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13. SUPPLEMENTARY NOTES

14. ABSTRACT: In the previous two reports, Tasks 1, 2, and 3 (Aim 1) were completed. Most of Takes 4, 5, 6, and 7 (Aim 2a, 2b, 2c) were accomplished. Tasks 8, 9, 10 (Aim3) were initiated. In brief, post approval for IACUC and IRB protocols, we completed the development of the 2nd and 3rd generation human CAR constructs, confirmed the sequences, generated the CAR T cells, and successfully completed cytolysis assays against TNBC cell lines in an antigen-specific manner without any toxic effects to normal epithelial cells. We initiated the in vivo experiments outlined in specific aim 2. Most of the data is now published. We completed the efficacy study of human CAR T+anti-PD1 blocking antibody treatment in the NSG mouse model of human TNBC. We successfully generated the murine CAR t cells and conducted in vitro functional assays using murine breast cancer cell lines. We started the efficacy evaluation of murine CAR t cells in an immune competent spontaneous PyVMT tumor model in vivo. In this cycle, we report the progress made in Tasks 6 through 10. We complete the efficacy study of mouse CAR t cells in immune competent human MUC1.Tg mice. No significance is observed from mouse CAR t cell treatment, which we believe is largely due to CAR t cell penetration into tumor in this aggressive orthotopic model. We achieve a significant reduction for tumor growth and progression as well as overall mouse survival in mouse CAR t cell group in spontaneous PyVMT tumor model, which lasts over 5 months. We receive five breast cancer patient PDX samples and complete their MUC1 expression profile. We establish the human tumor explant model with 2 PDX samples.

15. SUBJECT TERMS None listed.

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1. **Introduction:** Antibody-derived chimeric antigen receptor (CAR) T cell therapy has achieved gratifying breakthrough in hematologic malignancies but has shown limited success in solid tumor immunotherapy. Monoclonal antibody, TAB004, specifically recognizes the aberrantly glycosylated tumor form of MUC1 (tMUC1) in all subtypes of breast cancer including 95% of triple-negative breast cancer (TNBC) while sparing recognition of normal tissue MUC1. We transduced human T cells with MUC28z, a chimeric antigen receptor comprising of the scFv of TAB004 coupled to CD28 and CD3ζ. MUC28z was well expressed on the surface of engineered activated human T cells. MUC28z CAR T cells demonstrated significant target-specific cytotoxicity against a panel of human TNBC cells. Upon recognition of tMUC1 on TNBC cells, MUC28z CAR T cells increased production of Granzyme B, IFN-γ and other Th1 type cytokines and chemokines. Furthermore, we found that a single dose of human MUC28z CAR T cells significantly reduced HCC70 TNBC tumor growth in a xenograft model. Murine MUC28z was successfully expressed on mouse primary $CD8^+$ T cells. In vitro, those mouse MUC28z CAR t cells recognized tMUC1, lysed mouse breast cancer cells, and released IFN-γ and IL-2 in a tMUC1-antigen highly specific manner. In vivo, mouse MUC28z CAR t cells control spontaneous MMT tumor growth and progression, with survival benefit. No adverse effects were noticed with human or mouse CAR T/t cell treatment in all the in vivo animal models. Thus, MUC28z CAR T cells have high therapeutic potential against tMUC1-positive TNBC tumors with minimal damage to normal breast epithelial cells.

2. **Keywords:** Triple-negative breast cancer, Immunotherapy, MUC28z CAR T cells

3. **Accomplishments**

What were the major goals of the project?

The Specific Aims were:

Aim 1. Demonstrate tMUC1-CAR-T/t cell mediated killing *in vitro* of human and mouse TNBC cells.

Aim 2a. Demonstrate 2^{nd} and 3^{rd} generation tMUC1-CAR-T mediated killing *in vivo* in xenograft model of human metastatic TNBC.

Aim 2b. Demonstrate tMUC1-CAR-t mediated killing *in vivo* in orthotopic model of mouse metastatic TNBC in human MUC1.Tg syngeneic (immune competent) mice.

Aim 2c. Demonstrate tMUC1-CAR-t cell mediated killing of tumors in MMT bitransgenic mice that develop spontaneous mammary gland tumors and express human MUC1.

Aim 3. Demonstrate tMUC1-CAR-T mediated killing in human tumor explant models of metastatic, treatment refractory TNBC.

What was accomplished under these goals?

Specific Aim 1. Demonstrate tMUC1-CAR-T/t cell mediated killing *in vitro* of human and mouse TNBC cells.

Task 1: IRB/IACUC approval (Months 1-2).

Task 2: tMUC1-CAR-T mediated TNBC killing in vitro (Months 1-4).

Task 3: TAB-CAR-t mediated murine breast cancer cell killing in vitro (Months 4-8).

Task 1 Progress: IRB/IACUC approval (Months 1-2).

We completed this task and received UNCC IRB number: #18-0227, and this study was also approved through HRPO. UNCC IACUC number was also received (#19-018) and the study in animal subjects was approved through ACURO.

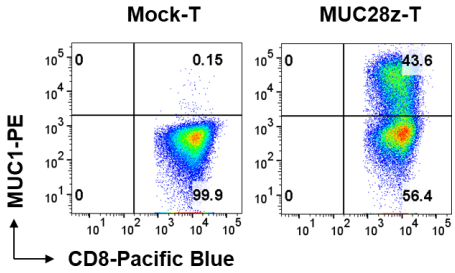
Task 2 Progress: tMUC1-CAR-T mediated TNBC killing in vitro (Months 1-4).

We have completed majority of this task to best of our abilities. The results are now published (Front. Immunol., 24 May 2019 | https://doi.org/10.3389/fimmu.2019.01149 and Front. Immunol., 07 December 2020 | https://doi.org/10.3389/fimmu.2020.628776.)

