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PRINCIPAL INVESTIGATOR: Benjamin Franc

CONTRACTING ORGANIZATION: University of California San Francisco
San Francisco, CA 94143

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Benjamin Franc

E-Mail: Benjamin.Franc@ucsf.edu

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The Regents of the University of California, San Francisco
3333 California Street, Suite 315
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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.

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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 (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.

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	80
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		25

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	50
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.

Major Activities:

- 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).
- Assessing the benefit of TGFβ inhibition in the context of IGRT
- Tumor microenvironment (TME) and immune system characterization as a function of radiotherapy and in combination with TGFβ blockade (1D11).

Specific Aim 2: Characterize functional imaging methods to assess drug distribution, tumor burden and immunological response to RT

Major Activities:

- Synthesis and characterization of 89Zr-DFO-fresolimumab
- In vivo characterization of 89Zr-DFO-fresolimumab
- Imaging of tumor growth over natural course of BCBM using MRI (control arm)
- Imaging of tumor-related immune response over natural course of BCBM (control arm)
- Translated manual synthesis of 5-[^{18}F]fluoro-α-methyl tryptophan (5-[^{18}F]F-AMT) to automated synthesis
- Imaged 5-[^{18}F]F-AMT metabolism as a means of assessing indoleamine 2,3-dioxygenase 1 (IDO1) activity which promotes tumor growth and immune system suppression (control arm)

ii. Specific objectives

Assessing the benefit of TGFβ inhibition in the context of IGRT. **(Barcellos-Hoff)**

Intracranial murine models of breast cancer metastasis, 4T1-BrA, genetically modified to constitutively expressed luciferase were stereotactically inoculated into the right striatum nucleus of female syngeneic mice. Tumor growth was quantified by measuring bioluminescence (BLI) using IVIS-Xenogen. Image-guided radiation therapy (IGRT) using an Xstrahl small animal radiation research platform (SARRP) and Muriplan planning software was used to deliver a single dose of 10 Gy (sRT). Murine TGFβ neutralizing monoclonal antibody, 1D11, was administered i.p. (20 mg/kg) 24 hr before RT, and repeated every 3 days for 4 weeks. Mice were monitored by BLI and physical symptoms. A subset of mice (5 per group) were selected for brain, spleen and blood collection 6 days post-RT to characterize tumor microenvironment (TME) and immune response as a function of RT and combination treatment. Kaplan-Meier survival analysis was calculated for the remaining mice in each group. Murine brains from different treatment groups were FFPE and the immune system populations were characterized by immunofluorescence. Mice that showed complete rejection of tumor by IVIS were re-challenged with subcutaneous injections of the same tumor cells, 4T1-BrA.

TSA-BrA characterization. As previously described, 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. We monitored tumor progression by BLI using IVIS and performed histology of the resulting brains at the moment of termination.

We initiated an experiment to determine latency of tumor growth with the second brain metastasis model, TSA-BrA. TSA-BrA cells were injected into the brain of Balb/c 6-7 weeks old female mice with three groups (5 mice per group) $1x10^4$, $1x10^5$, and$1x10^6$ cells. Tumor growth was monitored with BLI and Kaplan-Meier survival curves were generated. Murine brains from different groups were collected for FFPE.

Characterizing functional imaging methods to assess drug distribution, tumor burden and immunological response to RT ***(Franc)***

^{89}Zr-DFO-fresolimumab synthesis and characterization **–** We successfully modified fresolimumab with1-(4-isothiocyanatophenyl)-3-[6,17-dihydroxy-7,10,18,21-tetraoxo-27-[N-acetylhydroxylamino)-6,11,17,22-tetrazaheptaeicosine]-thiourea (DFO-CNS) and radiolabeled it with with Zr-89. Using size-exclusion HPLC and iTLC, ^{89}Zr-DFO-fresolimumab was successfully purified and the number of DFO per fresolimumab was determined. ^{89}Zr-DFO-fresolimumab radiolabeling yield and radiochemical purity were determined as well the biological activity of fresolimumab after conjugation and radiolabeling. In vivo characterization of ^{89}Zr-DFO-fresolimumab was accomplished. The specificity of ^{89}Zr-DFO-fresolimumab for immuno-PET imaging of active TGFβin a preclinical model was tested.

Tumor growth was imaged and quantified using MRI (control arm). In addition, imaging of tumor-related immune response of BCBM (control arm) was performed. Specifically, BCBM were imaged with ^{18}F FDG PET regularly beginning 7 days following implantation to evaluate combined metabolic signal from tumor and immune response. BCBM were imaged with ^{18}F F-AraG PET regularly beginning 7 days following implantation to evaluate levels of activated T-cells associated with the tumor. Finally, BCBM were imaged with ^{89}Zr-DFO-fresolimumab PET regularly beginning

7 days following implantation to evaluate combined TGFβsignal from tumor and immune response.

The synthesis of 5-[^{18}F]fluoro-α-methyl tryptophan (5-[^{18}F]F-AMT) was adapted to conditions in our laboratory. Subsequently, we translated synthesis of 5-[^{18}F]fluoro-α-methyl tryptophan (5-[^{18}F]F-AMT) to an automated platform. Specifically, the BPin precursor was prepared in 5 steps from methyl tryptophan as described in Giglio et. al. (3). The original labeling procedure utilized tetrabutyl ammonium [^{18}F]fluoride, however in our hands the labeling was inconsistent. We employed a labeling technique published by Mossine et. al. (4, 5) to achieve consistent labeling. Labeling using the KOTf/ K_2CO_3 system allowed us to concentrate the [^{18}F]fluoride ion on a QMA sep pak without adding large amounts of K_2CO_3 into the BPin/ copper catalyst reaction. Larger amounts of DMF (350 μL: 50 μL) was needed for the [^{18}F]fluorination step, thus a C18 sep pak was required to lower the total reaction volume for the TFA decyclization step. DMF (50 μL) was back into the 500 μL of TFA to keep the hydrophobic compound soluble for the reaction. The TFA was evaporated and NaOH was added to the reaction for deprotection. The reaction mixture was neutralized and injected for purification onto an analytical HPLC. The product was collected, diluted up, and loaded onto a C18 light sep pak. The final purified product was eluted with ethanol and taken to dryness. The final formulation of 0.9% saline was added and imaging studies were conducted.

Imaging of 5-[^{18}F]F-AMT metabolism using small animal PET as a means of assessing indoleamine 2,3-dioxygenase 1 (IDO1) activity which promotes tumor growth and immune system suppression (control arm).

iii. Significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative)

4T1-BrA intracranial tumor model (Barcellos-Hoff) During year 1 of funding we reported that combined treatment of RT and 1D11 provided benefit to fractionated RT (5 x 6 Gy) in the 4T1-BrA intracranial tumor model. 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 (**Figure 1**).

Kaplan-Meier survival plots demonstrate that the double treatment was superior to RT alone as demonstrated by an increase in median survival. 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 (**Figure 2**).

To test for immune memory, long-term survivors were challenged with subcutaneous tumor injection. These tumors were rejected in 2/2 from RT and 3/4 mice treated with RT+1D11 (**Figure**

3). These results are contrary to the results obtained with 5 fractions of 6 Gy in which none of the long-term survivors succeeded in rejecting subcutaneous tumor re-challenge.

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.

Our original plan was to use flow cytometry (FACS) for immune profiling, as reported in the first year. 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 brief, specimens are dissociated, the single cells are incubated with a cocktail of labeled antibodies specific to selected proteins and the cytometer separates each cell from the suspension into a single droplet that is vaporized. The ion cloud is passed through a quadrupole to enrich for heavy-metal reporter ions that are separated by their charge-to-mass ratio in a time-of-flight mass spectrometer. These signals are integrated into single-cell events for analysis, in which 100,000 or more such events are compiled for each specimen.

