

AWARD NUMBER: W81XWH-17-1-0033

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

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REPORT DATE: February 2018

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;  
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# REPORT DOCUMENTATION PAGE

*Form Approved*  
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<b>1. REPORT DATE</b> February 2018		<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED</b> 1 Feb 2017 - 31 Jan 2018	
<b>4. TITLE AND SUBTITLE</b> Dual Benefit of TGFβ Inhibition on Tumor Control in the Context of Radiotherapy for Breast Cancer Brain Metastases				<b>5a. CONTRACT NUMBER</b>	
				<b>5b. GRANT NUMBER</b> W81XWH-17-1-0033	
				<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b> Benjamin Franc  E-Mail: Benjamin.Franc@ucsf.edu				<b>5d. PROJECT NUMBER</b>	
				<b>5e. TASK NUMBER</b>	
				<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> University of California, San Francisco 3333 California Street, Suite 315 San Francisco, CA 94143-0962				<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>	
				<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited					
<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> This project evaluates whether TGFβ inhibition during radiation therapy (RT) to breast cancer brain metastases (BCBM) provides greater therapeutic benefit than RT alone using a proof-of-concept therapeutic protocol in combination with innovative functional imaging that can demonstrate efficacy in an in vivo system. In year 1 of this two-year project, we generated and characterized two syngeneic mouse models of triple negative breast cancer (TNBC) brain metastasis. We provided image-guided radiotherapy (IGRT) to murine BCBM using the small animal radiation research platform (SARRP) and assessed the benefit of TGFβ inhibition in the context of IGRT. We assessed tumor microenvironment (TME) and immune system characterization as a function of radiotherapy and in combination with TGFβ blockade (1D11). In order to monitor active TGFβ in vivo, we synthesized and characterized 89Zr-DFO-fresolimumab in vivo. We imaged tumor metabolism, tumor growth, and activated T cells associated with immunological response to tumor over the natural progression of the untreated (control) arm of the study. In year 2 of this project, we will ascertain the benefit of including TGFβ inhibition in addition to targeted radiation in these BCBM models and determine whether this endorses response to immunotherapy. We will complete our functional imaging methods to assess drug distribution, tumor burden and immunological response to therapy in the treatment arm.					
<b>15. SUBJECT TERMS</b> breast cancer brain metastases, transforming growth factor beta (TGFβ), immunotherapy, radiation therapy (RT), gamma-knife stereotactic radiosurgery (GKSRS), molecular imaging, positron emission tomography (PET)					
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT</b>	<b>18. NUMBER OF PAGES</b>	<b>19a. NAME OF RESPONSIBLE PERSON</b>
<b>a. REPORT</b>	<b>b. ABSTRACT</b>	<b>c. THIS PAGE</b>			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	25	<b>19b. TELEPHONE NUMBER</b> (include area code)

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1. **INTRODUCTION:** The goal of this project is to provide the rationale to revolutionize therapy regimens for women with brain metastases and thereby reduce the mortality associated with metastatic breast cancer. This project evaluates whether TGF $\beta$  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 that can demonstrate efficacy in an in vivo system. Successful demonstration that transforming growth factor beta (TGF $\beta$ ) inhibition provides durable RT response and augmented immunotherapy response in preclinical BCBM models will provide a strong rationale for clinical trials of gamma-knife stereotactic radiosurgery (GKSRS) for women with metastatic disease. The use of molecular imaging to assess drug delivery, therapeutic response via tumor metabolism, and identify potential immune – mediated predictors of response during this project will enable rapid clinical translation of combined RT and immunotherapy regimens.
2. **KEYWORDS:** breast cancer brain metastases, transforming growth factor beta (TGF $\beta$ ), 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
<b>Specific Aim 1:</b> Ascertain the benefit of TGF $\beta$ 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.			
<b>Major Task 1:</b> Evaluate TRI-Modal Therapy			
Subtask 1: Establish brain tumor metastasis models <ul style="list-style-type: none"> <li>Establish and characterize the brain metastasis models</li> </ul>	1-3	1-6	100
Subtask 2: Establish 2 cohorts of 80 mice <ul style="list-style-type: none"> <li>Image mice using bioluminescence and ascertain tumor burden</li> <li>Randomize to treatment arms</li> <li>Design single fraction treatment plan for each mouse</li> <li>Irradiate and monitor mice</li> <li>Transfer mice for functional imaging studies</li> <li>Complete imaging-based tumor response and immune modulation assessments in all cohorts and collect tissue at morbidity</li> </ul>	3-6	6-18	50%
<b>Major Task 2:</b> Correlation of biological processes with outcome			
Subtask 1: Preparation of tissues for immunoscore and tumor analysis (e.g. embedding, sectioning)	6-7	12-24	35%
Subtask 2: Analyze splenic immune repertoire and circulating cells by FACS	4-6	6-18	25%
<b>Major Task 3:</b> Replicate experiment using 2 fractionated radiation protocols			
Subtask 4: <ul style="list-style-type: none"> <li>Establish second brain metastasis model</li> <li>Use optimized protocol in second brain metastasis model</li> </ul>	12-15	6-18	20%

	<b>Proposed Timeline (Months)</b>	<b>Revised Timeline (Months)</b>	<b>% Complete to Date</b>
<b>Specific Aim 2:</b> Characterize functional imaging methods to assess drug distribution, tumor burden and immunological response to RT			
<b>Major Task 1:</b> 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 <ul style="list-style-type: none"> <li>Complete imaging-based tumor response and immune modulation assessments in all cohorts and collect tissue at morbidity</li> </ul>	4-12	9-20	33%
<b>Milestone #1:</b> Prepare manuscript on RT responses mediated by TGF $\beta$	12-15	20-22	20%
Subtask 3: Evaluate immunological responses mediated by RT <ul style="list-style-type: none"> <li>Assess systemic and localized processes that associate with decreased tumor burden following various arms of therapy</li> <li>Re-evaluate experimental design and optimize protocol</li> </ul>	12-15	12-16	20%
<ul style="list-style-type: none"> <li>Subtask 4: Assess best evidence and predictors for biological efficacy of combination.</li> </ul>	18-24	18-24	20%
<b>Milestone #2:</b> Prepare manuscript on use of the TRI-MODAL therapy in pre-clinical studies	18-24	18-24	5%

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

i. Major activities

**Ascertain the benefit of TGF $\beta$  inhibition in preclinical immunocompetent BCBM (Barcellos Hoff)**

**Specific Aim 1:** Ascertain the benefit of TGF $\beta$  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 TGF $\beta$  inhibition in the context of IGRT
- Tumor microenvironment (TME) and immune system characterization as a function of radiotherapy and in combination with TGF $\beta$  blockade (1D11).

**Molecular Imaging (Franc)**

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

**Major Activities:**

- Synthesis and characterization of <sup>89</sup>Zr-DFO-fresolimumab
- In vivo characterization of <sup>89</sup>Zr-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)

ii. specific objectives

**Ascertain the benefit of TGF $\beta$  inhibition in preclinical immunocompetent BCBM (Barcellos Hoff)**

*Generation of murine BCBM model.* 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.

*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. TGF $\beta$  activity was assessed using immunofluorescence.