The publication abstract is provided below:

Antibody-derived chimeric antigen receptor (CAR) T cell therapy has achieved gratifying breakthrough in hematologic malignancies but has shown limited success in solid tumor immunotherapy. Monoclonal antibody, TAB004, specifically recognizes the aberrantly glycosylated tumor form of MUC1 (tMUC1) in all subtypes of breast cancer including 95% of triple-negative breast cancer (TNBC) while sparing recognition of normal tissue MUC1. We transduced human T cells with MUC28z, a chimeric antigen receptor comprising of the scFv of TAB004 coupled to CD28 and CD3ζ. MUC28z was well-expressed on the surface of engineered activated human T cells. MUC28z CAR T cells demonstrated significant target-specific cytotoxicity against a panel of human TNBC cells. Upon recognition of tMUC1 on TNBC cells, MUC28z CAR T cells increased production of Granzyme B, IFN-γ and other Th1 type cytokines and chemokines. A single dose of MUC28z CAR T cells significantly reduced TNBC tumor growth in a xenograft model. Thus, MUC28z CAR T cells have high therapeutic potential against tMUC1-positive TNBC tumors with minimal damage to normal breast epithelial cells.

Task 3 Progress: TAB-CAR-t mediated murine breast cancer cell killing in vitro (Months 4-8).

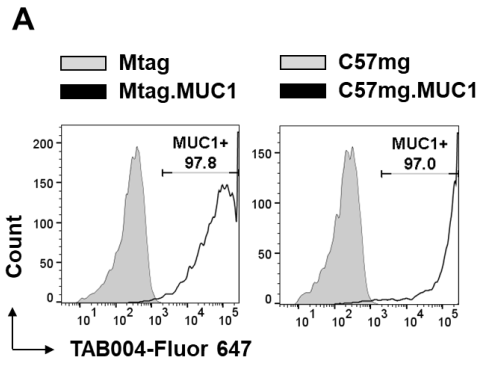


We successfully expressed mouse MUC28z CAR on the cell surface of primary mouse CD8+ T cells (Figure 1).

Figure 1. Mouse MUC28z CAR expression in activated mouse $CD8^+$ T cells after retrovirus transduction, as determined by flow cytometry analysis of MUC1-biotin binding in $CD8^+$ T cells. Dead cells were excluded by 7-AAD staining.

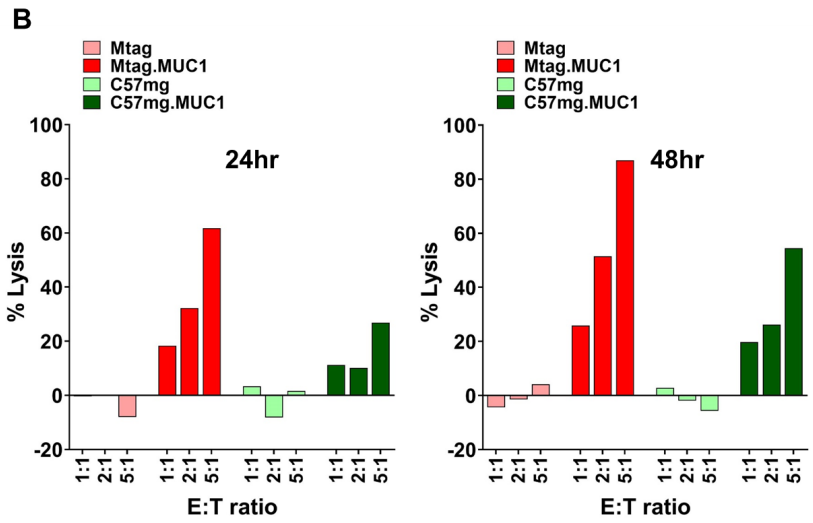
We next assessed the level of tMUC1 on the cell surface of a panel of mouse breast cancer cell lines by flow cytometry. The percentages of cells that express tMUC1 is shown in Figure 2A. The counterpart wildtype cell lines are shown in gray histograms to serve as human tMUC1-null controls. We had proposed to use MMT and

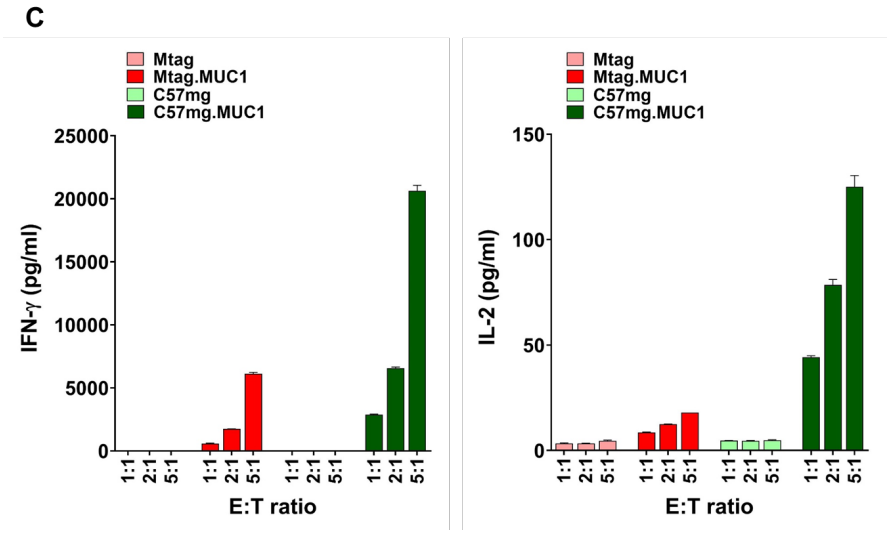
MMT-Lung. Even though MMT tumor cells showed high expression of tMUC1 when freshly isolated, the MMT cells lost their MUC1 expression after in vitro passages (data not shown here). Thus, we stably transfected the Mtag cell lines derived from the PyVMT tumors with the full-length MUC1 gene and designated the cell line Mtag.MUC1 cells. We also included another C57BL/6 mouse syngeneic mammary gland cell line, C57mg and C57mg.MUC1 cells that stably expresses full-length human MUC1.



We then assessed tMUC1-CAR-t mediated murine breast cancer cell killing in vitro.

The four cell lines (Mtag, Mtag.MUC1, C57mg, and C57mg.MUC1) were co-cultured with mouse MUC28z CAR t cells at E:T ratios of 1:1, 2:1, and 5:1 for 24h and 48h. There was a significant lysis of Mtag.MUC1 cells in vitro by MUC28z CAR t cells (Figure 2B). Even though only about 30% of the breast line C57mg.MUC1 were lysed by MUC28z CAR t cells within 24h co-culture, the lysis of C57mg.MUC1 was increase to approximately 60% after 48h co-culture with CAR t cells (Figure 2B). Importantly, the CAR t cells did not lyse tMUC1-null cell lines, suggesting the tumor killing was highly tMUC1 antigen dependent. All lysis data presented here was normalized to its own mock T cell lysis. We also included MMT cell line in the killing assay.





