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13. SUPPLEMENTARY NOTES

14. ABSTRACT: In the previous three 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 started and partially finished. In brief, we successfully generated the 2nd generation CAR T cells, and completed cytolysis assays against TNBC cell lines in an antigen-specific manner. Part of the in vivo experiments outlined in specific aim 2 had been completed and is 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. The efficacy of murine CAR t cells in orthotopic implantation model were completed using MUC1.Tg mice and no significant tumor reduction was observed. We achieved a significant reduction for tumor growth and progression as well as overall mouse survival in mouse CAR t cell group in spontaneous MMT tumor model, which lasted over 5 months. We received five breast cancer patient PDX samples and complete their MUC1 expression profile. We established the human tumor explant model with 2 PDX samples. **In this cycle**, we report the progress we made to complete the project. We successfully made the 3rd generation CAR T cells, and completed cytolysis assays against TNBC cell lines. The rest portion of work in Task 4 and 5 are started. We completed the efficacy and toxicity evaluation of murine CAR t cells in spontaneous MMT tumor model in vivo, and its associated endpoint studies in Task 7. We completed in vivo passage of 2 TNBC PDX samples and made sufficient tissue stocks for Task 10. We made sufficient human CAR T cells for Task 10.

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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 MUC1 CAR, a chimeric antigen receptor comprising of the scFv of TAB004 coupled to CD28 and CD3ζ (we also named this MUC1 CAR construct as MUC28z). MUC1 CAR was well expressed on the surface of engineered activated human T cells. MUC1 CAR T cells demonstrated significant target-specific cytotoxicity against a panel of human TNBC cells. Upon recognition of tMUC1 on TNBC cells, MUC1 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 MUC1 CAR T cells significantly reduced HCC70 TNBC tumor growth in a xenograft model. Murine MUC1 was successfully expressed on mouse primary enriched T cells as well as isolated $CD8^+$ T cells. In vitro, those murine MUC1 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, murine MUC1 CAR t cells controlled spontaneous MMT tumor progression and improved survival benefit. No adverse effects were noticed with human or mouse CAR T/t cell treatment in all the in vivo animal models. The liver and renal toxicity of murine MUC1 CAR t cells were evaluated in immunocompetent MMT disease model and the murine CAR t cells were well tolerated. Mechanistically, gene microarray data showed the remodeling of tumor microenvironment by murine CAR t cells when proper host immunity was present. Together, MUC1 CAR T cells have high therapeutic potential against tMUC1-positive TNBC tumors and possibly other tMUC1-expressing epithelial malignancies.

2. **Keywords:** Triple-negative breast cancer, Immunotherapy, MUC1 CAR T cells (same as 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.

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.

We have completed this task to best of our abilities. Part of 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.

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

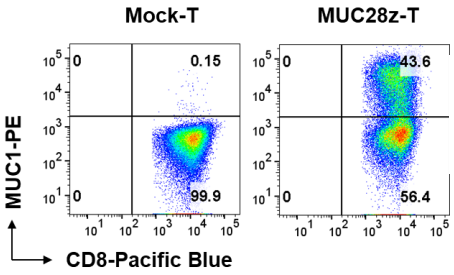


Figure 1. Mouse MUC28z CAR expression in activated mouse CD8+ T cells after retrovirus transduction, as determined by flow cytometry analysis of MUC1-biotin binding in CD8+ T cells. Dead cells were excluded by 7-AAD staining.

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 as 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.