A second BCBM model has been generated using TSA-LUC breast cancer cells by direct injection into the brain of Balb/c female mice. Upon tumor formation, brains were collected for histology or enzymatically digested. Breast cancer cells were sorted based on EpCAM expression and expanded as a new cell line (TSA-BrA).

*Treatment of BCBM with IGRT.* 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. CD3+ tumor-infiltrating T-cells were measured as a function of treatment.

*Assessing the benefit of TGF $\beta$  inhibition in the context of IGRT.* 4T1-BrA cells were injected into the brain and mice were randomized to control or treated arms based on BLI. Mice were treated with 1D11 TGF $\beta$ -neutralizing monoclonal antibody or isotype control IgG prior to irradiation with 5 daily fractions of 6Gy or sham irradiated controls. A subset of mice (n=3) were euthanized 5 days after completion of the treatment to collect specimens. Tumor growth was monitored by BLI and Kaplan-Meier survival curves were generated. Tumor microenvironment (TME) and immune system characterization as a function of radiotherapy and in combination with 1D11 TGF $\beta$  neutralization. Murine brains from different treatment groups were FFPE and the immune system populations were characterized by immunofluorescence. Myeloid-derived suppressive cells (MDSC) were assessed by CD11b/Ly6G-Gr1 positivity. Tumor-infiltrating CD3+ T-cells were quantified. Surviving, tumor-free mice were re-challenged with subcutaneous implantation of parental 4T1-BrA tumor cells.

### **Molecular Imaging (Franc)**

*<sup>89</sup>Zr-DFO-fresolimumab synthesis and characterization* – We successfully modified fresolimumab with 1-(4-isothiocyanatophenyl)-3-[6,17-dihydroxy-7,10,18,21-tetraoxo-27-[N-acetylhydroxylamino)-6,11,17,22-tetrazaheptaecosine]-thiourea (DFO-CNS) and radiolabeled it with Zr-89. Using size-exclusion HPLC and iTLC, <sup>89</sup>Zr-DFO-fresolimumab was successfully purified and the number of DFO per fresolimumab was determined. <sup>89</sup>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 <sup>89</sup>Zr-DFO-fresolimumab was accomplished. The specificity of <sup>89</sup>Zr-DFO-fresolimumab for immuno-PET imaging of active TGF $\beta$  in 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 <sup>18</sup>F FDG PET regularly beginning 7 days following implantation to evaluate combined metabolic signal from tumor and immune response. BCBM were imaged with <sup>18</sup>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 <sup>89</sup>Zr-DFO-fresolimumab PET regularly beginning 7 days following implantation to evaluate combined TGF $\beta$  signal from tumor and immune response.

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

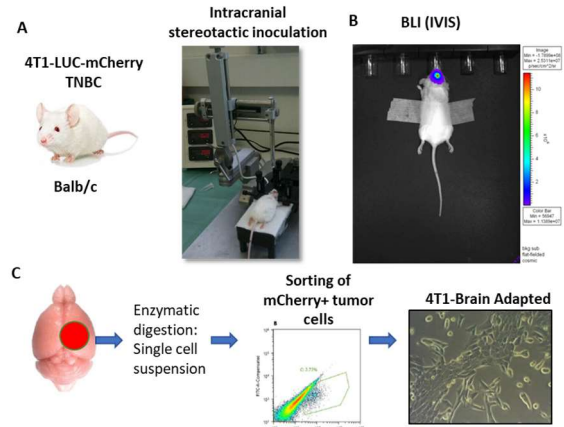


## Barcellos Hoff

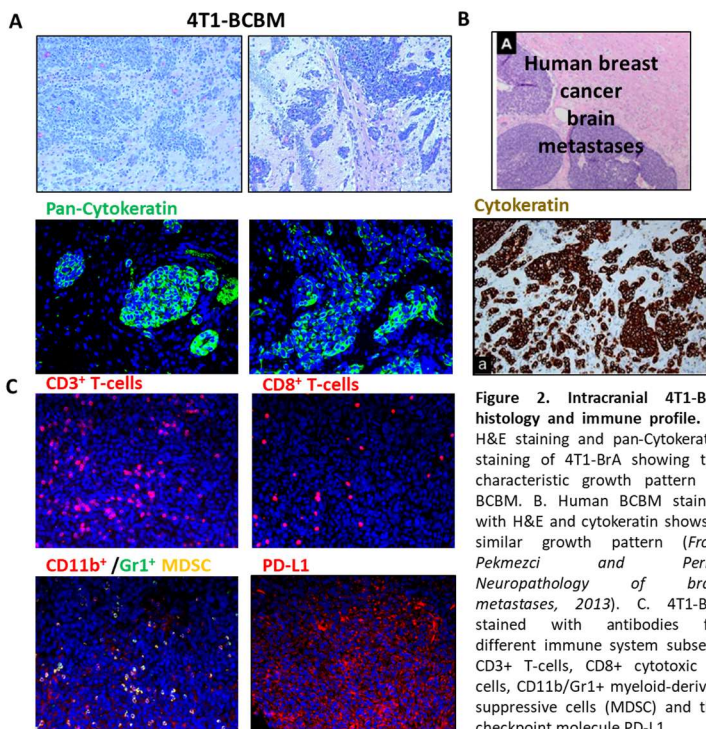
### Ascertain the benefit of TGF $\beta$ inhibition in preclinical immunocompetent BCBM

We successfully generated 2 syngeneic mouse models of brain adapted breast cancer cells (**Figure 1**). Median survival for mice harboring intracranial 4T1-BrA was 20 days. The TSA-BrA exhibits a similar growth pattern as 4T1BrA. This model has a similar latency and a median survival of 21 days but seems to be less aggressive than 4T1BrA, as are the orthotopic parental cells. These new models will allow us to characterize the response of breast cancer in the brain parenchyma to treatment.

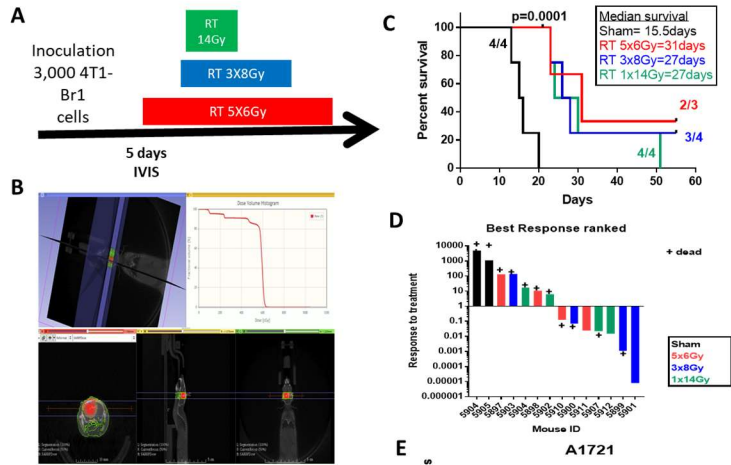
These tumors recapitulate the growth pattern, histology and some of the main features of human BCBM. Both TSA-BrA and 4T1-BrA tumors form invasive nests into the brain parenchyma (**Figure 2**). The 4T1-BrA form highly immune cell-infiltrated tumors in the brain parenchyma, similar to parental 4T1 subcutaneous 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. All 3 RT schedules controlled 4T1-BrA tumors and significantly increasing median survival to 27-31 days compared to 15 days for the sham-irradiated mice. (**Figure 3A**). RT alone increased the CD3+ T-cell infiltration (**Figure 3B**).