The combination of multiplexed parameterization of many events at single cell resolution across hundreds of thousands cells provides previously unattainable capacity to study multicellular biological processes. The depth and detail as applied to the immune system, which is complex and highly heterogeneous, provides an innovative tool to analyze specimens generated in our studies.

Aw preliminary CyTOF analysis was conducted in collaboration with Dr. M. Spitzer (UCSF) using spleen samples collected five days post RT (**Figure 4**). 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 (red, left panel), whereas there was an increase in PD1 (blue, right panel) compared to RT alone.

TS/1-BrA intracranial tumor model (Barcellos-Hoff)

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 (Figure 5). 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. Currently, the TSA-BrA cells re-selected with puromycin and plan to perform additional studies with the cell line.

Molecular Imaging (Franc)

^{89}Zr-DFO-fresolimumab synthesis and characterization - We have successfully radiolabeled fresolimumab with zirconium-89 (Zr-89/^{89}Zr) by conjugating the antibody with 1-(4-isothiocyanatophenyl)-3-[6,17-dihydroxy-7,10,18,21-tetraoxo-27-[N-acetylhydroxylamino)-6,11,17,22-tetrazaheptaeicosine]-thiourea (DFO). DFO is the most widely used bifunctional chelating agent for stable complexation of Zr-89 **(Figure 6).**

The radiolabeling of DFO-fresolimumab conjugate was performed in aqueous conditions and room temperature. Characterization of the ^{89}Zr-DFO-fresolimumab was done using size-exclusion HPLC and iTLC. Quantification of the number of DFO per fresolimumab molecule was determined by isotopic dilution assay as described previously in the literature. ^{89}Zr-DFO-fresolimumab radiolabeling yield was > 76% and after purification the radiochemical purity of ^{89}Zr-DFO-fresolimumab was higher than 98% and specific activities ranging from 3.2 -732 MBq/mg were obtained. After conjugation and radiolabeling the biological activity of fresolimumab may be affected.

Therefore, the immunoreactivity of ^{89}Zr-DFO-fresolimumab was assessed in a competition assay with unmodified fresolimumab using TGF-ß3 as a target antigen. TGF-ß3 was selected because it has the highest affinity toward fresolimumab. Competition of ^{89}Zr-DFO-fresolimumab against unlabeled fresolimumab yield a half maximum inhibitory concentration of 16 nM, which shows no loss of immunoreactivity after conjugation and radiolabeling. Several batches of ^{89}Zr-DFO-fresolimumab were prepared and their characterization parameters were reproducible.

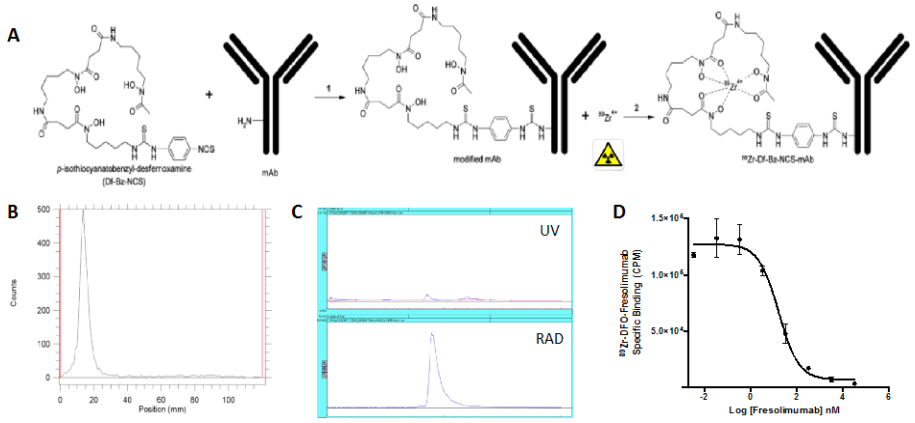


Figure 6. Synthesis and validation of 89Zr-Fresolimumab. A. Schematic representation of conjugation and radiolabeling of fresolimumab. B. Radio thin-layer chromatography of purified 89Zr-DFO-fresolimumab showing radiochemical purity higher than 98%. C. HPLC chromatogram of purified 89Zr-DFO-fresolimumab. D. Competition binding assay of 89Zr-DFO-fresolimumab against unlabeled fresolimumab (IC50 = 16nM).

In vivo characterization of ^{89}Zr-DFO-fresolimumab - We tested the feasibility of ^{89}Zr-DFO-fresolimumab for immuno-PET imaging of active TGFβin a preclinical model of breast cancer brain metastasis **(Figure 7).** We generated a murine model of 4T1 triple negative breast cancer cells selected for their capacity to grow in the brain of immunocompetent balb/c mice. The mice were injected with ^{89}Zr-DFO-fresolimumab with different specific activities and µPET/CT images were acquired at 24 and 96h post-injection. High tumor uptake was observed as early as 24h post-injection of ^{89}Zr-DFO-fresolimumab in this preclinical model allowing for clear tumor visualization, which demonstrates that ^{89}Zr-DFO-fresolimumab is capable of crossing the damaged blood brain barrier. Preparations of ^{89}Zr-DFO-fresolimumab with lower specific activity showed higher tumor uptake in mice bearing breast cancer brain metastasis. As a control, we

performed similar experiments in mice injected with PBS instead of 4T1 triple negative breast cancer cells. No brain uptake was observed in control mice.

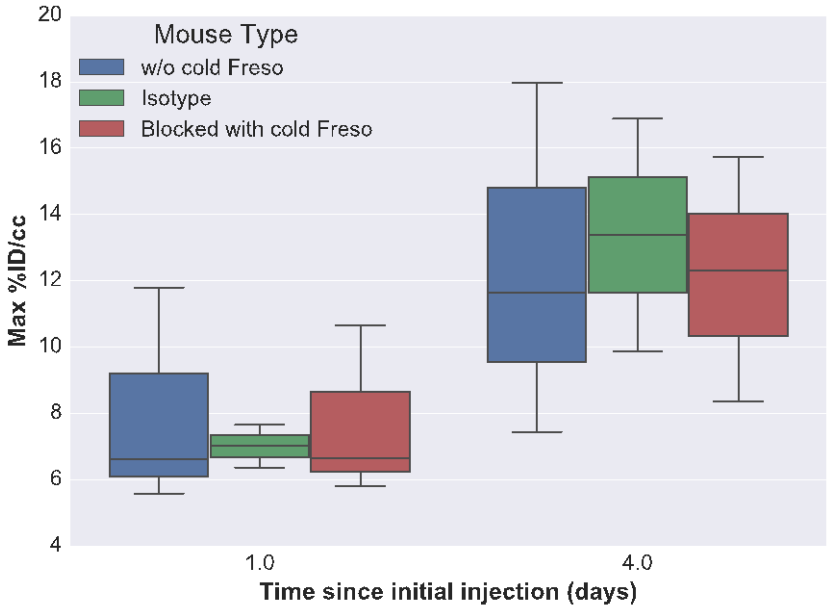


Figure 7. Biodistribution of 89Zr-Fresolimumab in BCBM as a function of time.