However, there was minimal cell death observed in MMT cells since they lost tMUC1 (data not shown).

Besides tumor cell lysis, the engagement of murine MUC28z CAR t cells with tMUC1-expressing murine tumor cell lines led to the IFN-γ and IL-2 production (Figure 2C) in an antigen-dependent and dose-dependent manner.

Figure 2. The mouse MUC28z CAR t cells target on tMUC1-expressing tumor cells for lysis in vitro. (A) Percentage of cells expressing tMUC1, determined by TAB004-Fluor 647 staining and flow cytometry analysis. (B) Percentage of mouse tumor cell lysis by MUC28z CAR t cells. Cells were co-cultured at the indicated E:T ratio for 24hr and 48hr. The lysis of tumor cells was determined by MTT assay. Data are presented as the mean ± SD. (C) IFN-γ and IL-2 production by MUC28z CAR t cells. Data are presented as the mean ± SD.

Milestones for Aim 1: Tasks 1-3: Test 15 human breast cancer cell lines for the 3 TAB-CAR-T cells (TAB 28z; TAB28BBz, and TAB28OXz) killing in vitro. 10 TNBC, 3 luminal and Her-2 type, and 2 normal mammary epithelial lines. Test 6 murine cell lines (syngeneic to C57BL/6) for tMUC1-CAR-t cell killing in vitro. This will determine the optimal breast cancer cell lines for in vivo experiment.

This was mostly accomplished. We tested 6 murine cell lines for tumor lysis, but two cell lines lost their tMUC1 expression in vitro. Nevertheless, this did not change the direction of the overall goals. We were able to identify the cell lines to be used for in vivo studies.

Specific Aim 2a: Demonstrate 2nd and 3rd generation tMUC1-CAR-T mediated killing *in vivo* in xenograft model of human metastatic TNBC.
Task 4: Test the treatment efficacy of the 2nd and 3rd generation TAB-CAR-T cells in orthotopic implantation model in NSG mice (Months 8-18).
Task 5: Combining CAR T cells with anti-PD1 antibody will offer the potential to improve antitumor effects.

Task 4 Progress: Test the treatment efficacy of the 2nd and 3rd generation TAB-CAR-T cells in orthotopic implantation model in NSG mice.

To determine the anti-tumor effect of MUC28z CAR T cells on TNBC tumor growth, the HCC70 cells were inoculated in NSG female mice, followed by a single injection of human MUC28z CAR T cells 6 days after tumor cell injection. Compared to the vehicle control, MUC28z CAR T cells effectively reduced the HCC70 tumor growth till the experiment endpoint on day 81 (Figure 3A). The insert in Figure 3A showed the wet weights of tumors resected from NSG mice at the endpoint. The tumor weights in the MUC28z CAR T cell-treated group was significantly lower than the vehicle group that received PBS. However, it must be noted that even though there was a significant difference between control and treated groups, the tumors treated with MUC28z CAR T cells did start to progress faster after ~60 days post treatment suggesting that a) a single injection of CAR T cells may not be sufficient, b) tMUC1 is lost in the remaining tumor that progressed, and c) blocks anti-tumor immune response and therefore a combination therapy together with CAR T cells is needed.

We investigated tMUC1 expression in tumors post MUC28z CAR T cell treatment in vitro and in vivo. The level of tMUC1 on HCC70 cells remained unchanged post co-culture with MUC28z CAR T cells or mock T cells in vitro (Figure 3B). In addition, the tumor sections from MUC28z CAR T cells and vehicle treated mice were stained with TAB004 for tMUC1. Surprisingly, there was increased tMUC1 staining in the group treated with MUC28z CAR T cells than in the vehicle group (Figure 3C) suggesting that tMUC1 loss is not a factor for tumor out-growth post CAR T treatment.