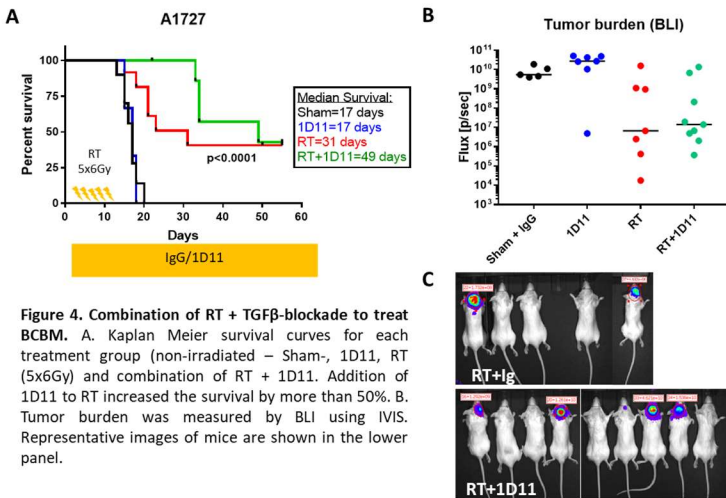
**Figure 1.** Generation of a syngeneic mouse model of BCBM. A. 4T1 murine TNBC cells were injected intracranially into female Balb/c immunocompetent mice using stereotactic device. B. Tumor growth was monitored by bioluminescence (BLI) using IVIS Xenogen. C. Upon tumor growth, brain was collected and digested using collagenase + hyaluronidase + DNase enzymatic cocktail. Tumor cells were sorted by mCherry positivity and a novel cell line 4T1 brain-adapted was generated.



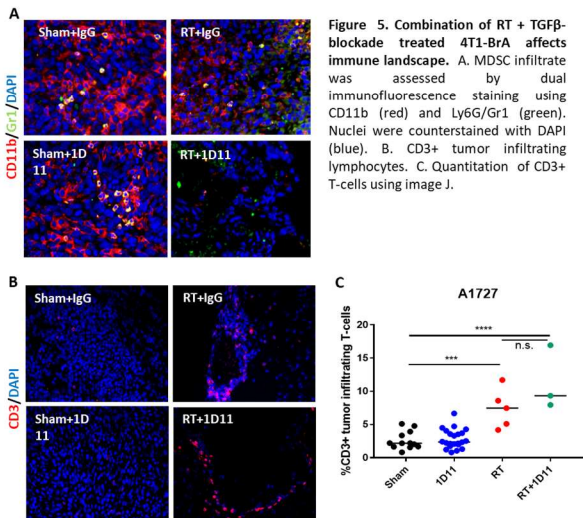
Combined treatment with RT and 1D11 provides therapeutic benefit to fractionated RT (**Figure 4**). Addition of 1D11 increased median survival by 58% compared to fractionated RT alone (31 days for RT vs 49 days for RT+1D11). Approximately 50% of mice of both RT (n=7) and RT+1D11 (n=9) survived more than 50 days without BLI evidence of tumor regrowth. Combination of RT+1D11 significantly reduced the amount of infiltrating MDSC (**Figure 5A**) and increased CD3+ T-cell infiltrate (**Figure 5B,C**), suggesting a shift towards anti-tumor immunity. However, surviving mice (n=3) that were rechallenged did not reject 4T1-BrA injected in the flank, indicating that TGF $\beta$  inhibition is likely acting by increasing the efficacy of radiation. Nonetheless, the immune landscape may be evidence that combination with immunotherapy will increase tumor eradication.



**Figure 3. Comparison of different protocols of radiation on a brain-adapted breast cancer model.** Intracranial 4T1-BrA cells were treated with equivalent protocols of radiation. B. Example of treatment plan using Muriplan and SARRP. C. Kaplan-Meier survival curves for non-irradiated and irradiated mice. D. Waterfall plot showing the best response by BLI. E. CD3+ T-cell infiltrate was assessed by immunofluorescence and quantified by ImageJ.



**Figure 4. Combination of RT + TGF $\beta$ -blockade to treat BCBM.** A. Kaplan Meier survival curves for each treatment group (non-irradiated - Sham-, 1D11, RT (5x6Gy) and combination of RT + 1D11. Addition of 1D11 to RT increased the survival by more than 50%. B. Tumor burden was measured by BLI using IVIS. Representative images of mice are shown in the lower panel.



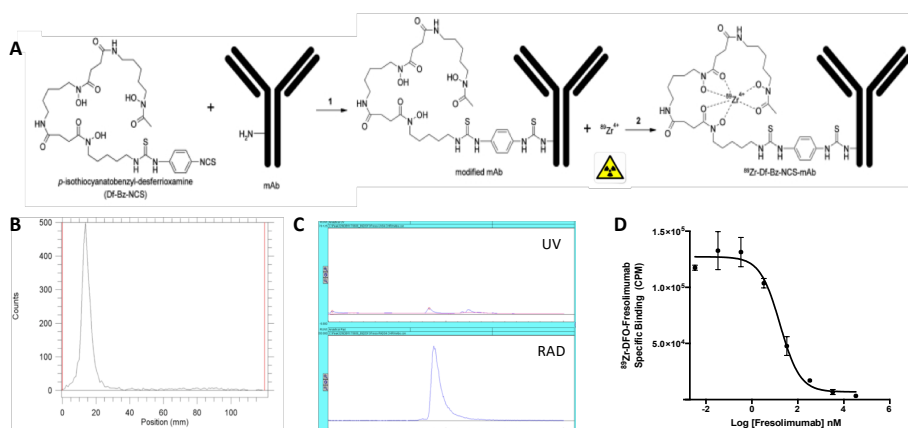
**Figure 5. Combination of RT + TGF $\beta$ -blockade treated 4T1-BrA affects immune landscape.** A. MDSC infiltrate was assessed by dual immunofluorescence staining using CD11b (red) and Ly6G/Gr1 (green). Nuclei were counterstained with DAPI (blue). B. CD3+ tumor infiltrating lymphocytes. C. Quantitation of CD3+ T-cells using ImageJ.

## **Franc** **Molecular Imaging**

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

The radiolabeling of DFO-fresolimumab conjugate was performed in aqueous conditions and room temperature. Characterization of the  $^{89}\text{Zr}$ -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.  $^{89}\text{Zr}$ -DFO-fresolimumab radiolabeling yield was > 76% and after purification the radiochemical purity of  $^{89}\text{Zr}$ -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  $^{89}\text{Zr}$ -DFO-fresolimumab was assessed in a competition assay with unmodified fresolimumab using TGF- $\beta$ 3 as a target antigen. TGF- $\beta$ 3 was selected because it has the highest affinity toward fresolimumab. Competition of  $^{89}\text{Zr}$ -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  $^{89}\text{Zr}$ -DFO-fresolimumab were prepared and their characterization parameters were reproducible.