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 2 4T1-BrA flank tumors. One tumor in each mouse was treated with 15 Gy, the mice were imaged and then the tumors harvested (Figure **Figure 8A**). Immunohistochemistry was performed in the tumor slices. Tumor slices from irradiated tumors showed high levels of active TGFβ and induction of phosphorylated Smad 2/3, indicative of TGFβ activation (**Figure 8B**). As a control we used ^{89}Zr-DFO-isotype and ^{89}Zr-DFO-PEG in order to assess the specificity of ^{89}Zr-DFO-fresolimumab toward active TGF-ß. *Ex vivo* biodistribution studies were also conducted in order to quantify and correlated the µPET/CT images. ^{89}Zr-DFO-fresolimumab uptake in treated tumors were significantly higher than that for untreated tumors. After the *in vivo* studies, tumors were sliced and counted for radioactivity (**Figure 8C)**. The slices from irradiated tumors showed higher ^{89}Zr-DFO-fresolimumab uptake when compared to non-treated tumors. These results correlated with those in the *ex vivo* biodistribution studies.

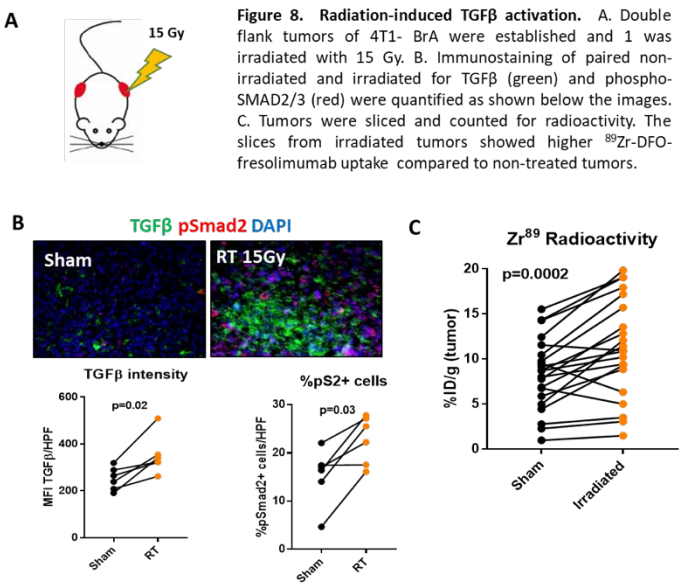


Figure 8. Radiation-induced TGFβ activation. A. Double flank tumors of 4T1- BrA were established and 1 was irradiated with 15 Gy. B. Immunostaining of paired non-irradiated and irradiated for TGFβ (green) and phospho-SMAD2/3 (red) were quantified as shown below the images. C. Tumors were sliced and counted for radioactivity. The slices from irradiated tumors showed higher ^{89}Zr-DFO-fresolimumab uptake compared to non-treated tumors.

Imaging of tumor growth using MRI (control arm) - From MRI measurements, the average untreated tumor doubling time was approximately 2 days.

Imaging of tumor-related immune response of BCBM (control arm) Mouse BCBM models were imaged at regular intervals during the course of BCBM growth to characterize the natural course of immune-targeting radiopharmaceutical uptake without therapeutic intervention. Serial images of the BCBM models were obtained every 3-4 days until the animal required euthanasia under pre-determined mouse health conditions. For a given mouse, raw PET data was converted to %ID/cc by scaling by the injected dose at scan time. The PET/CT images of each respective radiopharmaceutical acquired at the final imaging timepoint prior to euthanasia were utilized to identify the location of and signal from each imaged tumor. PET/CTs acquired at prior timepoints were co-registered to this final dataset and regions-of-interest were propagated across imaging datasets using a publically-available software tool (AMIDE). For each tumor, a cylinder or ellipsoid volume of interest (VOI) was generated, the VOI was propagated across imaging datasets from all time points. Derived VOI imaging statistics were then analyzed using Python.

Image BCBM with ^{18}F FDG PET regularly beginning 7 days following implantation to evaluate combined metabolic signal from tumor and immune response – Significantly increased uptake of ^{18}F FDG was observed in the intracranial tumor over that observed in sham mice who had received an intracranial injection of PBS, suggesting ^{18}F FDG uptake due to a combination of tumor metabolism and tumor-directed immunity rather than due to non-specific localization. ^{18}F FDG signal continued to increase over the approximate 2-week course of disease **(Figure 9).**

Image BCBM with ^{18}F-AraG PET regularly beginning 7 days following implantation to evaluate levels of activated T-cells associated with the tumor – Although there was a small degree of quantifiable signal in the region containing the intracranial tumor, there was no visible focal uptake and no significant difference in ^{18}F-AraG uptake in tumor versus PBS control over the course of the disease in this cohort of untreated mice.

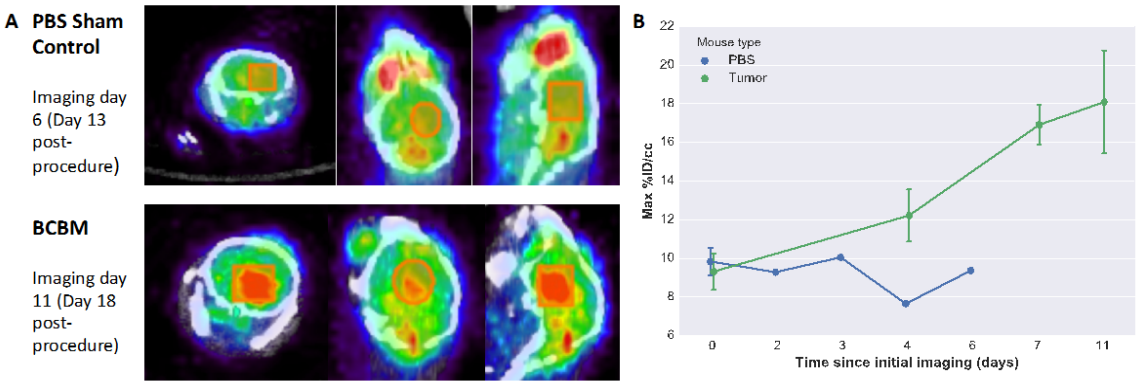


Figure 9. ^{18}F FDG uptake over the natural course of BCBM growth. A. axial, coronal, and sagittal fused PET/CT images demonstrating volumes of interest from which statistics were derived using AMIDE. Maximum injected dose per volume measured from VOIs of PET/CT images.

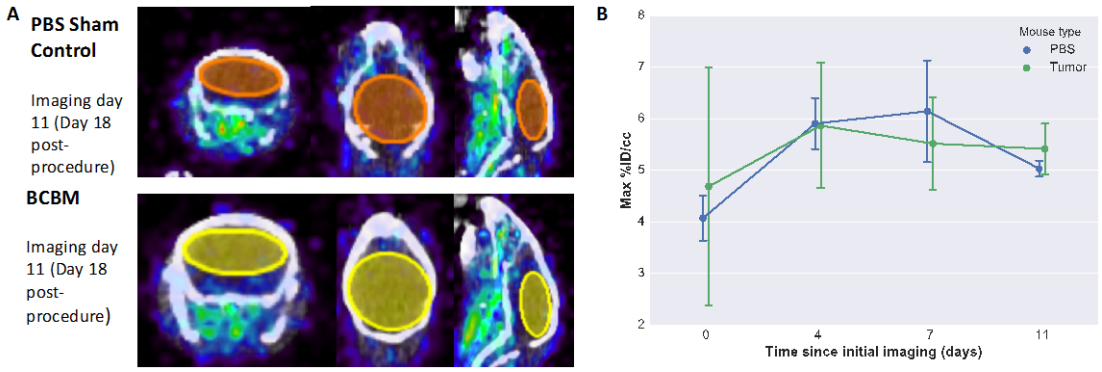


Figure 10. ^{18}F F AraG uptake over the natural course of BCBM growth. A. axial, coronal, and sagittal fused PET/CT images demonstrating volumes of interest from which statistics were derived using AMIDE B. Maximum injected dose per volume measured from VOIs of PET/CT images