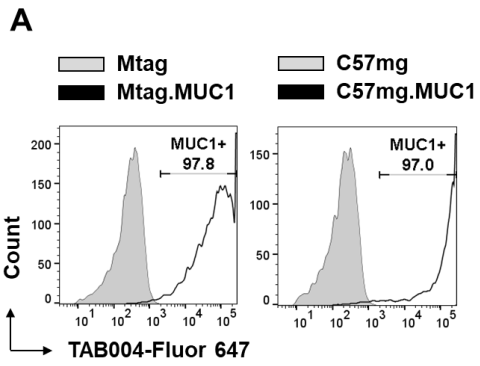
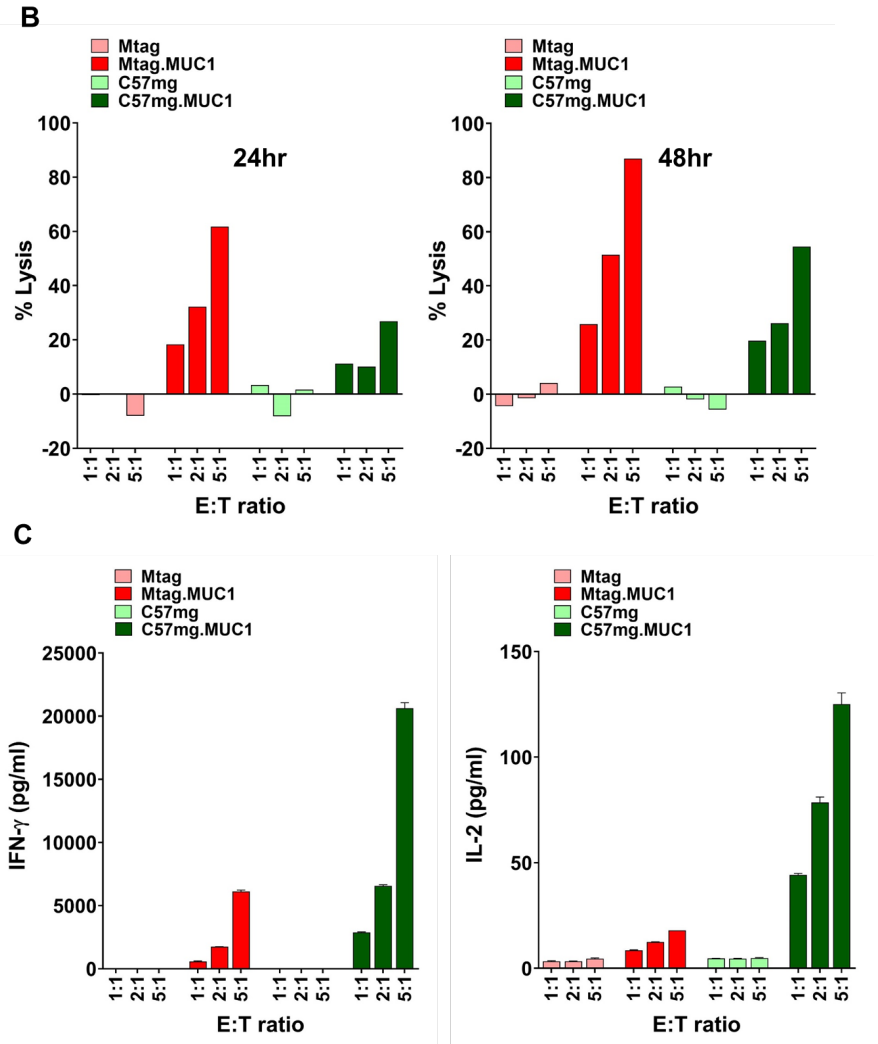


Figure 2. The mouse MUC28z CAR t cells target on tMUC1-expressing tumor cells for lysis in vitro. (A) Percentage of cells expressing tMUC1, determined by TAB004-Fluor 647 staining and flow cytometry analysis. (B) Percentage of mouse tumor cell lysis by MUC28z CAR t cells. Cells were co-cultured at the 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.



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.

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.