**Figure 6. Synthesis and validation of  $^{89}\text{Zr}$ -Fresolimumab.** A. Schematic representation of conjugation and radiolabeling of fresolimumab. B. Radio thin-layer chromatography of purified  $^{89}\text{Zr}$ -DFO-fresolimumab showing radiochemical purity higher than 98%. C. HPLC chromatogram of purified  $^{89}\text{Zr}$ -DFO-fresolimumab. D. Competition binding assay of  $^{89}\text{Zr}$ -DFO-fresolimumab against unlabeled fresolimumab ( $\text{IC}_{50} = 16\text{nM}$ ).

*In vivo characterization of  $^{89}\text{Zr}$ -DFO-fresolimumab* - We tested the feasibility of  $^{89}\text{Zr}$ -DFO-fresolimumab for immuno-PET imaging of active TGF $\beta$  in 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  $^{89}\text{Zr}$ -DFO-fresolimumab with different specific activities and  $\mu\text{PET}/\text{CT}$  images were acquired at 24 and 96h post-injection. High tumor uptake was observed as early as 24h post-injection of  $^{89}\text{Zr}$ -DFO-fresolimumab in this preclinical model allowing for clear tumor visualization, which demonstrates that  $^{89}\text{Zr}$ -DFO-fresolimumab is capable of crossing the damaged blood brain barrier. Preparations of  $^{89}\text{Zr}$ -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.

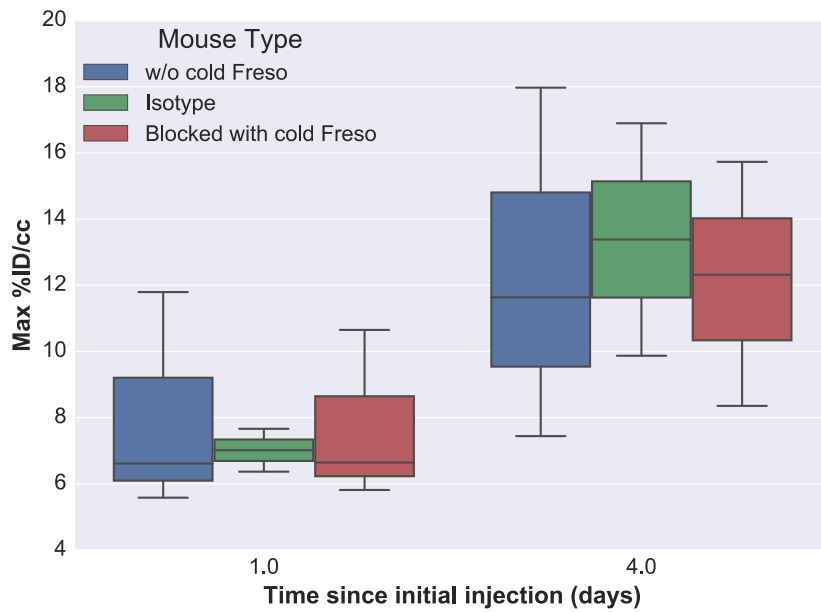


Figure 7. Biodistribution of <sup>89</sup>Zr-Fresolimumab in BCBM as a function of time.

In order to evaluate the feasibility of immuno-PET imaging of active TGFβ with <sup>89</sup>Zr-DFO-fresolimumab we performed μPET/CT studies of <sup>89</sup>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 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 <sup>89</sup>Zr-DFO-isotype and <sup>89</sup>Zr-DFO-PEG in order to assess the specificity of <sup>89</sup>Zr-DFO-fresolimumab toward active TGF-β. *Ex vivo* biodistribution studies were also conducted in order to quantify and correlated the μPET/CT images. <sup>89</sup>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 <sup>89</sup>Zr-DFO-fresolimumab uptake when compared to non-treated tumors. These results correlated with those in the *ex vivo* biodistribution studies.

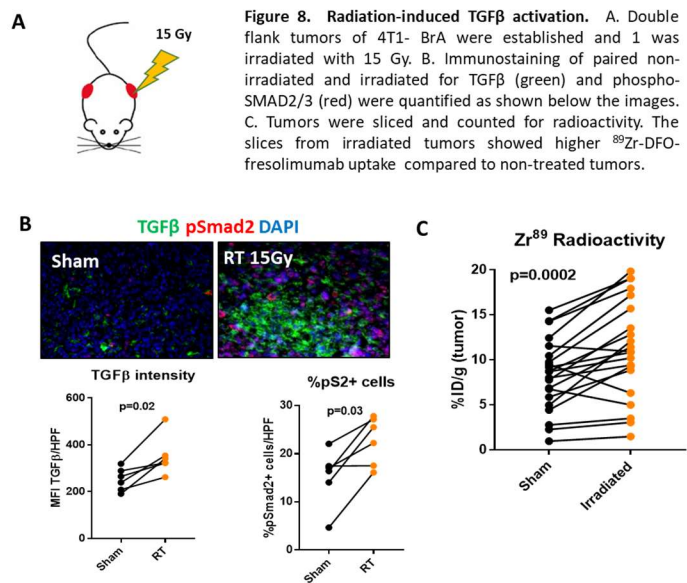


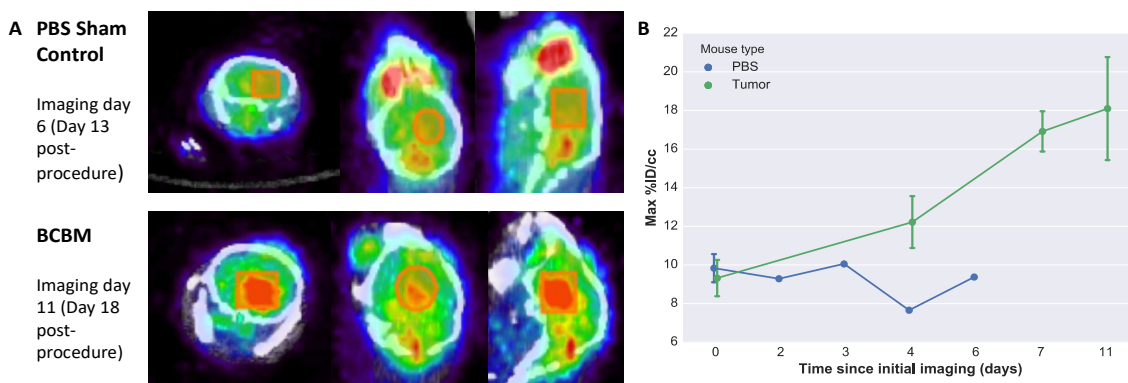
Figure 8. Radiation-induced TGFβ activation. A. Double flank tumors of 4T1- Bra were established and 1 was irradiated with 15 Gy. B. Immunostaining of paired non-irradiated and irradiated for TGFβ (green) and phospho-SMAD2/3 (red) were quantified as shown below the images. C. Tumors were sliced and counted for radioactivity. The slices from irradiated tumors showed higher <sup>89</sup>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  $^{18}\text{F}$  FDG PET regularly beginning 7 days following implantation to evaluate combined metabolic signal from tumor and immune response* – Significantly increased uptake of  $^{18}\text{F}$  FDG was observed in the intracranial tumor over that observed in sham mice who had received an intracranial injection of PBS, suggesting  $^{18}\text{F}$  FDG uptake due to a combination of tumor metabolism and tumor-directed immunity rather than due to non-specific localization.  $^{18}\text{F}$  FDG signal continued to increase over the approximate 2-week course of disease (**Figure 9**).