This result is what would be expected in untreated mice as robust T-cell activation would not necessarily occur in the absence of an immune modulating drug. We have observed a similar low level of signal in untreated sarcomas in mice and untreated bladder and breast cancers in humans **(Figure 10).**

Image BCBM with ^{89}Zr-DFO-fresolimumab PET to evaluate combined TGFβ signal from tumor and immune response – Uptake of ^{89}Zr-DFO-fresolimumab was significantly increased in tumor over PBS control. Each mouse received a single injection of ^{89}Zr-DFO-fresolimumab and was imaged at early (1 day) and late (4 day) timepoints, enabling determination of an appropriate length of time following injection when the signal-to-background uptake would be sufficient to detect TGFβ activity in the tumor **(Figure 11).**

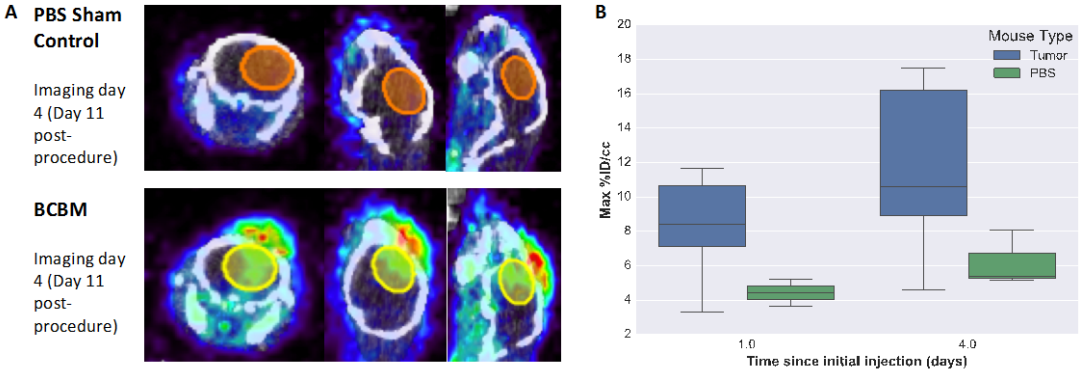
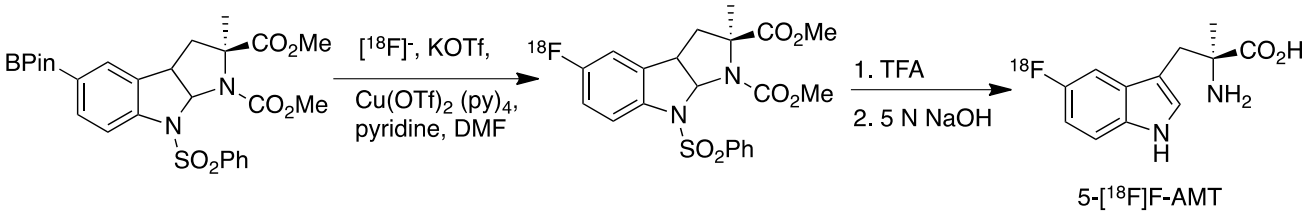


Figure 11. ^{89}Zr – Fresolimumab anti-TGF- β uptake over the natural course of BCBM growth. A. axial, coronal, and sagittal fused PET/CT images demonstrating volumes of interest from which statistics were derived using AMIDE. B. Maximum injected dose per volume measured from VOIs of PET/CT images

Manual synthesis of 5-[^{18}F]fluoro-α-methyl tryptophan (5-[^{18}F]F-AMT) and translation of 5-[^{18}F]F-AMT to an automated platform - Incorporation yields (analyzed by radioTLC, non-decay corrected) were sufficient and consistent (25% ± 5%, n = 5). The automation on the ELIXYS required volume and reaction condition adjustments to achieve suitable synthetic yields. The decyclization and deprotection scheme was one of the most challenging parts of the automation with various partially protected species being present in the HPLC purification. All of the possible species formed from various partial reactions are all separable on the HPLC using the gradient solvent system of 5: 95% acetonitrile with 0.1% TFA over 30 mins. The addition of the 50 μL of DMF to the TFA led to more efficient decyclization. NaOH was substituted for KOH in the

deprotection reaction as older KOH did not accomplish complete deprotection. Automation of 5-[^{18}F]F-AMT was completed on the Sofie Biosciences ELIXYS FLEX/ CHEM module and the synthesis takes 120 ± 10 minutes, with a yield of 1.52 ± 1.04% (n = 3, decay corrected). Adequate amounts of 5-[^{18}F]F-AMT (37- 185 MBq) were formulated and injected into the metastatic tumor model in mice to perform imaging studies.



Imaging 5-[^{18}F]F-AMT metabolism as a means of assessing indoleamine 2,3-dioxygenase 1 (IDO1) activity which promotes tumor growth and immune system suppression – 4T1 BCBM models were imaged with 5-[^{18}F]F-AMT PET regularly beginning 7 days following implantation to evaluate levels of indoleamine 2,3-dioxygenase 1 (IDO1) activity within the tumor – Although there was a small degree of quantifiable signal in the region containing the intracranial tumor, there was no visible focal uptake and no significant difference in 5-[^{18}F]F-AMT uptake in tumor versus PBS control over the course of the disease in this cohort of untreated mice.

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

Completion of the objectives for second year of funding were delayed by 3 major events. The SARRP was down for approximately 12 weeks due to technical problems that resulted in loss of experimental time. In addition, Dr. Alba Gonzalez-Junca, the postdoctoral fellow who led this project on year one and half of year two, accepted a permanent research position at biotech company. I hired a new postdoctoral fellow, Dr. Luis Borrero-Garcia, who received a doctorate in Cancer Biology at the University of Puerto Rico in 2018 for his thesis research in breast cancer. He joined the lab in August and was trained by Dr. Gonzalez-Junca and lab manager Mr. William Chou. A third challenge was the erratic behavior of TSA-BrA experimental model, which required troubleshooting that identified loss of the luciferase reporter gene, also delayed experiments in the phase.

This confluence of events precluded molecular imaging characterization of animals AFTER receiving radiation treatment. Therefore, baseline control evaluation was completed, but post-therapy characterization was, for the most part, precluded.

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

The project has provided the postdoctoral fellow Dr. Gonzalez-Junca the opportunity to share her work at symposiums and conferences, which included presenting at the UCSF radiology symposium, imaging conference, and breast oncology program symposium, as well as attending the AACR immunobiology of CNS meeting in February 2018. In addition, Dr. Gonzalez-Junca trained Dr. Borrero-Garcia, who is new to the field. He has now completed 2 experiments and

presented the research at the UCSF Breast Oncology Program Scientific retreat. Notably he was selected for an oral presentation of the work and received an award for the abstract.

The project provided the opportunity for a master's student, Niecholle Roco, to complete her thesis on the radiolabeling of ^{89}Zr-DFO-fresolimumab. In the process of working alongside senior members of our team, she learned how to perform radiolabeling of molecules using positron emitters, use of HPLC and thin layer chromatography for purification, and using immune-based assays to assess biologic activity. In addition, she learned about PET imaging and was also able to perform small animal anatomic-based imaging with MRI.

The project also provided the opportunity for a medical student, Nathan Jenkins, to learn about the fundamentals of medical imaging, understand the use of time activity curves to in PET imaging, and assist senior members of the team in analyzing uptake of various radiopharmaceuticals on PET images over time.