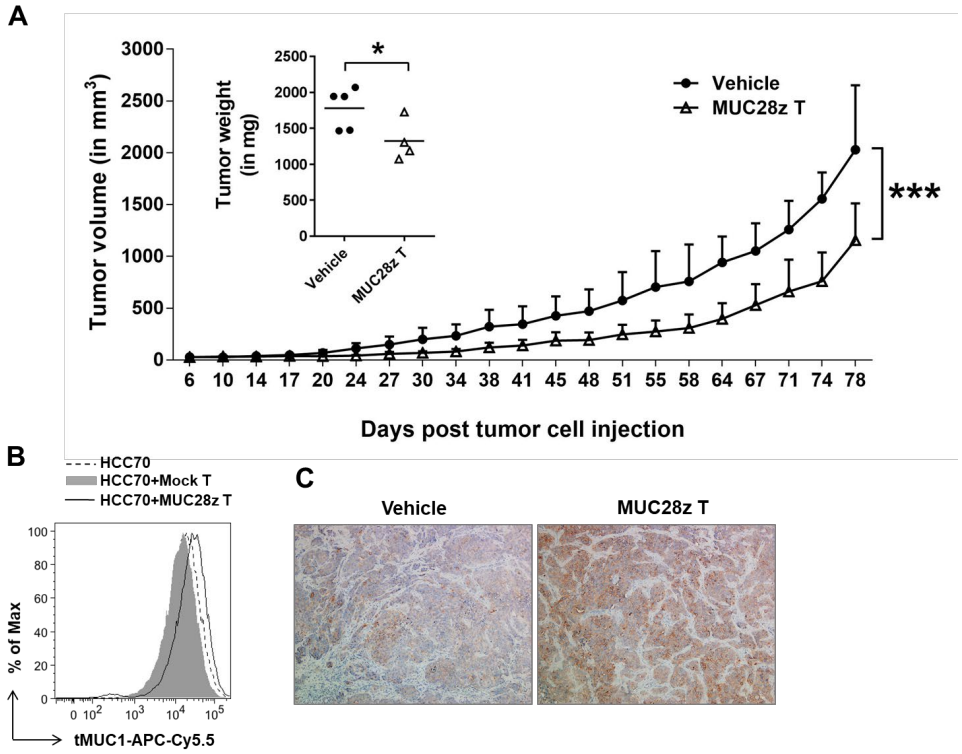


Figure 3. MUC28z CAR T cells have long-term efficacy for HCC70 tumor reduction in vivo. (A) Decrease of HCC70 tumor burden by a single injection of MUC28z CAR T cells in vivo. HCC70 cells were orthotopically injected into the mammary fat pad of female NSG mice. When tumors were palpable, mice were randomized and received a single i.v. injection of PBS as vehicle control, or MUC28z CAR T cells on day 6 post tumor cell challenge. Tumor growth was monitored by caliper measurement. Data are presented as mean ± SD. The statistical analysis was performed by two-way ANOVA. ***, $p<0.001$. The insert shows the wet weight of resected tumor mass on day 81 at endpoint. *$p<0.05$ (student t-test). (B) No tumor antigen loss while MUC28z CAR T cells were present in vitro. HCC70 cells were cultured alone or co-cultured with the mock T cells or MUC28z CAR T cells (E:T = 2:1) for 24hr. The viable HCC70 cells were analyzed for tMUC1 level. (C) Increased intensity of tMUC1 expression in MUC28z CAR T cells-treated HCC70 tumors. HCC70 tumor sections were prepared on day 81 at endpoint. Immunohistochemistry staining of tMUC1 was performed with TAB004 antibody. The brown staining shows tMUC1 positivity (100x magnification).

Some parts of Task 4 are now published in the Frontiers in Immunology paper. We used the 2nd generation CAR T cells for the in vivo experiments. (Front. Immunol., 24 May 2019 https://doi.org/10.3389/fimmu.2019.01149 and Front. Immunol., 07 December 2020 | https://doi.org/10.3389/fimmu.2020.628776.) *We are working on the 3rd generation CAR T cells now. The retrovirus for 3rd generation CAR has been made and used for primary human CAR T cell generation.*

Task 5 Progress: Combining CAR T cells with anti-PD1 antibody will offer the potential to improve antitumor effects.

We checked the MUC28z CAR T cells for PD1 expression right before the adoptive transfer and on the day of the experimental endpoint. Data in Figure 4A showed the changes within CD8+ MUC28z CAR T cells. Approximately 50 days after surviving in vivo, the tumor-infiltrating CD8+ MUC28z CAR T cells expressed very high level of PD1 (Figure 4A), suggesting their further activation by in vivo tMUC1 tumor antigen stimulation.

To assess if in vivo combining MUC28z CAR T cells with anti-PD1 antibody may be a better strategy for tumor eradication, we i.p. injected anti-human PD1 antibody at 10mg/kg once weekly for about 8 weeks. Combing anti-PD1 antibody with human MUC28z CAR T cells did not show improvement for tumor reduction compared to CAR T cell alone (Figure 4B). We will optimize the PD1 blocking antibody dose and treatment schedule to

see whether we can improve the outcome. Alternatively, we will block PD-L1 on TNBC tumor along the PD1-PD-L1 checkpoint signaling axis, which could possibly enhance our CAR T cell efficacy.

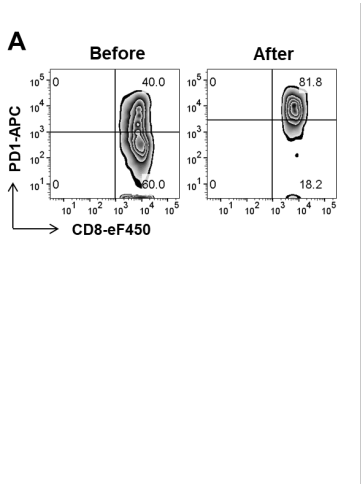
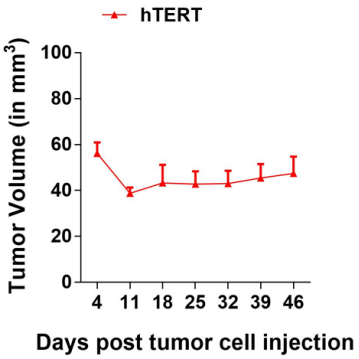


Figure 4. PD1 blockade did not enhance tumor reduction by human MUC28z CAR T cells under the indicated conditions. (A) Increase of PD1, an activation/exhaustion marker, on CD8+ MUC28z CAR T cells. MUC28z CAR T cells were stained right before i.v. injection and right after tumor infiltrating lymphocytes analysis from tumor mass. Cells were gated on $CD8^+$ T cells. (B) Combining anti-PD1 blocking antibody with MUC28z CAR T cells showed no synergistic effect for tumor reduction. HCC70 tumors were inoculated same as Figure 3A. When tumors were palpable, mice were randomized and received a single i.v. injection of PBS as vehicle control, or MUC28z CAR T cells. Anti-human PD1 antibody were i.p. injected once weekly till the endpoint. Tumor growth was monitored by caliper measurement. Tumors were resected and weighed at endpoint on Day 57. The statistical analysis was performed by Student t-test. **, $p<0.01$; ***, $p<0.001$.

Milestones for Aim 2a: Tasks 4 and 5: Test n=6 human TNBC and n=1 normal epithelial cell in vivo in NSG mice using 3 preparation of TAB-CAR T cells and two sources of T cells (one source from normal donor and one from TNBC patient).

We have completed most of the milestone with the 2nd generation CAR T cells. Experiments with the 3rd generation CAR T cells are still ongoing.



We had proposed to use a normal mammary gland epithelial cells in vivo but we found that the normal hTERT-HME1 didn't form tumors that were capable of progress in the NSG mouse (Figure 5). Therefore, we will not use this cell line for in vivo control.

Figure 5. hTERT-HME1 cells were not able to form tumor in vivo. hTERT-HME1 cells were injected into mammary fat pad of NSG mice same as the procedure for HCC70 cells in Figure 3 and 4.

During this report period (10/15/2020-10/14/2021), we have achieved the following:

Specific Aim 2a: Demonstrate 2nd and 3rd generation tMUC1-CAR-T mediated killing *in vivo* in xenograft model of human metastatic TNBC.

Task 4 Progress: Test the treatment efficacy of the 2nd and 3rd generation TAB-CAR-T cells in orthotopic implantation model in NSG mice.

Task 5 Progress: Combining CAR T cells with anti-PD1 antibody will offer the potential to improve antitumor effects.