*Image BCBM with  $^{18}\text{F}$ -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  $^{18}\text{F}$ -AraG uptake in tumor versus PBS control over the course of the disease in this cohort of untreated mice.



**Figure 9.**  $^{18}\text{F}$  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.

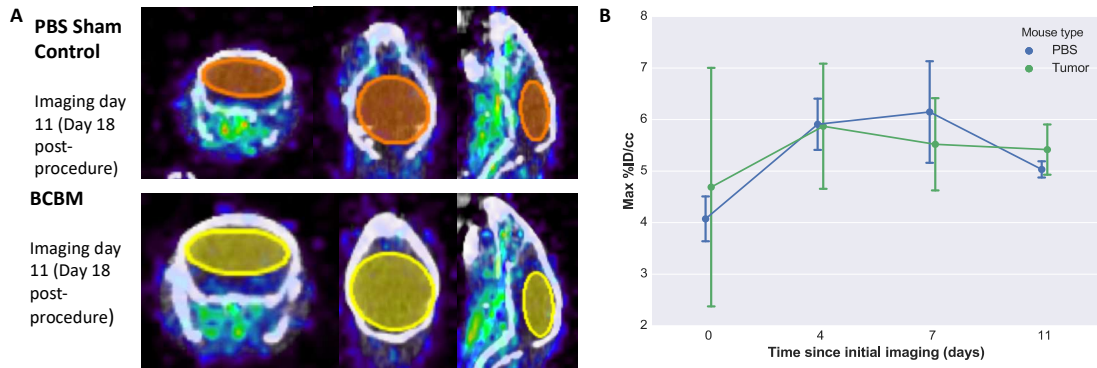


Figure 10.  $^{18}\text{F}$  AraG uptake over the natural course of BCBM growth. A. axial, coronal, and sagittal fused PET/CT images demonstrating volumes of interest from which statistics were derived using AMIDE. B. Maximum injected dose per volume measured from VOIs of PET/CT images

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  $^{89}\text{Zr}$ -DFO-fresolimumab PET to evaluate combined  $\text{TGF}\beta$  signal from tumor and immune response* – Uptake of  $^{89}\text{Zr}$ -DFO-fresolimumab was significantly increased in tumor over PBS control. Each mouse received a single injection of  $^{89}\text{Zr}$ -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  $\text{TGF}\beta$  activity in the tumor (Figure 11).

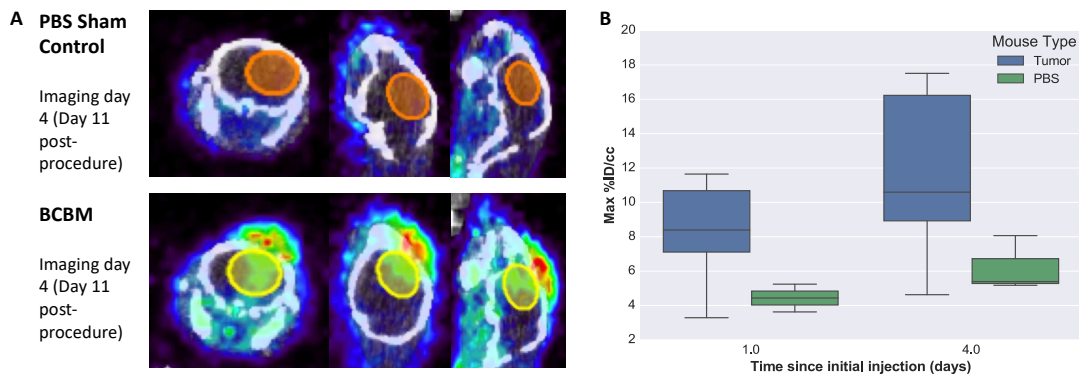


Figure 11.  $^{89}\text{Zr}$  – Fresolimumab anti-TGF- $\beta$  uptake over the natural course of BCBM growth. A. axial, coronal, and sagittal fused PET/CT images demonstrating volumes of interest from which statistics were derived using AMIDE. B. Maximum injected dose per volume measured from VOIs of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

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

### Ascertain the benefit of $\text{TGF}\beta$ inhibition in preclinical immunocompetent BCBM (Barcellos Hoff)

In the first year of the DOD funding, we were delayed by the poor quality of established mouse models of syngeneic brain metastatic breast cancer. Our initial intent was to use a TNBC 4T1 murine cell line that has been selected for its spontaneous brain-metastatic capacity [cite]. We received the 4T1-BR3 and 4T1-BR5 cell lines from 2 independent laboratories. We routinely perform authentication and testing of all cell lines and we discovered that both cell lines were positive for mycoplasma. Given our time line and objective, we chose to establish two brain-adapted breast cancer cell lines (4T1-BrA and TSA-BrA), which also express luciferase for monitoring. These models are now set up and characterized but delayed our initial aims by 6 months.

We tested the tumor response to several radiation doses and fractionation schemes in both models and then combined fractionated protocol with TGF $\beta$  inhibition in the 4T1-BrA. This combination provides substantial increase in survival, consistent with our primary hypothesis that TGF $\beta$  inhibition compromises tumor DNA damage response, and thus will augment response to RT.

Notably, RT alone appeared to provide durable response, i.e. survival in half of the mice. To further explore this therapeutic benefit, we will repeat the experiments using a sub-optimal radiation dose of 10 Gy, to be able to study whether the addition of TGF $\beta$  blockade provides long term benefit.

### **Molecular Imaging (Franc)**

In Year 1, we characterized the growth and immune response to a BCBM model over the course of the disease in an untreated control cohort using PET with a variety of radiopharmaceuticals. In Year 2, we will repeat these experiments on animals that have received the optimal combined radiotherapy and immune therapy regimen.

The original research plan included an exploratory aim to evaluate the relationship between indoleamine 2,3-dioxygenase (IDO) expression and the level of measured clinical response to therapy. IDO expression was to be assessed with PET imaging using  $\alpha$ -[<sup>11</sup>C]methyl-L-tryptophan (<sup>11</sup>C-AMT). IDO catalyzes tryptophan degradation through the kynurenine pathway. The level of IDO expression in many human tumors has been linked to tumoral immunoresistance via arrest of T lymphocyte proliferation in the presence of tryptophan shortage. In the period since our original proposal was written, the synthesis of 5-[<sup>18</sup>F]F-L- $\alpha$ -methyl tryptophan (5-[<sup>18</sup>F]F-AMT) has been reported (Giglio BC, et al., Synthesis of 5-[<sup>18</sup>F]Fluoro- $\alpha$ -methyl Tryptophan: New Trp Based PET Agents, *Theranostics*, 2017. 7(6): 1524-1530). Given the advantages of this newer agent including improved ease of synthesis and longer half-life to facilitate imaging, we will utilize this agent to assess IDO expression in Year 2 of this project.

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

##### **Ascertain the benefit of TGF $\beta$ inhibition in preclinical immunocompetent BCBM (Barcellos Hoff):**

The project has provided the postdoctoral fellow Dr. Gonzalez-Junca the opportunity to share her work at symposiums and conferences that include 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.