The project provided the opportunity for a new UCSF faculty member, Denis Beckford-Vera, to lead the authorship of papers related to molecular imaging and obtain preliminary data for additional future investigations in immune-based small animal PET imaging.

d. **How were the results disseminated to communities of interest?** The early phase of these studies were disseminated within our institution as follows:

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, 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)- Poster presentation Mary Helen Barcellos-Hoff *This presentation reported on the use of imaging agent developed by partnering PI Dr. Benjamin Franc (#BC160513P1) and his team.*

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) – Oral presentation Luis D. Borrero-Garcia

Positron emission tomography imaging of TGF- ß **using ^{89}Zr-DFO-Fresolimumab.**

Denis R. Beckford-Vera, Alba Gonzalez-Junca, Tony L. Huynh, Niecholle Roco, Jessica S Janneck, Joseph E. Blecha , Mary H Barcellos-Hoff, Benjamin L. Franc, and Henry F. VanBrocklin; Society of Nuclear Medicine and Molecular Imaging 2018 – Oral presentation by Denis Beckford-Vera.

Automated synthesis of 5-[^{18}F]fluoro-α-methyl tryptophan (5-[^{18}F]F-AMT)

Joseph E. Blecha, Thomas Hayes, Tony Huynh,Luis Borrero Garcia, William Chou, Mary Helen Barcellos-Hoff, Benjamin Franc, and Henry F. VanBrocklin; (accepted ISRS May 2019)

e. **What do you plan to do during the next reporting period to accomplish the goals?**
(Barcellos-Hoff) - In Year 2, we showed that effective intracranial control was achieved by a single fraction of RT of 10 Gy and TGFβ inhibition. Moreover, subsequent rejection of tumor re-challenge indicates effective intracranial tumor control can elicit immune memory. Our focus in the next term will be on repeat studies using the TSA-BrA model to evaluate the therapeutic benefit of combine treatment of TGFβ inhibition and RT. The unexpected difference between the effect of TGFβ inhibition in combination with single dose vs fractionated RT in the 4T1-BrA model warrants further exploration. TGFβ inhibition increased tumor control by both RT regimens as evidenced a median survival. However, in those mice in which tumors were eliminated, only the mice treated with a single dose were able to reject tumor rechallenge, supporting immunity as a mode of action. If confirmed upon repetition, this could suggest that GKSRS would be more effective than conventional radiation treatment plans as a means to promote immune response. Hence in NCE year 3, the Barcellos-Hoff laboratory plans to repeat experiments with 4T1-BrA explicitly comparing single 10 Gy versus 5 fraction of 6 Gy, as well as complete experiments with TS/A-BrA.

(Franc) – In Year 2, we completed the synthesis of all molecular imaging agents that we sought to characterize the immune response of combined radiation therapy and TGFβ inhibition – targeting therapy. We used PET imaging to characterize the activity of each of these tracers within the models generated by the Barcellos-Hoff laboratory. The Franc laboratory will not be continuing beyond Year 2 as the end of Year 2 was the end of the grant period and the Franc laboratory did not file for an extension. Dr. Franc moved to Stanford University in December 2018. Dr. Franc will work with the entire team to author and publish manuscripts outlined in the major objectives for this project.

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.

The automation of 5-[^{18}F]F-AMT synthesis on the Sofie Biosciences ELIXYS FLEX/ CHEM module is potentially relevant to human studies of response to therapy. This may be useful in clinical trials, not only at our own institution, but at any institution that utilized the Sofie Biosciences box in the cyclotron laboratory.

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

Nothing to report

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 SARRP needed for mouse irradiation
      ii. Cell line needed to be re-transfected with reporter
      iii. Installation of Sofie PET/CT in February but not commissioned for radioactivity by EH&S until November
      iv. These issues delayed execution of experiments and prompted the request for a no-cost extension on 10/23/18. NOTE Partnering PI, Dr. Benjamin Franc (#BC160513P1) did not request a NCE.
   b. Personnel changes
      i. Dr. Alba Gonzalez-Junca left for an industry position in July, 2018
         1. Dr. Gonzalez-Junca assisted in training new postdoc
         2. She continues to contribute to data analysis
      ii. Dr. Luis Borrero-Garcia joined August, 2018
         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
      iii. Dr. Benjamin Franc closed out the partnering grant (#BC160513P1) in imaging upon moving to a faculty position at Stanford in December

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

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

f. What individuals have worked on the project?

Name	Role	Person Months Worked	Contribution	Funding Support
Mary Helen Barcellos-Hoff, Ph.D	PI	1.2	Designed expts and analyzed experimental data	
Alba Gonzalez-Junca, Ph.D.	Postdoctoral Fellow	5.16	Generation of pre-clinical BCBM models. Treatment and characterization.	
Luis Borrero-Garcia, Ph.D.	Postdoctoral Fellow	7	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	
Benjamin Franc	PI	0.96	Oversaw molecular imaging portion of project	
Henry VanBrocklin	Co-investigator	0.60	Oversaw development of ^{89}Zr-DFO-fresolimumab	
Youngho Seo	Co-investigator	0.36	Oversaw quantitation of PET images	
Joe Blecha	Assoc Specialist	4.8	Synthesis, purification and analysis of ^{89}Zr-DFO-fresolimumab	
Shih-ying Huang	Assoc Specialist	5.3	PET image analysis and correlation with histology	Replaced Roy Harnish on this project
Niecholle Roco	Masters Student	1.07	Assisted in MRI acquisition and analysis	

Nathan Jenkins	Medical Student	1.61	Assisted in PET analysis	
Tony Hyunh	Asoc Specialist	5 (approx.)	Acquired PET images	Paid by Department through Equipment recharge

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

Completed: Varian Medical Systems 12/01/2017 - 1/31/2019 1.2 calendar

TRI-Modal Therapy Phase 2

Pilot project focused on TGFβ inhibition during radiotherapy to define the specific conditions under which TGFβ inhibition acts in concert with immunotherapy in glioblastoma.

Role: P.I.

Added:
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.

R01NS109911 02/01/2019-01/31/2024 1.80 calendar
NIH/NCI $372,639
Role: MPI (Contact PI)
Reorienting the Glioblastoma Microenvironment to Respond to Immunotherapy
Goal: We hypothesize that clinically viable TGFβ blockade could both eliminate local immunosuppression by reducing MDSC viability and promote T cell infiltration by breaking down HA and TNC-driven stiff ECM.

h. What other organizations were involved as partners?
Nothing to Report.

8. **SPECIAL REPORTING REQUIREMENTS**

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ers.amedd.army.mil for each unique award. QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

Dr. Benjamin Franc (#BC160513P1) did not request a NCE, and moved to Stanford University in December 2018.

