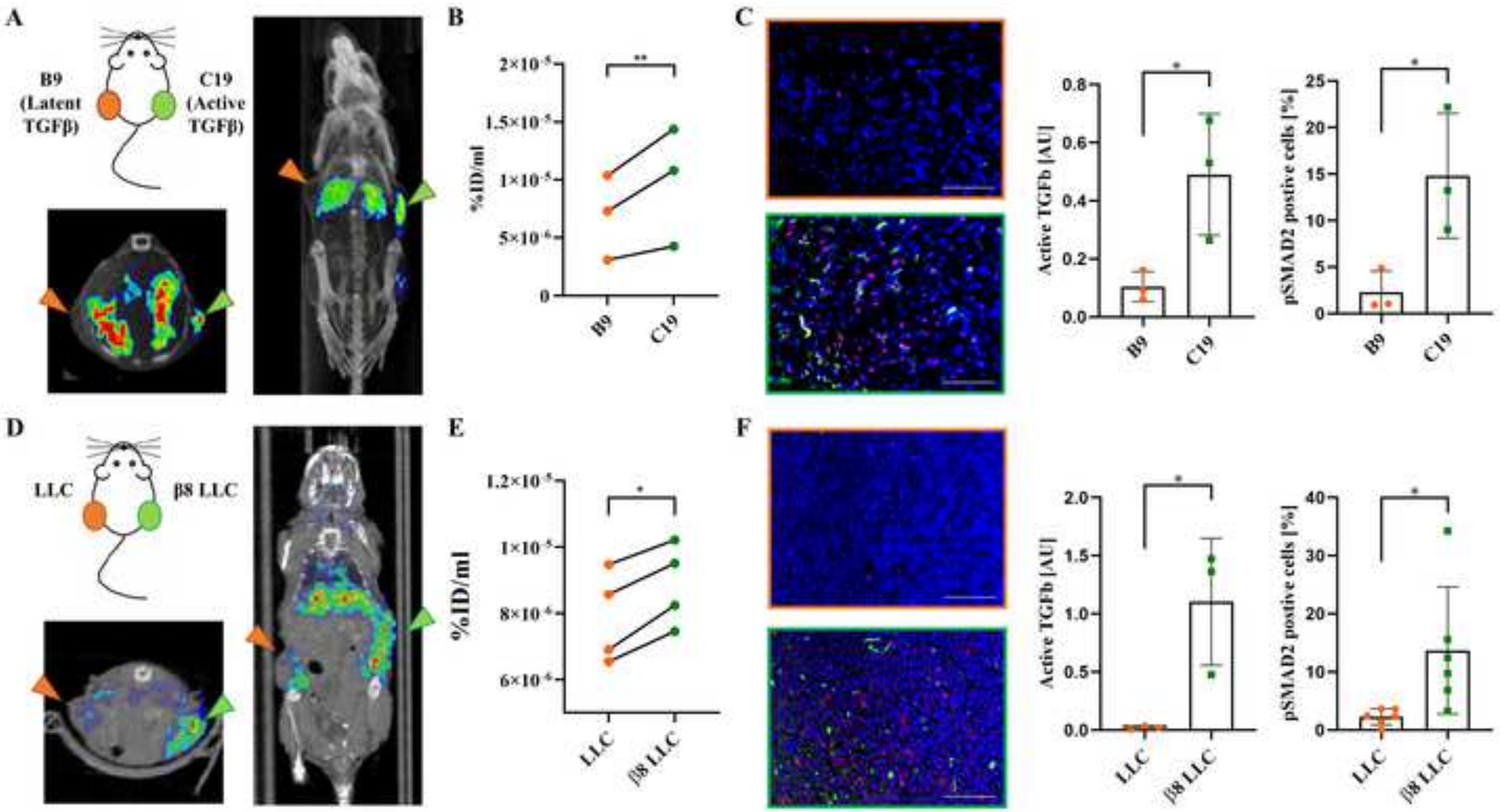


Figure 2