**Molecular Imaging:** The project provided the opportunity for a master's student, Niecholle Roco, to complete her thesis on the radiolabeling of <sup>89</sup>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 in PET imaging, and assist senior members of the team in analyzing uptake of various radiopharmaceuticals on PET images over time.

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

1. UCSF Precision Cancer Imaging Retreat and Symposium 2017 – Oral presentation Alba Gonzalez-Junca and Denis Beckford-Vera.
2. UCSF Radiology Retreat – Poster presentation Alba Gonzalez-Junca and Denis Beckford-Vera
3. UCSF Breast Oncology Retreat (BOP Retreat – February 2018) – Poster
4. UCSF Biomedical Sciences Faculty speaker 2018 - Mary Helen Barcellos-Hoff
5. AACR- Immunobiology of CNS malignancies – Poster presentation Alba Gonzalez-Junca

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

**Ascertain the benefit of TGFβ inhibition in preclinical immunocompetent BCBM (Barcellos Hoff)**

In Year 1, we showed that TGFβ inhibition not only improves tumor control by RT but facilitates a shift in the immune landscape that we predict could synergize with immunotherapy. We will repeat this study with TSA-BrA. We will also extend the 4T1-BrA to use a single dose of 10 Gy, similar to radiosurgery or gamma-knife treatment, to determine whether inhibition of TGFβ provides durable responses, and to combine this protocol with anti-PD1 immunotherapy checkpoint inhibitors. Our publication showed that the triple-therapy provided prolonged control in subcutaneous 4T1 tumors under this regimen [Vanpouille-Box, 2015 #19614]. The mice in each study will be imaged in collaboration with Dr. Franc.

**Molecular Imaging (Franc)**

In Year 1, we characterized the growth and immune response to a BCBM model over the course of the disease in an untreated control cohort using PET with a variety of radiopharmaceuticals. In Year 2, we will repeat these experiments on animals that have received the optimal combined radiotherapy and immune therapy regimen as determined by the Barcellos - Hoff laboratory. We will evaluate the relationship between indoleamine 2,3-dioxygenase (IDO) expression and the level of measured clinical response to therapy using [<sup>18</sup>F]F-AMT PET. We will evaluate drug delivery using higher doses of <sup>89</sup>Zr-DFO-fresolimumab combined with PET

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 <sup>89</sup>Zr-DFO-fresolimumab is potentially relevant to human studies of response to therapy and can be readily moved to preliminary assessment in humans.

- 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:**



**Experimental Model:** We intended to use 4T1-BR5 murine breast cancer cells that were selected for brain-metastasis capacity in vivo [Lockman, 2010 #20928]. 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 parental 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 also generated a brain-adapted model from the TSA triple-negative murine breast cancer cell line.

**IDO Imaging:** The original research plan included an exploratory aim to evaluate the relationship between indoleamine 2,3-dioxygenase (IDO) expression and the level of measured clinical response to therapy. IDO expression was to be assessed with PET imaging using  $\alpha$ -[<sup>11</sup>C]methyl-L-tryptophan (<sup>11</sup>C-AMT). IDO catalyzes tryptophan degradation through the kynurenine pathway. The level of IDO expression in many human tumors has been linked to tumoral immunoresistance via arrest of T lymphocyte proliferation in the presence of tryptophan shortage. In the period since our original proposal was written, the synthesis of 5-[<sup>18</sup>F]F-L- $\alpha$ -methyl tryptophan (5-[<sup>18</sup>F]F-AMT) has been reported (Giglio BC, et al., Synthesis of 5-[<sup>18</sup>F]Fluoro- $\alpha$ -methyl Tryptophan: New Trp Based PET Agents, Theranostics, 2017. 7(6): 1524-1530). Given the advantages of this newer agent including improved ease of synthesis and longer half-life to facilitate imaging, we will utilize this agent to assess IDO expression in Year 2 of this project.

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

Gonzalez-Junca A, Beckford-Vera D, Roco N, Hyunh T, Korenchan D, Flavell R, Franc BL, Barcellos-Hoff MH. Development of a multimodal functional imaging platform to monitor progression and response to therapy in a pre-clinical model of breast cancer brain metastasis. UCSF Precision Cancer Imaging Retreat and Symposium 2017.

Beckford-Vera DR, Gonzalez-Junca A, Huynh TL, Roco N, Janneck JS, Blecha JE, Barcellos-Hoff MH, Franc BL, VanBrocklin HF. Positron emission tomography imaging of TGF $\beta$  using 89Zr-DFO-Fresolimumab. UCSF Radiology and Biomedical Imaging Symposium 2017.

**7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

**a. What individuals have worked on the project?**

Name	Role	Person Months Worked	Contribution	Funding Support
Mary Helen Barcellos-Hoff	PI	1.2	Designed expts and analyzed experimental data	
Steve Braunstein	Co-Investigator	0.5	Provided clinically relevant data in analysis of experimental data	
Alba Gonzalez-Junca		5.16	Generation of pre-clinical BCBM	

			models. Treatment and characterization.	
Lin Ma	Postdoctoral Fellow	3	Generation of pre-clinical BCBM models. Treatment and characterization	
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	
<b>Benjamin Franc</b>	PI	0.96	Oversaw molecular imaging portion of project	
Henry VanBrocklin	Co-investigator	0.60	Oversaw development of <sup>89</sup> 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 <sup>89</sup> 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

- b. 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**

**Added: Varian Medical Systems, Barcellos-Hoff, M H**

12/01/2017 - 11/31/2018

*TRI-Modal Therapy Phase 2*

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

**Role: P.I.**

**COMPLETED:**

**Varian Medical Systems, Barcellos-Hoff, M H**

6/01/2016 - 9/31/2017

*TRI-Modal Therapy*

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

**Role: P.I.**

**PI: FRANC, BENJAMIN**

**ADDED OVER INTERVAL:**

Title: Imaging Latent HIV.

Time: 2% effort (0.24 mo).

Role: Co-Inv

Supporting Agency: American Foundation for AIDS Research

Performance Period: 01/01/2016-12/31/2020

Level of Funding (Total): \$1,000,000

Goals/Aims: This grant establishes the San Francisco amFAR Institute that brings together researchers from UCSF and the Blood Systems Research Center at the Blood Centers of the Pacific, San Francisco, to identify novel strategies to cure HIV. Our (VanBrocklin, Henrich, Franc) module aims to develop methods of monitoring HIV reservoirs and immune cell interactions using imaging, enabling treatments targeted to increase the immune reaction to HIV.

I am the imaging clinician scientist and develop and test these imaging strategies with my colleagues in radiochemistry and HIV medicine.

Overlap: None

Title: Imaging HIV.

Time: 2% effort (0.24 mo).

Role: Co-Inv

Supporting Agency: National Institutes of Health

Performance Period: 01/01/2017-12/31/2020

Level of Funding (Total): \$2,000,000

Goals/Aims: This grant aims to develop methods of monitoring the immune system, HIV reservoirs and immune cell interactions using imaging, enabling treatments targeted to increase the immune reaction to HIV.

I am the imaging clinician scientist and develop and test these imaging strategies with my colleagues in radiochemistry and HIV medicine.