Due to the failure of hTERT-HME1 cells to form tumor in NSG mice, so before we carried out the large groups of CAR T cell treatment, we did pilot experiments to test the tumor formation kinetics for BT549 (high MUC1; for Task 4), HCC1806 (medium to high MUC1, but relatively resistant to CAR T killing; for Task 5), and MDA-MB-453 (low MUC1 as replacement for hTERT-HME1 cells; for Task 4). Data are shown as Figure 6. To our surprise, BT549 cells did not form progressive tumors in NSG mice (Figure 6, left), so this cell line is excluded from further study. HCC1806 cells formed aggressive large tumors (Figure 6, middle). MDA-MB-453 cells formed tumors, even though they were small and slow growing (Figure 6, right). HCC1806 and MDA-MB-453 cells will be tested for CAR T cell killing in vivo.

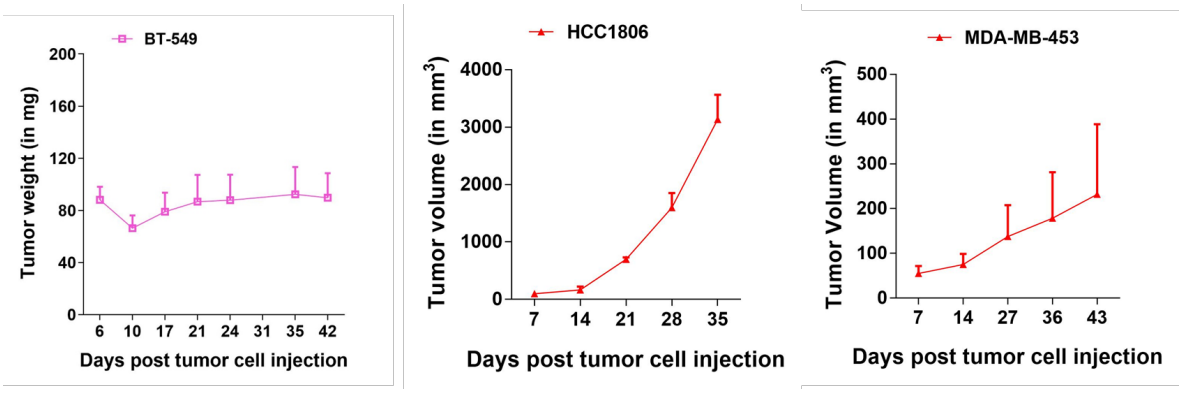


Figure 6. Tumor formation by three cells in vivo. The indicated three TNBC cells were injected into mammary fat pad of NSG mice same as the procedure for HCC70 cells in Figure 3 and 4.

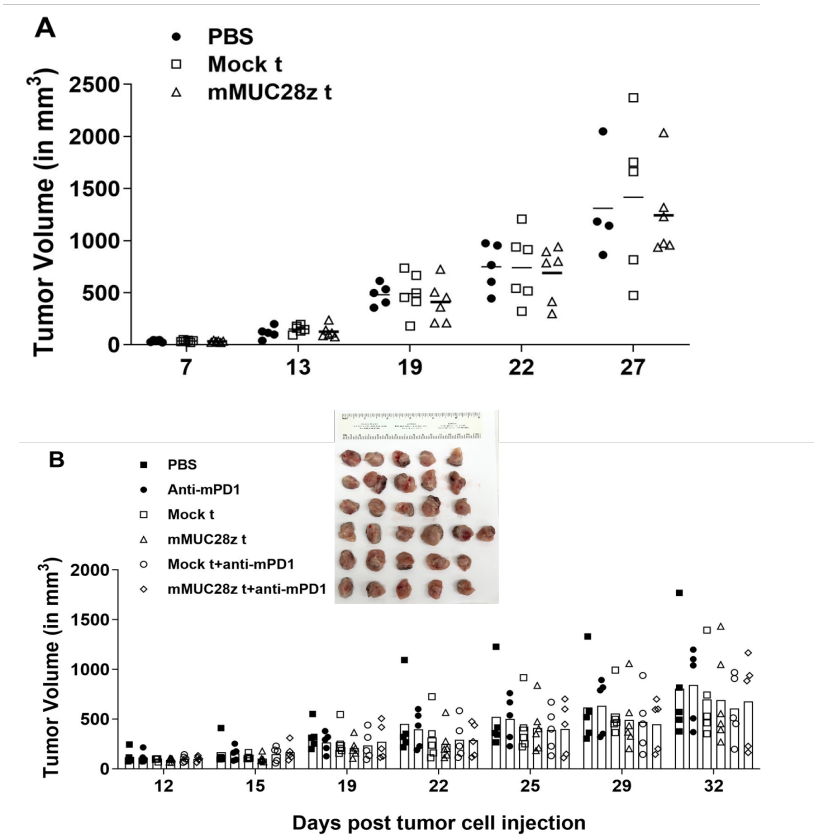
Specific Aim 2b: Demonstrate tMUC1-CAR-t mediated killing *in vivo* in orthotopic model of mouse metastatic TNBC in human MUC1.Tg syngeneic (immune competent) mice.

Task 6: Test the treatment efficacy of 2nd and 3rd generation TAB-CAR-t in orthotopic implantation model in MUC1.Tg mice (Months 12-18).

Task 6 Progress: Test the treatment efficacy of 2nd and 3rd generation TAB-CAR-t in orthotopic implantation model in MUC1.Tg mice.

So far, we used 2nd generation mouse MUC28z CAR t cells for Task 6. We orthotopically injected Mtag.MUC1 cells into mammary fat pad of immune competent MUC1.Tg mice. A single dose of CAR t cells was i.v. injected to Mtag.MUC1 tumor bearing mice. We observed a trend towards tumor control by mouse MUC28z CAR t cells as compared to mock t cell control or vehicle PBS control under current conditions (Figure 7A); however, the results were not significant. There is limited research using mouse CAR t cells in immune competent mice. We expected the difficulties we would encounter. To improve the outcome, we included the anti-mouse PD1 blocking antibody as a synergistic combination for mouse CAR t cells. We once again did observe decreased tumor growth in the combination treatment (Figure 7B; the insert shows the resected tumors at the endpoint). With most if not all possibilities considered, this failure of mouse CAR t cells to fully control tumor growth is likely due to the inadequate penetration of CAR t cells into the MTag.MUC1 tumor in this particularly

aggressive orthotopic tumor model within an immune competent host. Further, we may have to inject less tumor cells to begin with so that the tumors are slow growing and CARt cells can penetrate. We injected 1 million cells and the tumors grew too rapidly. We therefore, went directly to conducting the experiment in the bi-transgenic MMT model (Specific Aim 2c, Task 7).