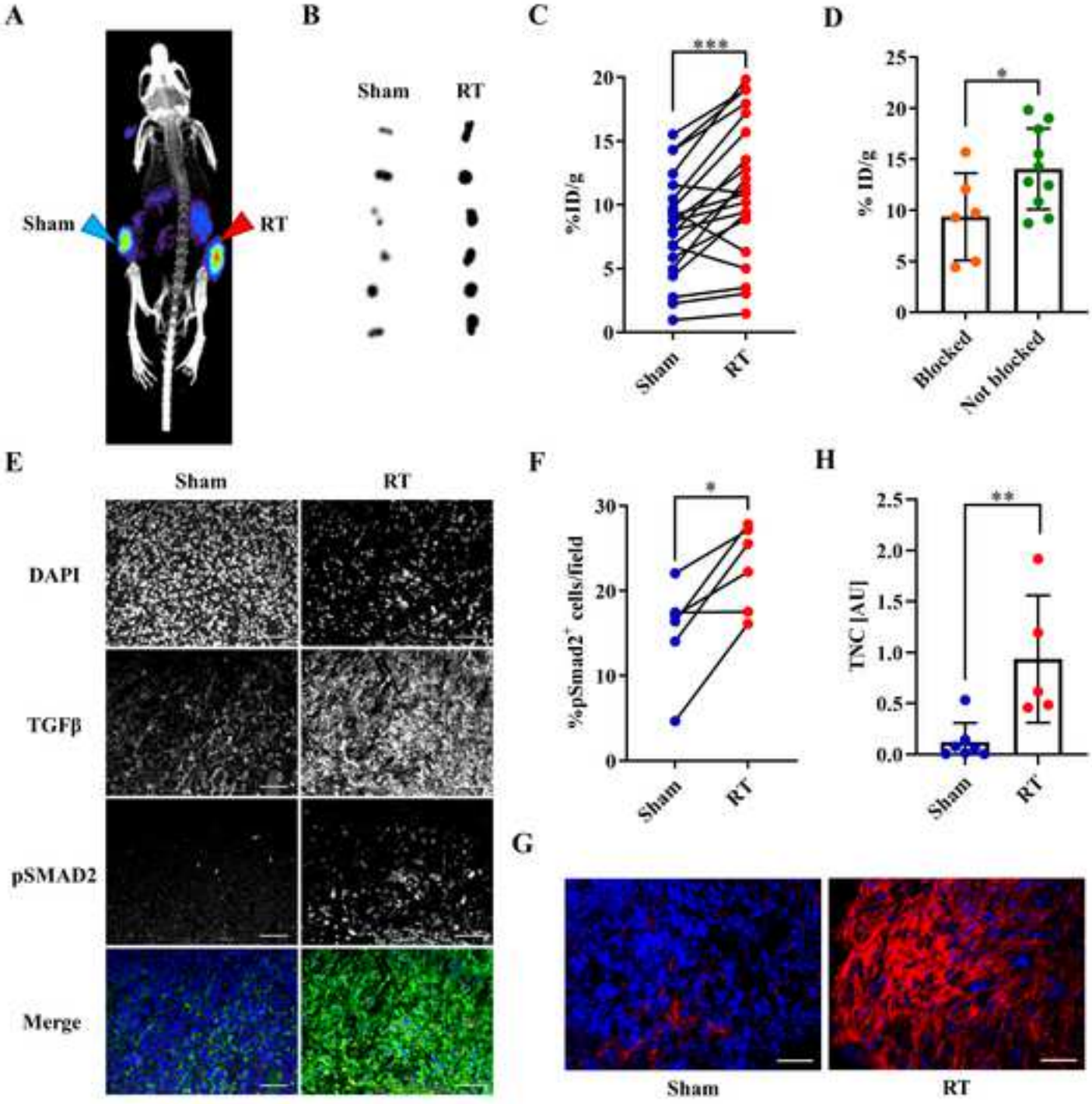


Figure 3