Overlap: None

Title: Variation in Provider Breast Cancer Surveillance Strategies Following Initial Treatment: Contribution of Patient and Provider Factors, Association with Outcomes, and Stakeholder Insights.

Time: 35% effort (4.2 mo).

Role: Co-Inv

Supporting Agency: Agency for Health Quality Research

Performance Period: 10/01/2016-09/30/2020

Level of Funding (Total): \$1,600,000

Goals/Aims: This grant aims to identify characteristics of oncology providers that may drive imaging utilization in breast cancer following its initial treatment.

Overlap: None

### **ENDED OVER INTERVAL:**

Title: PET Radiopharmaceuticals for Tumor Necrosis Factor alpha (TNF-a) Imaging of Rheumatoid Arthritis.

Time: No salary support.

Role: PI

Supporting Agency: UCSF Resource Allocation Program (Internal University Grant)

Performance Period: 07/01/2015-06/30/2017

Level of Funding (Total): \$30,000

Goals/Aims: The goal of this project is to develop a positron-emitting PET imaging agent to monitor immune-mediated therapies in rheumatoid arthritis.

Overlap: None

### **c. What other organizations were involved as partners?**

Nothing to Report.

### **8. SPECIAL REPORTING REQUIREMENTS**

COLLABORATIVE AWARDS: As per DOD guidelines, a duplicative report has been prepared for this collaborative award (from BOTH the Initiating PI and the Collaborating/Partnering PI). The reports have A report been submitted to <https://ers.amedd.army.mil> for each unique award.

## **9. APPENDICES:**

### **APPENDIX A: RELATED ABSTRACTS**

## **ABSTRACT**

Gonzalez-Junca A, Beckford-Vera D, Roco N, Hyunh T, Korenchan D, Flavell R, Franc BL, Barcellos-Hoff MH. Development of a multimodal functional imaging platform to monitor progression and response to therapy in a pre-clinical model of breast cancer brain metastasis. UCSF Precision Cancer Imaging Retreat and Symposium 2017.

### **Development of a multimodal functional imaging platform to monitor progression and response to therapy in a pre-clinical model of breast cancer brain metastasis**

**Alba Gonzalez-Junca, Denis Vera, Niecholle Roco, Tony Hyunh, Dave Korenchan, Robert Flavell, Benjamin Franc, Mary Helen Barcellos-Hoff**

#### **INTRODUCTION:**

Breast Cancer Brain Metastasis (BCBM) occurs in 30% of breast cancer patients and represents a clinical challenge due to the lack of effective treatments. Moreover, as a result of the prolonged survival among breast cancer patients, the incidence of BCBM has increased in the recent years. Current efforts to control the disease are focused on radiotherapy (RT), which can be administered on a high single dose (SBRT or radio-surgery) or with whole-brain radiation, according to the number of metastatic foci at the time of diagnosis. However, despite the clinical improvement in the symptomatology, this treatment is not curative.

We have previously shown that radiation therapy promotes the activation of TGF $\beta$ , a pleiotropic cytokine which activation is tightly regulated and restricted to the tumor micro-environment. Increased TGF $\beta$  activity compromises the efficacy of RT in different cancer models, not only by regulating the DNA-damage response, but also through the suppression of immune response.

#### **METHODS:**

In order to characterize the progression and therapy response of BCBM, we developed a syngeneic mouse model of brain-adapted triple negative breast cancer, which effectively recapitulates some of the histopathological and clinical features of human BCBM. We used the Small Animal Radiation Research Platform (SARRP) to perform image-guided RT localized into the intracranial tumoral lesions and compared different protocols (1x14Gy, 3x8Gy, 5x6Gy). We have implemented a multi-modal functional imaging platform to monitor tumor progression as well as response to different radiotherapy protocols and combinations with immunotherapy.

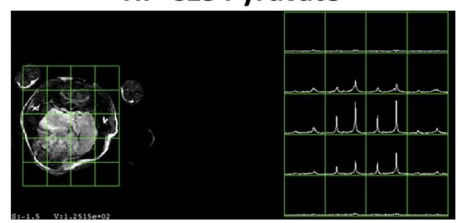
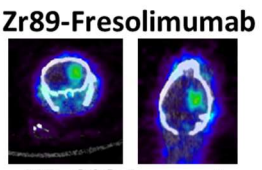
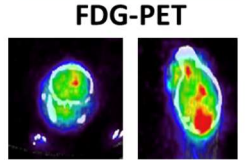
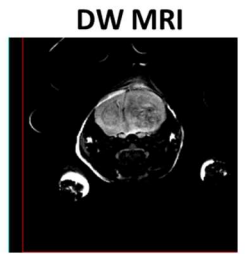
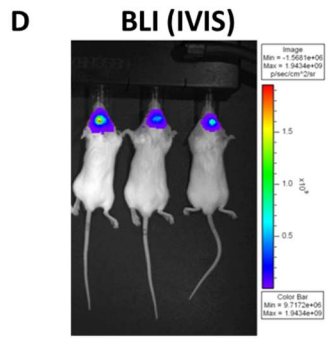
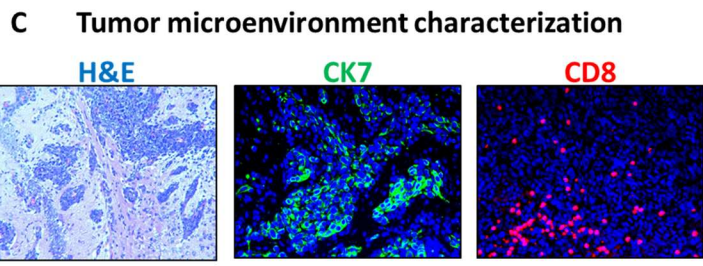
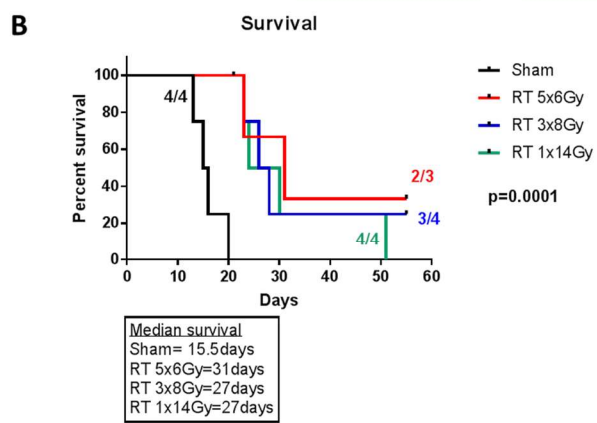
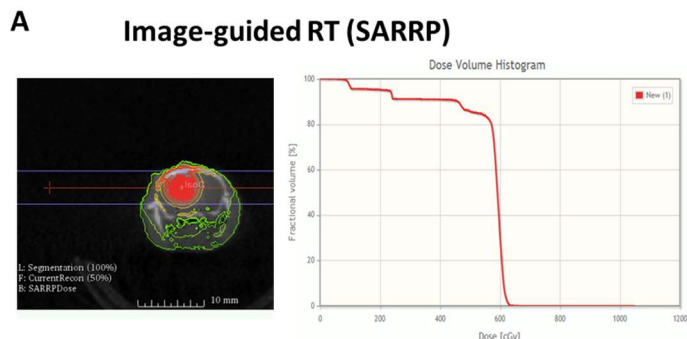
#### **RESULTS:**

We have been able to quantify the growth of BCBM using bioluminescence and diffusion weight imaging (DWI) by MRI. We were also able to detect the BCBM inside the brain by using FDG-PET-CT, observing tumor uptake. Additionally we determined metabolic changes within the tumor, using hyper-polarized pyruvate.