The experiments with the 3rd generation CAR t cells are still ongoing.

Figure 7. Mouse MUC28z CAR t cells against mouse Mtag.MUC1 breast tumor in human MUC1.Tg syngeneic immune competent mice. Mtag.MUC1 cells were orthotopically injected into the mammary fat pad of female MUC1.Tg mice. When tumors were palpable, (A) mice were randomized and received a single i.v. injection of PBS as vehicle control, Mock t cells, or MUC28z CAR t cells. Tumor growth was monitored by caliper measurement; (B) mice were randomized and injected with Mock t cells, or MUC28z CAR t cells, once weekly for 3 consecutive weeks. Anti-mouse PD1 blocking antibody or PBS were i.p. injected into respective groups 1hr before the 1st dose of t cells adoptive transfer, and every 5 days thereafter till the endpoint.

Milestones for Aim 2b: Tasks 6: Test one murine cell line in vivo in MUC1.Tg mice. Identify the optimal dosing schedule. Confirm enhanced antitumor effect of the 3 CAR-T/t cell preparation (TAB28z, TAB28BBz, and TAB28OXz) in orthotopic implantation model.

We have tested 1 cell line with 2nd generation CAR-t cells in vivo. The experiments with 3rd generation CART/t cells have been challenging as in vitro experiments show no enhanced cytotoxicity as well as low expression levels of the CAR constructs on T/t cells. Nevertheless, the experiments are ongoing.

Specific Aim 2c: Demonstrate tMUC1-CAR-t cell mediated killing of tumors in MMT bitransgenic mice that develop spontaneous mammary gland tumors and express human MUC1.

Task 7: (Months 12-24). Two groups of MMT mice will be treated 1) starting at 8 weeks of age (at the mammary intraepithelial neoplasia (MIN), MIN stage)**,** a second cycle at 12 weeks of age, and a third cycle at 15 weeks of age**;** and 2) starting at 12 weeks of age (early carcinoma) and a second cycle at 15 weeks of age. This will determine if treatment early during tumor progression is more efficacious. Three formulations of TAB-CAR-t cells will be injected **(12-30 months)**

Task 7 Progress: Demonstrate tMUC1-CAR-t cell mediated killing of tumors in MMT bitransgenic mice that develop spontaneous mammary gland tumors and express human MUC1.

So far, we have used 2nd generation mouse MUC28z CAR t cells and without combination with anti-PD1 antibody for Task 7. We used the immune competent MMT mice that spontaneously develop mammary tumors. The tMUC1 expression on two freshly isolated MMT tumors were evaluated and the data in Figure 8A confirming high tMUC1 expression.

MMT mice treated with MUC28z CAR t cell every two weeks showed significantly slower tumor growth as compared to age-matched MMT mice that received mock t cells (Figure 8B shows individual mouse data; 8C shows average growth). The mouse survival was significantly improved with mouse MUC28z CAR t cells (Figure 8D). When further separating mice in Figure 8B as early intervention group (9-11 weeks of age) and late intervention group (14-15 weeks of age) for analysis, we found that late intervention showed more dramatic difference between the two treatment groups with the same treatment frequency (data not shown here). This is likely due to short persistence of mouse CAR t cells in vivo. In another word, the early intervention of CAR t cells indicated their later absence while spontaneous MMT tumor progressed rapidly at late stage.

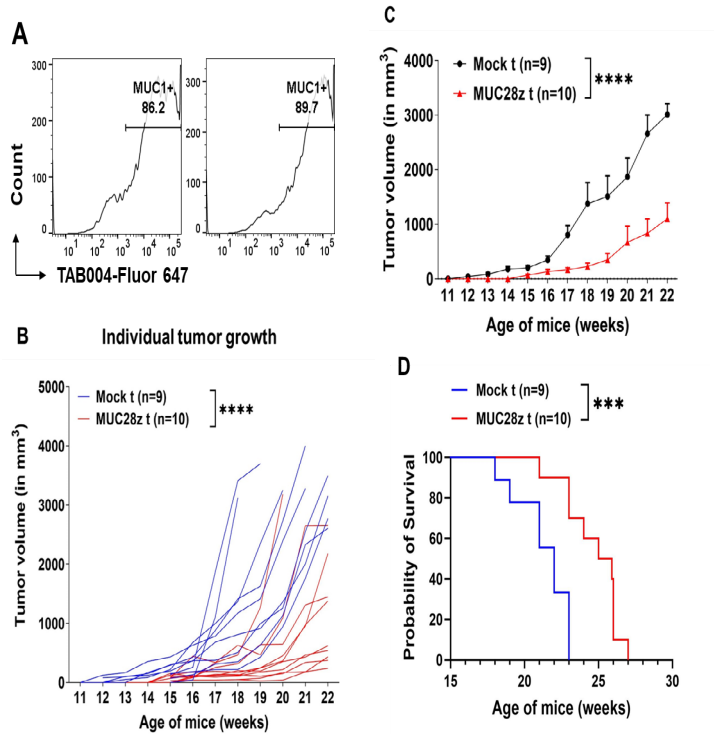


Figure 8. MUC28z CAR t cells control spontaneous MMT tumor growth. (A) Tumors from two female MMT mice (~20 weeks of age) were resected and single cell suspensions were prepared. Cells were then promptly stained with TAB004-Fluor 647 and analyzed for MUC1 level by flow cytometry. (B, C) Female MMT mice at approximately 9-11 weeks of age or 14-15weeks of age were pooled and randomized into two groups. One group received i.v. injection of mock t cells as control, and the other group received mouse MUC28z CAR t cells. Three additional t cell injections were administered at a 2-weeks interval afterwards. N=9 mice for mock t cell group; N=10 mice for mouse MUC28z CAR t cells. ****, p<0.0001 (two-way ANOVA). (B) shows individual mouse tumor growth; (C) shows averaged tumor growth as a group. (D) Improved mouse survival with mouse MUC28z CAR t cell treatment. ***, p<0.001 (Log-rank test).