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

UCSF Breast Oncology Retreat San Francisco February 19, 2019

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

Department of Radiation Oncology and Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, CA, USA

Breast cancer brain metastases (BCBM) are associated with poor prognosis and limited therapeutic options. Current efforts focus on developing approaches to improve response to radiation therapy (RT) to test whether inhibition of transforming growth factor beta (TGFβ) improves response of brain adapted (BrA) breast cancer to radiation therapy. The rationale for this comes from previous studies that showed that TGFβ is activated in irradiated tissue affecting the composition of the tumor microenvironment and enhancing the ability of tumor cells to survive DNA damage. We first image TGFβ activity in situ using fresolimumab (GC1008), the humanized 1D11 TGFβ neutralizing antibody, radiolabeled with ^{89}Zr for PET-CT imaging (^{89}Zr-fresolimumab). Mice harboring irradiated (15 Gy) 4T1-BrA flank tumors displayed a heightened PET/CT signal compared to un-radiated tumors. We collected irradiated and non-irradiated tumors and perform dual immunofluorescence staining for active TGFβ and phospho-SMAD2. We found TGFβ intensity correlated with the radioactivity of each tumor, which shows specificity of ^{89}Zr-fresolimumab to detect TGFβ activity in vivo. Next, we tested if inhibition of TGFβ improves response of 4T1-BrA intracranial tumor models to RT. Tumor growth was quantified by measuring bioluminescence (BLI) using IVIS-Xenogen. Image-guided radiation therapy using an Xstrahl small animal radiation research platform and Muriplan planning software was used to deliver a single dose of 10 Gy (sRT) or 5 daily fractions of 6 Gy (fRT). Murine TGFβ neutralizing monoclonal antibody, 1D11, was administered i.p. and mice were monitored by BLI and physical symptoms. Combine treatment with 1D11 and RT led to an increase in median survival compared to RT alone using fRT (49 vs 31 days) or sRT (41 vs 33 days). fRT eliminated tumors in 4/9 mice whereas sRT eliminated 2/12. Double treated mice had similar response by fRT (3/8), but increased with sRT (5/13). Mice that showed complete rejection of tumor were re-challenged with subcutaneous injections of the same tumor cells. Re-challenge showed that only sRT double-treated 4T1-BrA rejected newly tumors. Effective intracranial control of BCBM was achieved by

RT and TGFβ inhibition of intracranial tumors and subsequent rejection of tumor re-challenge indicates effective intracranial tumor control can elicit immune memory.

Positron emission tomography imaging of TGF- ß **using ^{89}Zr-DFO-Fresolimumab.**

Denis R. Beckford-Vera, Alba Gonzalez-Junca, Tony L. Huynh, Niecholle Roco, Jessica S Janneck, Joseph E. Blecha , Mary H Barcellos-Hoff, Benjamin L. Franc, and Henry F. VanBrocklin

Transforming growth factor-ß (TGF- ß) leads to more invasive and metastatic tumor phenotypes. Hence, TGF- ß is a rational target for therapy monitoring in highly invasive or metastatic tumors such as glioblastomas and metastatic breast cancer. Clinical imaging of TGF- ß can have a relevant role in patient selection and therapy monitoring. Therefore, our aim is to demonstrated the feasibility of using ^{89}Zr-DFO-fresolimumab for immunoPET imaging of TGF- ß.

Fresolimumab was modified with 1-(4-isothiocyanatophenyl)-3-[6,17-dihydroxy-7,10,18,21-tetraoxo-27-[N-acetylhydroxylamino)-6,11,17,22-tetrazaheptaeicosine]-thiourea (DFO-CNS) and radiolabeled with Zr-89. Quantification of the number of DFO per fresolimumab molecule was determined by isotopic dilution. Immunoreactivity of ^{89}Zr-DFO-fresolimumab was assessed in a competition assay with unmodified fresolimumab using TGF-ß3 as a target antigen. We tested the feasibility ^{89}Zr-DFO-fresolimumab for immunoPET imaging of active TGF- ß in a preclinical model of breast cancer brain metastasis. We generated a murine model of triple negative breast cancer cells selected for their capacity to growth into the brain of immunocompetent balb/c mice. Mice were injected with ^{89}Zr-DFO-fresolimumab with different specific activities and microPET/CT images were acquired at 24 and 96h post i.v. injection.

^{89}Zr-DFO-fresolimumab was isolated with radiolabeling yields ranging from 76-90% and specific activities ranged from 3.2 -732 MBq/mg. Following size exclusion purification, the radiochemical purity of ^{89}Zr-DFO-fresolimumab was greater than 98%. After conjugation and radiolabeling, ^{89}Zr-DFO-fresolimumab exhibited high affinity and specific binding to TGF-ß (Fig 1 A). Competition of ^{89}Zr-DFO-fresolimumab against unlabeled fresolimumab yield a half maximum inhibitory concentration of 16 nM. High tumor

uptake was observed as early as 24h post injection of ^{89}Zr-DFO-fresolimumab in a preclinical model of breast cancer brain metastasis (Fig 1B) allowing for clear tumor visualization. Hence, demonstrating that ^{89}Zr-DFO-fresolimumab is capable of crossing the damaged blood brain barrier. Unexpectedly, higher tumor uptake was observed when ^{89}Zr-DFO-fresolimumab with low specific activity was administered. Clinical translation of non-invasive immuno-PET imaging of TGF- ß with ^{89}Zr-DFO-fresolimumab may be particular useful for patient selection and therapy monitoring due the dual functions of TGF- ß.

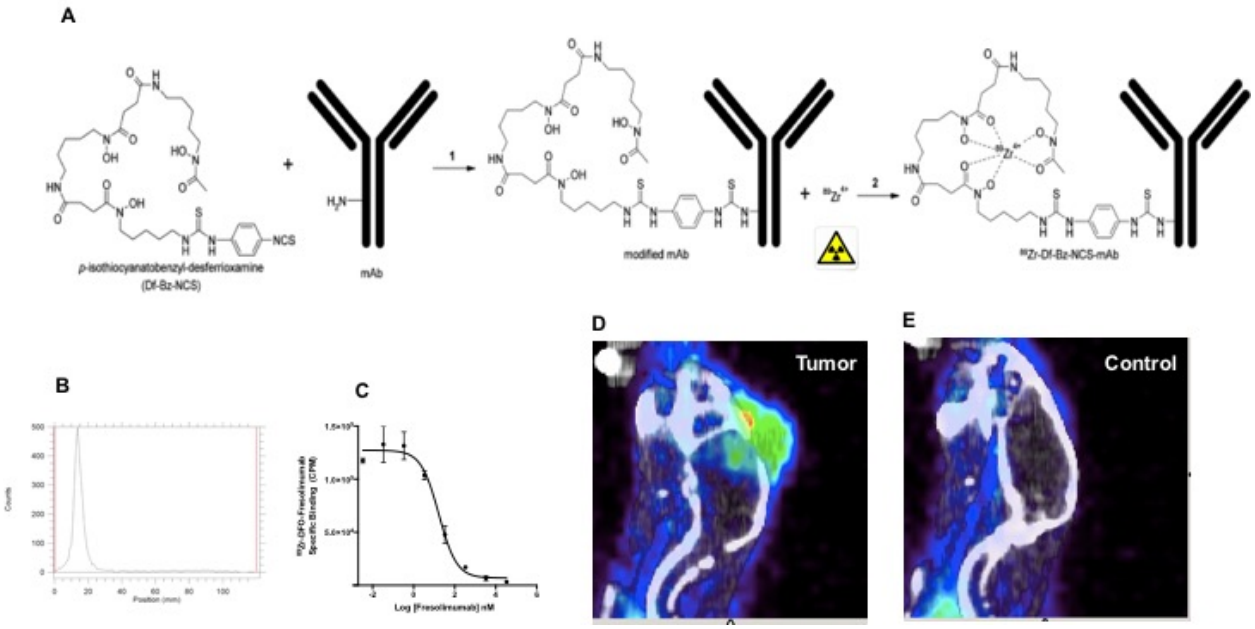


Figure 1 (A) Preparation of ^{89}Zr-DFO-fresolimumab. (B) Radio thin-layer chromatography of purified ^{89}Zr-DFO-fresolimumab showing high radiochemical purity. (C) Competition binding assay of ^{89}Zr-DFO-fresolimumab against unlabeled fresolimumab (IC50 = 16nM). (D) microPET/CT image of ^{89}Zr-DFO-fresolimuma in a preclinical model of breast cancer brain metastasis showing high tumor uptake and (E)) microPET/CT image of ^{89}Zr-DFO-fresolimumab in a control model.