Moreover, we used <sup>89</sup>Zr-Fresolimumab, a humanized antibody that recognizes the active form of TGF $\beta$ , to monitor the activity of this cytokine in BCBM. In our model, we successfully detected increased uptake of <sup>89</sup>Zr-Fresolimumab within the metastatic foci, correlating with high TGF $\beta$  activity.

#### **CONCLUSIONS:**

Our multi-modal functional imaging platform has been validated and will allow us to monitor and predict response to treatment with RT as well as combinations with immunotherapy.



A. SARRP was used to perform image-guided radiotherapy on the pre-clinical model of BCBM. B. Radiation therapy significantly improves survival of BCBM mice. C. Tumor microenvironment and immune system composition was characterized as a function of treatment by histology and immunofluorescence. D. Tumor progression and response to treatment assessed by multi-modal imaging including BLI, DW MRI, FDG-PET, 89Zr-Fresolimumab and HP-Pyruvate.

## ABSTRACT

Beckford-Vera DR, Gonzalez-Junca A, Huynh TL, Roco N, Janneck JS, Blecha JE, Barcellos-Hoff MH, Franc BL, VanBrocklin HF. Positron emission tomography imaging of TGF $\beta$  using  $^{89}\text{Zr}$ -DFO-Fresolimumab. UCSF Radiology and Biomedical Imaging Symposium 2017.

### **Positron emission tomography imaging of TGF $\beta$ using $^{89}\text{Zr}$ -DFO-Fresolimumab.**

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

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

Fresolimumab was modified with 1-(4-isothiocyanatophenyl)-3-[6,17-dihydroxy-7,10,18,21-tetraoxo-27-[N-acetylhydroxylamino)-6,11,17,22-tetrazaheptaecosine]-thiourea (DFO-CNS) and radiolabeled with Zr-89. Quantification of the number of DFO per fresolimumab molecule was determined by isotopic dilution. Immunoreactivity of  $^{89}\text{Zr}$ -DFO-fresolimumab was assessed in a competition assay with unmodified fresolimumab using TGF- $\beta$ 3 as a target antigen. We tested the feasibility of  $^{89}\text{Zr}$ -DFO-fresolimumab for immunoPET imaging of active TGF $\beta$  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 grow into the brain of immunocompetent balb/c mice. Mice were injected with  $^{89}\text{Zr}$ -DFO-fresolimumab with different specific activities and microPET/CT images were acquired at 24 and 96h post i.v. injection.

$^{89}\text{Zr}$ -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  $^{89}\text{Zr}$ -DFO-fresolimumab was greater than 98%. After conjugation and radiolabeling,  $^{89}\text{Zr}$ -DFO-fresolimumab exhibited high affinity and specific binding to TGF $\beta$ (Fig 1 A). Competition of  $^{89}\text{Zr}$ -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  $^{89}\text{Zr}$ -DFO-fresolimumab in a preclinical model of breast cancer brain metastasis (Fig 1B) allowing for clear tumor visualization. Hence, demonstrating that  $^{89}\text{Zr}$ -DFO-fresolimumab is capable of crossing the damaged blood brain barrier. Unexpectedly, higher tumor uptake was observed when  $^{89}\text{Zr}$ -DFO-fresolimumab with low specific activity was administered. Clinical translation of non-invasive immuno-PET imaging of TGF $\beta$  with  $^{89}\text{Zr}$ -DFO-fresolimumab may be particular useful for patient selection and therapy monitoring due the dual functions of TGF $\beta$ .

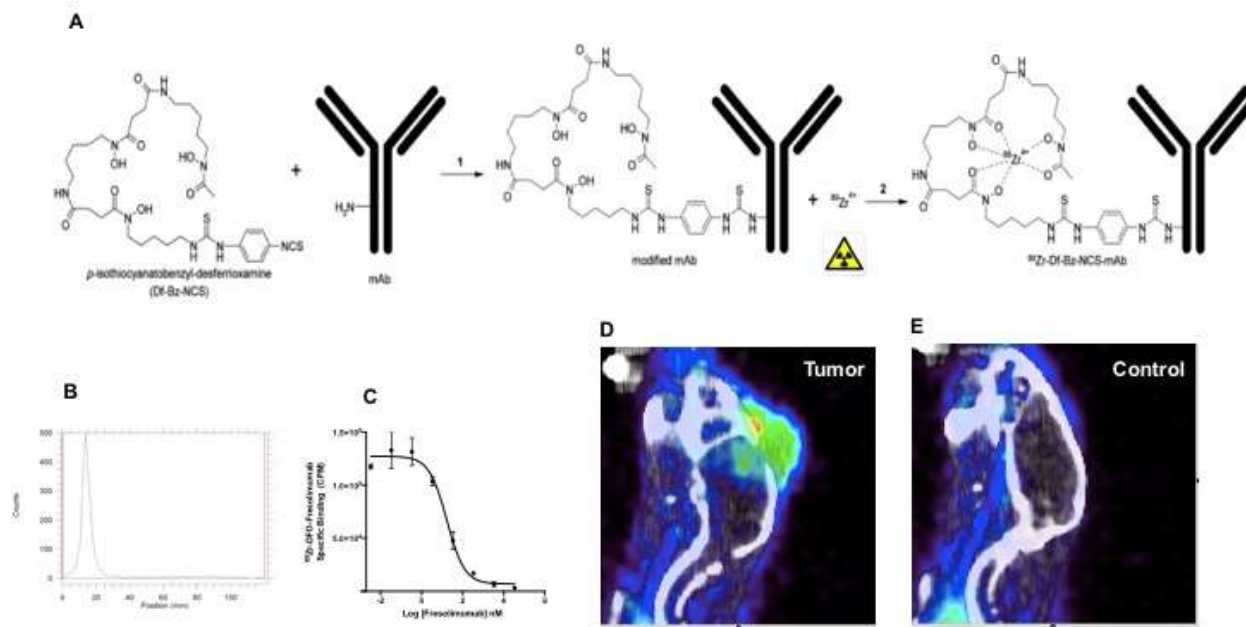


Figure 1 (A) Preparation of  $^{89}\text{Zr}$ -DFO-fresolimumab. (B) Radio thin-layer chromatography of purified  $^{89}\text{Zr}$ -DFO-fresolimumab showing high radiochemical purity. (C) Competition binding assay of  $^{89}\text{Zr}$ -DFO-fresolimumab against unlabeled fresolimumab ( $\text{IC}_{50} = 16\text{nM}$ ). (D) microPET/CT image of  $^{89}\text{Zr}$ -DFO-fresolimumab in a preclinical model of breast cancer brain metastasis showing high tumor uptake and (E) microPET/CT image of  $^{89}\text{Zr}$ -DFO-fresolimumab in a control model.