Milestones for Aim 2c: Tasks 7: Test the tMUC1-CAR-t in immune competent MMT model that develop spontaneous mammary gland tumors and express human MUC1 and mimics the human disease.

Thus far, the experiment was conducted with the MMT model using the 2nd generation CAR T cells. We achieved the success in spontaneous breast tumor control with our murine tMUC1-CAR-t cells. Since the spontaneous MMT mouse model well mimics the human disease, our data are highly encouraging for future

use of our tumor MUC1 targeting CAR T cells in breast cancer patients. Data from Tasks 6 and 7 suggests that spontaneous MMT mice respond well to CARt cells but cell lines-based homogenous tumors do not once again highlighting the importance of the appropriate models used for immunotherapy that mimic the human disease.

Specific Aim 3: Demonstrate tMUC1-CAR-T mediated killing in human tumor explant models of metastatic, treatment refractory TNBC.

Task 8: Receive cells generated from breast cancer specimens from patients with metastatic/treatment refractory breast cancer (anticipated receipt of cells will be Month 24).

Task 8 Progress: Receive cells generated from breast cancer specimens from patients with metastatic/treatment refractory breast cancer.

Since the collaborators from Duke University were not able to provide PDXs or PDX-derived primary tumor cells from human triple negative breast cancer patients, they referred us to Huntsman Cancer Institute from University of Utah. We received five PDX samples so far. By immunohistochemistry staining of the respective PDX slides from Utah, we found 3 PDX samples had various level of tumor MUC1 expression with TAB004 staining (Figure 9, HCI008, HCI009, HCI010), and other 2 PDX samples showed no expression (HCI002, HCI003; data not shown here) which were excluded from further study.

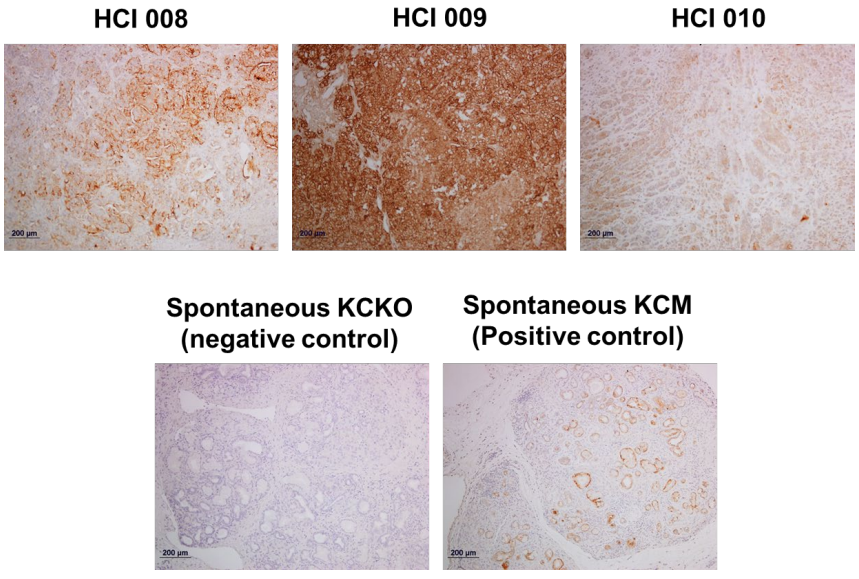


Figure 9. Tumor MUC1 expression in three primary human metastatic breast cancer PDX samples. Immunohistochemistry staining of tMUC1 was performed with TAB004 antibody. Spontaneous KCKO sample was used as negative control since KCKO mice are lack of both human and mouse MUC1. Spontaneous KCM samples was used as positive control since these KCM mice are transgenic for human MUC1 gene. The brown staining shows tMUC1 positivity (100x magnification).

Task 9: Determine tMUC1-CAR-T mediated killing of TNBC explant cells in vitro (Months 26-28).

No progress has been made on this task. We are working on during the final year.

Task 10: Determine tMUC1-CAR-T mediated killing of TNBC explant cells in vivo (Months 28-36).

Task 10 Progress: Determine tMUC1-CAR-T mediated killing of TNBC explant cells in vivo.

After confirming tumor MUC1 level in the received PDX samples (3 out of 5 samples), we implanted those 3 live PDX tumor samples (~2x4mm in size for each explant) into mammary fat pads of NSG mice for in vivo expansion. The individual tumor explant growth curves are shown as Figure 10 (9 implants for HCI009 and 3 implants for HCI100; both are TNBC). The HCI008 PDX implants did not progress in NSG mice, so it is excluded from CAR T efficacy evaluation. HCI009 PDX progressed rapidly, and HCI010 PDX grew but relatively slow. After further passaging their tumor explants/chunks in vivo, we will begin CAR T cell treatment.

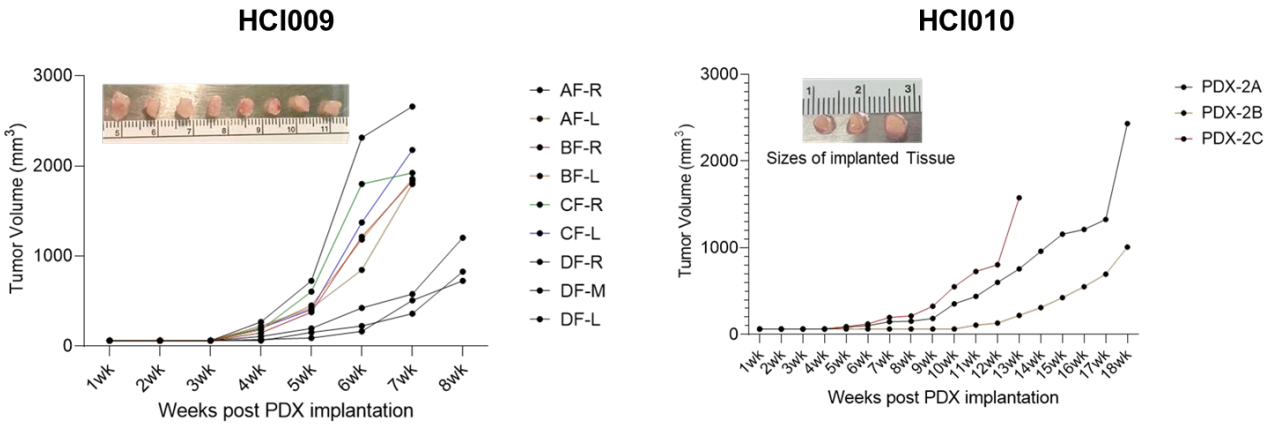


Figure 10. Human breast PDX tumor progression in NSG mice. The thawed human breast PDX tumor explants were surgically implanted into mammary fat pads of female NSG mice (two explants for two fat pads in same mouse). Tumor growths were measured by caliper. The inserts show the sizes of explants right before surgical implantation.