Automated synthesis of 5-[^{18}F]fluoro-α-methyl tryptophan (5-[^{18}F]F-AMT)

Joseph E. Blecha[1], Thomas Hayes[1], Tony Huynh[1],Luis Borrero Garcia[2], William Chou[2], Mary Helen Barcellos-Hoff[2], Benjamin Franc[1], and Henry F. VanBrocklin[1]

1. Department of Radiology and Biomedical Imaging, UCSF, SF CA 94107
2. Helen Diller Family Comprehensive Cancer Center, UCSF, SF CA 94115

Objectives:
Tryptophan is an essential amino acid that is metabolized in cancer by indoleamine 2,3-dioxygenase 1 (IDO1) to L-kynureine, promoting tumor growth and immune system suppression. (1) Carbon-11-α-methyl tryptophan was identified as an inhibitor of IDO1 and not a substrate for protein synthesis. (2) Recently, a fluorine-18 version, 5-[^{18}F]F-AMT (Figure), has been shown to be taken up in melanoma xenografts. (3) The inspiration behind the current project was to image brain metastatic cancer using 5-[^{18}F]F-AMT. Based on the foundational synthetic effort work (3), we sought to automate the production of 5-[^{18}F]F-AMT on the Sofie Biosciences ELIXYS FLEX/ CHEM module to prepare the tracer preclinical imaging studies and future human translational research studies.

Methods:
The BPin precursor was prepared in 5 steps from methyl tryptophan as described in Giglio et. al. (3). The original labeling procedure utilized tetrabutyl ammonium [^{18}F]fluoride, however in our hands the labeling was inconsistent. We employed a labeling technique published by Mossine et. al. (4, 5) to achieve consistent labeling. Labeling using the KOTf/ K_2CO_3 system allowed us to concentrate the [^{18}F]fluoride ion on a QMA sep pak without adding large amounts of K_2CO_3 into the BPin/ copper catalyst reaction. Larger amounts of DMF (350 μL: 50 μL) was needed for the [^{18}F]fluorination step, thus a C18 sep pak was required to lower the total reaction volume for the TFA decyclization step. DMF (50 μL) was back into the 500 μL of TFA to keep the hydrophobic compound soluble for the reaction. The TFA was evaporated and NaOH was added to the reaction for deprotection. The reaction mixture was neutralized and injected for purification onto an analytical HPLC. The product was collected, diluted up, and loaded onto a C18 light sep pak. The final purified product was eluted with ethanol and taken to dryness. The final formulation of 0.9% saline was added and imaging studies were conducted.

Results:
Incorporation yields (analyzed by radioTLC, non-decay corrected) were sufficient and consistent (25% $\pm$ 5%, n = 5). The automation on the ELIXYS required volume and reaction condition adjustments to achieve suitable synthetic yields. The decyclization and deprotection scheme was one of the most challenging parts of the automation with various partially protected species being present in the HPLC purification. All of the possible species formed from various partial reactions are all separable on the HPLC using the gradient solvent system of 5: 95% acetonitrile with 0.1% TFA over 30 mins. The addition of the 50 μL of DMF to the TFA led to more efficient decyclization. NaOH

was substituted for KOH in the deprotection reaction as older KOH did not accomplish complete deprotection.

Conclusions:
Automation of 5-[^{18}F]F-AMT was completed on the Sofie Biosciences ELIXYS FLEX/ CHEM module and the synthesis takes 120 ± 10 minutes, with a yield of 1.52 ± 1.04% (n = 3, decay corrected). Adequate amounts of 5-[^{18}F]F-AMT (37- 185 MBq) were formulated and injected into the metastatic tumor model in mice to perform imaging studies. The purification of [^{18}F]F-AMT is still under examination, when larger volumes of the reaction mixture are loaded onto the HPLC the yield is lower. These lower amounts of purified product injections led directly to the low yields presented.

Acknowledgements:
The work was supported by grant number W81XWH-17-1-0033 from the US Department of Defense Breast Cancer Research Program (BF).

References:

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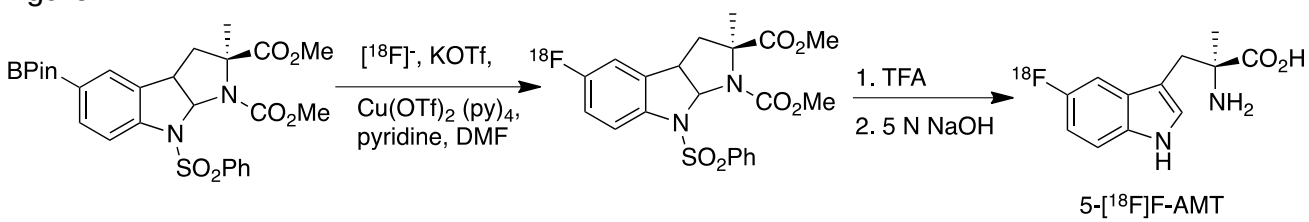
Figure:



5-[^{18}F]F-AMT

Keywords: 5-[^{18}F]F-AMT, boron pinacol ester, ELIXYS, copper- mediated labeling

APPENDIX B: FIGURES CITED IN TEXT

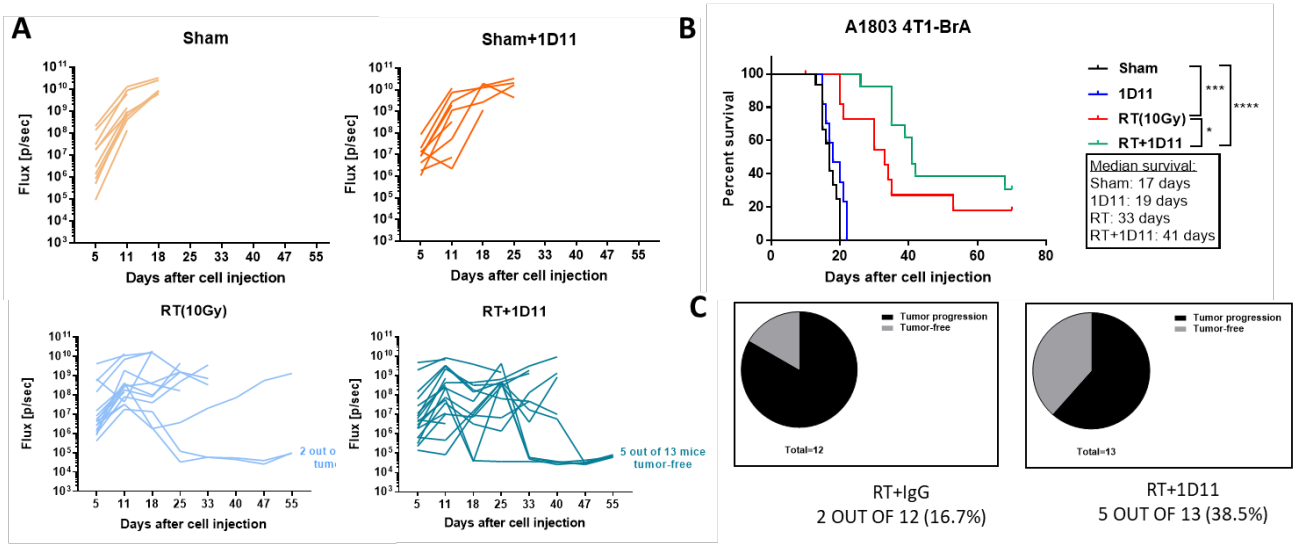


Figure 1. Treatment with RT (10 Gy) and/or IgG/1D11 in the 4T1-BrA murine model. A. Individual tumor growth curves of all mice according to treatment. B. Kaplan Meier survival plots of survival following IGRT and/or ID11. B. Tumor burden measure by BLI at 20 days. C. Frequency of tumor-free mice as a function of RT or RT and 1D11.