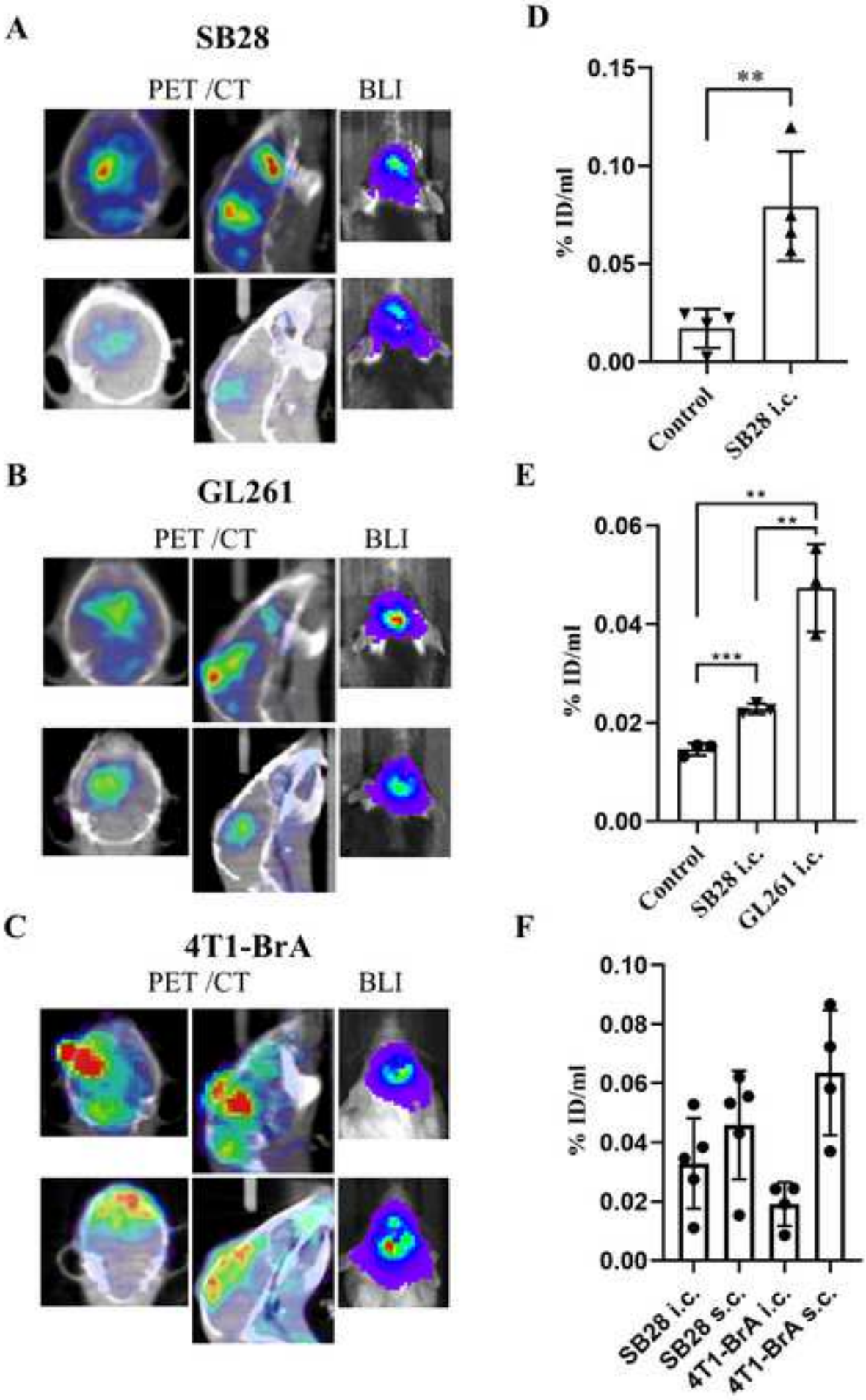
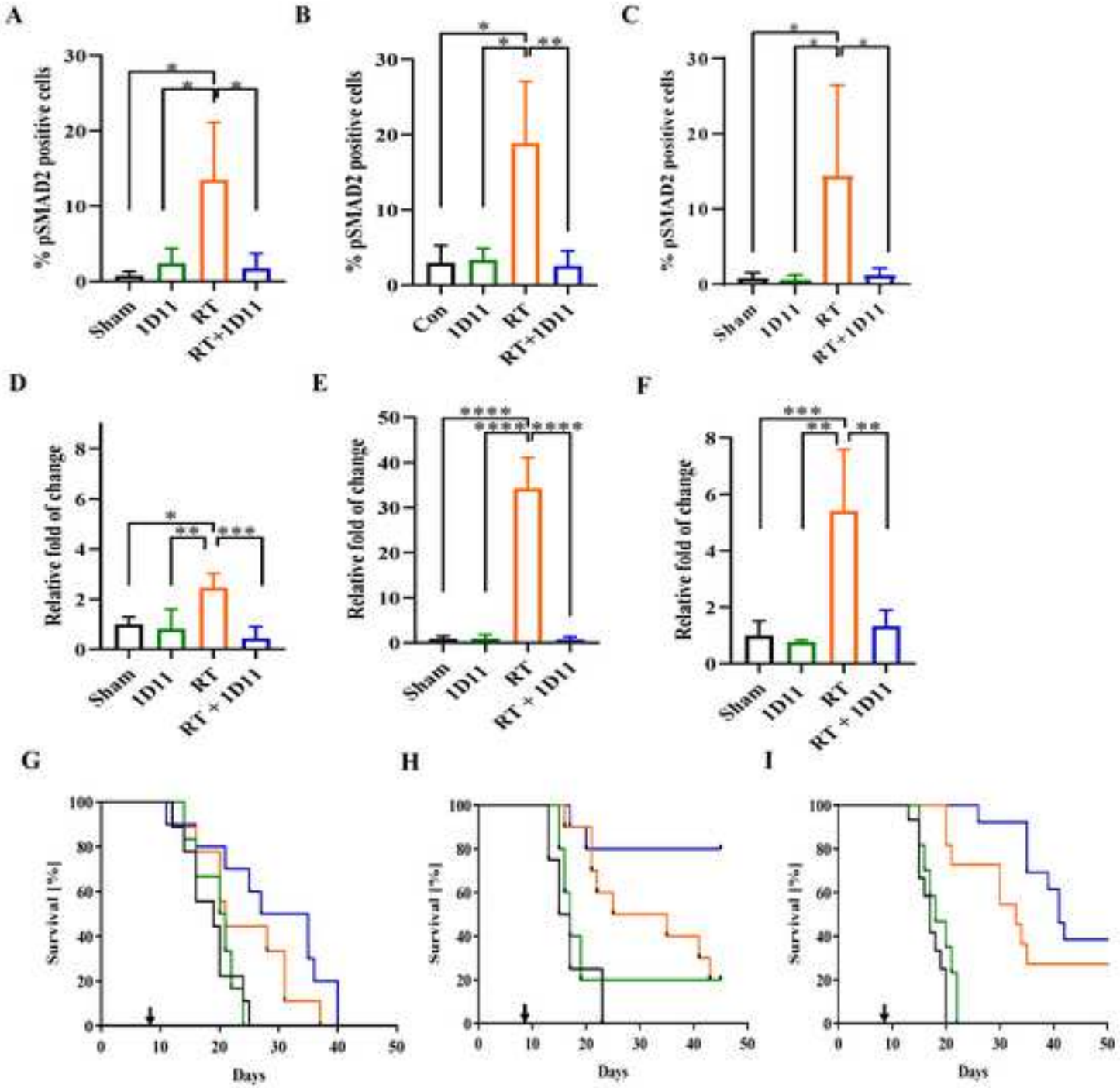


Figure 4



MHBH

OR

BF

AL

DBV

LBG

SB