Milestones: Duke University (Dr. Lyerly's group) will collect at least 15 breast cancer samples. They will establish at least 10 breast cancer explant in NSG mice. We will test n=6 TNBC explants in vitro and n=2 in vivo in the orthotopic model for treatment.

So far, we have two PDXs validated for tumor MUC1 expression and for in vivo tumor formation and progression. We will soon start the in vivo CAR T cell treatment in those two PDX orthotopic models. Additional different PDX samples will be obtained and validated for in vivo treatment.

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

We had PhD students and undergraduate students work on parts of this project. We also had the opportunity to train 2 undergraduate students belonging to underrepresented minority population. Dr. Ru Zhou was able to get her promotion from Research Assistant Professor to Research Associate Professor while working on this grant. Dr. Chandrav De was recruited as post-doc fellow to gain experience in the field of tumor immunotherapy and has produced the data presented for specific Aim 3.

How were the results disseminated to communities of interest?

Front. Immunol., 24 May 2019 | https://doi.org/10.3389/fimmu.2019.01149 and Front. Immunol., 07 December 2020 | https://doi.org/10.3389/fimmu.2020.628776.)

Due to the pandemic, we did not present at any conferences. Manuscript is under preparation with the potential title "Mouse *CAR t Cells Targeting the Tumor MUC1 Reduces Breast Cancer Growth in Immune Competent Host"*

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

We are planning to complete the rest of the objectives in the last period of this project. All the new data will be published in peer-reviewed journals and also will be shared in the conferences. We are concerned about the 3rd generation CAR T/t cells being less than ideal to move forward in vivo.

4. **Impact:** The proposed research has the potential to lead to revolutionary therapies that will not only eliminate the mortality associated with metastatic TNBC but also replace interventions that have life threatening toxicities with ones that are safe and effective, i.e.: novel immunotherapeutic strategies targeting only the specific tumor associated antigen on TNBC while sparing normal organs. Such therapies have the potential of controlling disease progression, prolonging time to recurrence and ultimately, even serving as a preventive measure or cure. If successful, this project will have a major impact and accelerate progress toward a clinical trial for metastatic TNBC. The impact will be significant and move much beyond an incremental advancement.

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

The fact that the murine CAR t cells did not cause toxicity in immune competent MUC1.Tg mice suggests that the TAB004 CARs will be safer than other CARs that are being developed and that these CAR T cells will not attack the normal epithelia that expresses normal MUC1. Targeting the tumor form of MUC1 was the overall innovation and goal of the project and thus far, it certainly seems that is the case.

There is limited publications reporting generating mouse CAR t cells and testing them in immune competent animals. To the best of our knowledge, the data presented with the mouse CAR t cells will be the only report that will show CAR t cells efficacy and safety profiles in the unique *spontaneous immune competent* MMT mice, in which the MMT mice mimic the tumor initiation, progression, and metastasis stages of human breast cancer.

What was the impact on other disciplines?

In general, we show the significance of the model system used to test immunotherapy strategies.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems:

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Established MMT cell line lost its MUC1. We will focus on stable Mtag.MUC1 cells. hTERT-HME1 cells were not able to form tumor in vivo. We will use other tMUC1 low expressing cells as replacement. We were not able to get isolated primary PDX tumor cells or tumor explants from Duke University. Instead, we obtained several patient PDX samples as tumor chunks for in vivo direct implantation from Huntsman Cancer Institute. This direct PDX tumor chunk implantation is better to keep its tumor heterogeneous in vivo, and also better for tumor explant survival and progression. These changes will not affect the overall impact.

Changes that had a significant impact on expenditures

None…However, due to the pandemic, we did lose months of in vivo mouse work although we still needed to keep the breeding and genotyping going.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. Products: murine CAR t cells were successfully generated and tested in vitro and in vivo in animal models.

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	Pinku Mukherjee	Ru Zhou	Chandrav De	Mukulika Bose	Sophia Shwartz
Project Role:	PI	Co-Investigator	Post-doc	Graduate Student	Technician
Researcher Identifier:	Orchid ID:0002-6782-3576	N/A	N/A	N/A	N/A
Nearest person month worked:	2 calendar months	12 months	4 months	6 months	5 months
Contribution to Project:	Supervise project	Perform tasks for mouse CAR t cells in vitro and in vivo	Perform tasks for human CAR T cells in vitro and in PDX model	Perform flow cytometry and helped with cell lines and transfections	Maintain and genotype mice
Funding Support:	NIH RO1, Belk Endowment	None	Belk Endowment	UNCC Grad School	NIH RO1

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

Pinku Mukherjee

1 R41 CA265619-01
Dates: 09/24/21 – 09/23/22
Agency: NIH/NCI National Institutes of Health (Candace Coffie (NIH/NCI), 9609 Medical Center Drive, Bethesda, MD 20892)
Title: An integrated strategy using a serum and imaging biomarker for the early detection of pancreatic cancer.
Objective: The objective is to enhance early detection of pancreatic ductal adenocarcinomas using the radio-imaging
Role: PI
Amount:
Effort: 1.00 calendar months
No Overlap

1 R41 CA265655-01
Dates: 09/24/21 – 09/23/22
Agency: NIH/NCI National Institutes of Health (Candace Coffie (NIH/NCI), 9609 Medical Center Drive, Bethesda, MD 20892)
Title: The use of tMUC1/CD3 bispecific antibody to control pancreatic ductal adenocarcinoma.
Objective: The objective is to develop treatment regimen for metastatic pancreatic cancer using T cell engager bispecific antibody

Role: PI
Amount:
Effort: 1.00 calendar months
No overlap

What other organizations were involved as partners?

Nothing to report

8. Special Reporting Requirements: Nothing to report

9. Appendices: none