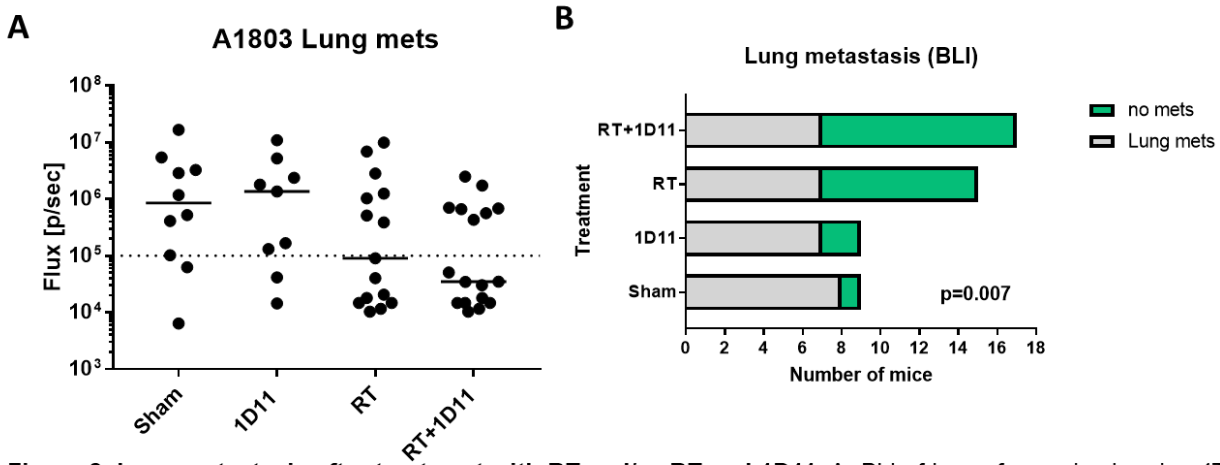


Figure 2. Lung metastasis after treatment with RT and/or RT and 1D11. A. BLI of lungs from mice bearing 4T1 tumors treated with 10 Gy RT with and without TGFβ inhibition. B. Frequency of metastasis-free mice as a function of RT or RT and 1D11.

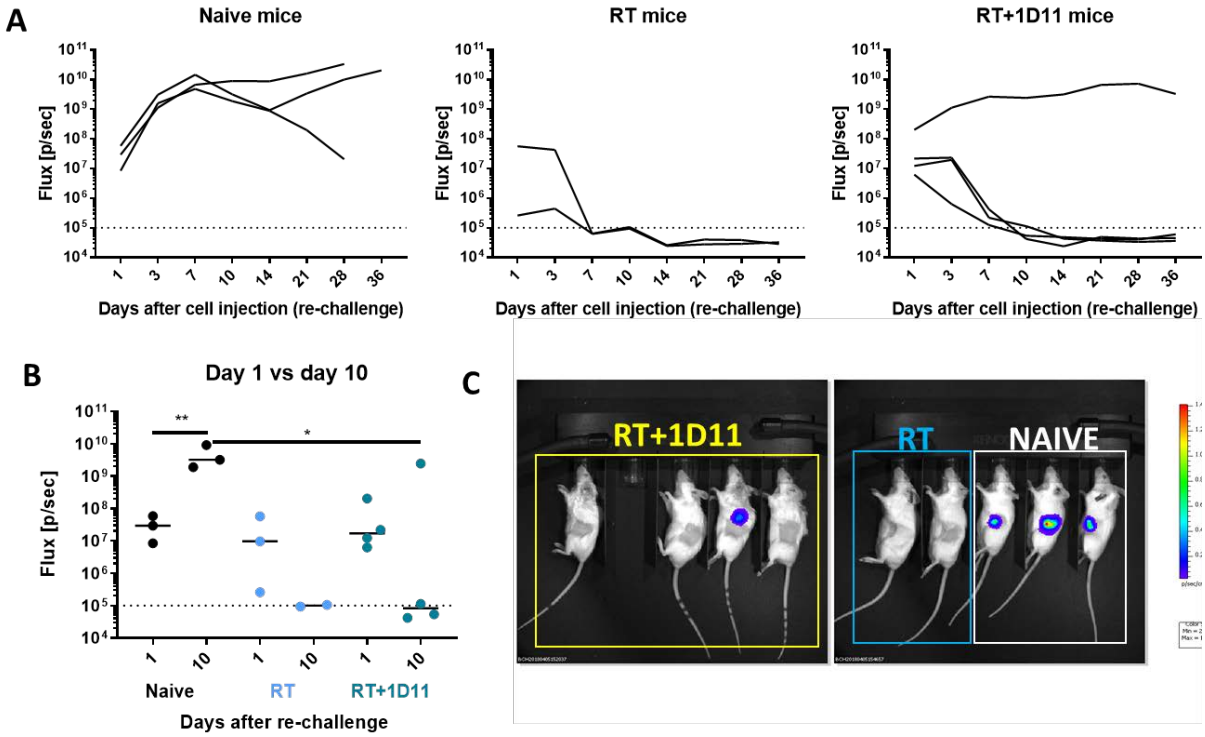


Figure 3. Rechallenge of tumor-free mice with subcutaneous tumors. A. BLI of subcutaneous tumors injection of naïve and mice who exhibited long term survival after indicated treatment B. BLI comparison of day 1 vs day 10 after rechallenge with flank tumors. C. Representative images of BLI of subcutaneous tumors.

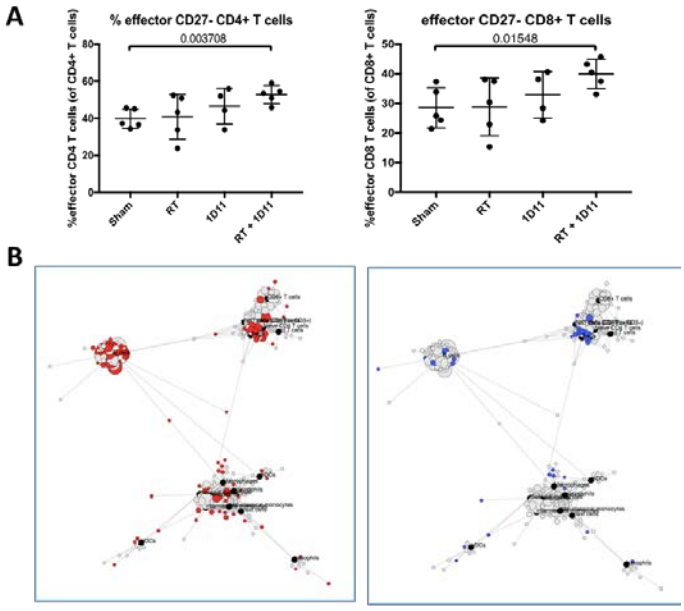


Figure 4. Preliminary CyTOF analysis of 4T1-BrA cells treated with RT (10 Gy) and /or IgG/1D11. Percentage of CD27-CD4+ T-cells and CD27-CD8+ T-cells in all treated groups. B. Scaffold maps of PD1 and PDL1 using semi-unbiased analysis where immune cells are binned into different clusters represented by the circles. The size of the circles represent number of cells in that cluster. The color (red vs. blue) represents the increase (red) vs. decrease (blue) of cells in condition 2 when compared to condition 1.