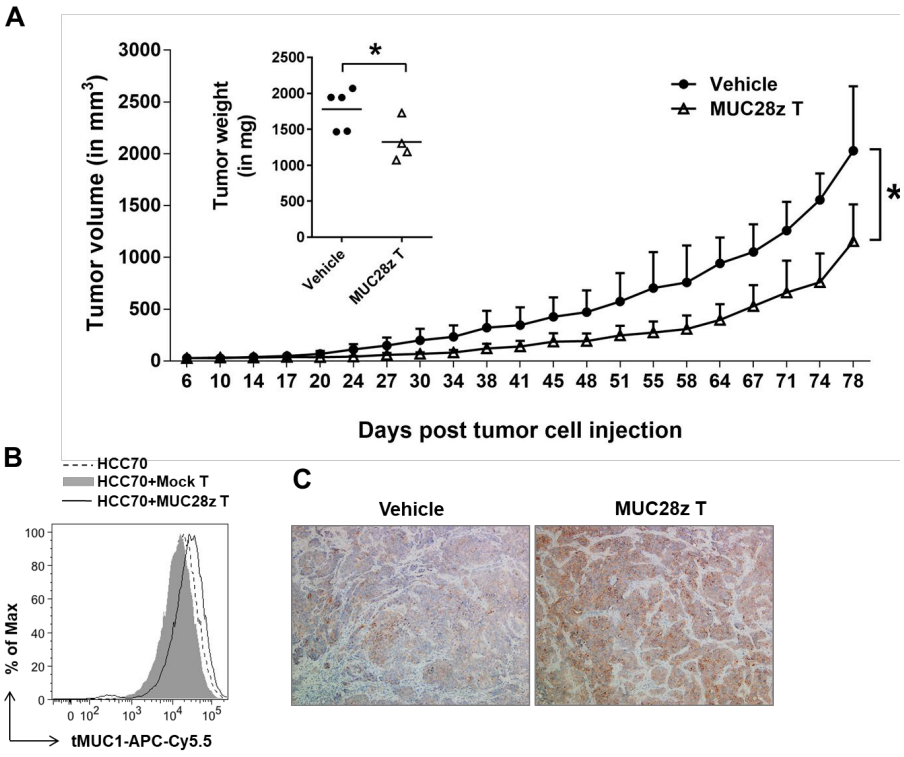


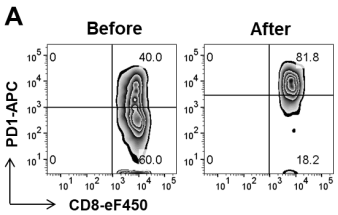
Figure 3. MUC28z CAR T cells have long-term efficacy for HCC70 tumor reduction in vivo. (A) Decrease of HCC70 tumor burden by a single injection of MUC28z CAR T cells in vivo. HCC70 cells were orthotopically injected into the mammary fat pad of female NSG mice. When tumors were palpable, mice were randomized 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. This will be accomplished by December of 2022.*

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.



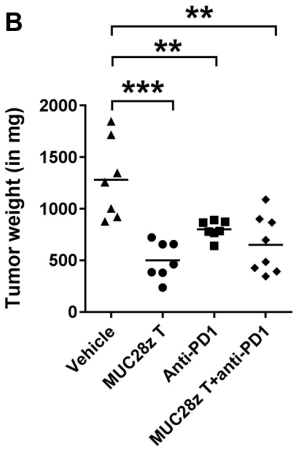


Figure 4. PD1 blockade did not enhance tumor reduction by human MUC28z CAR T cells under the indicated conditions. (A) Increase of PD1, an activation/exhaustion marker, on CD8+ MUC28z CAR T cells. MUC28z CAR T cells were stained right before i.v. injection and right after tumor infiltrating lymphocytes 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. **, $p<0.01$; ***, $p<0.001$.

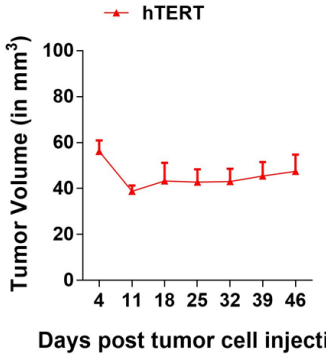


Figure 5. hTERT-HME1 cells were not able to form tumor in vivo. hTERT-HME1 cells were injected into mammary fat pad of NSG mice same as the procedure for HCC70 cells in Figure 3 and 4.

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. Pilot experiments with the 3rd generation CAR T cells had been attempted.

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.

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.

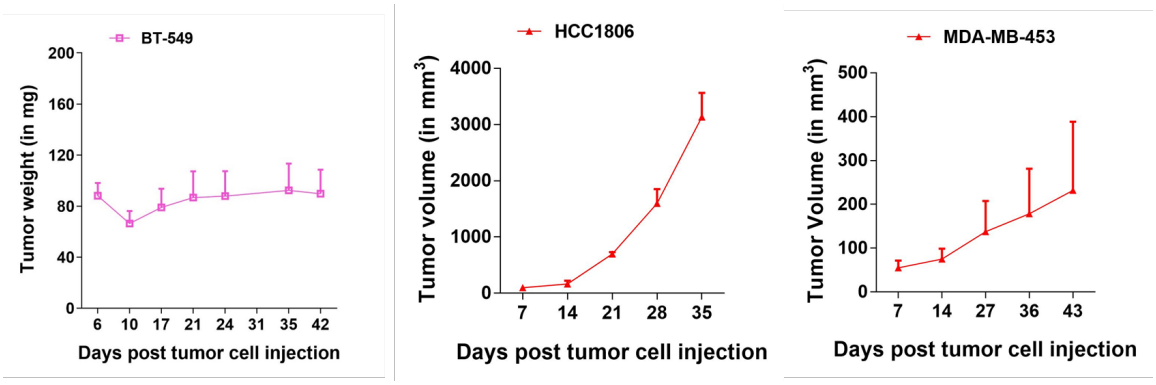


Figure 6. Tumor formation by three cells in vivo. The indicated three TNBC cells were injected into mammary fat pad of NSG mice same as the procedure for HCC70 cells in Figure 3 and 4.

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 CAR t 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).

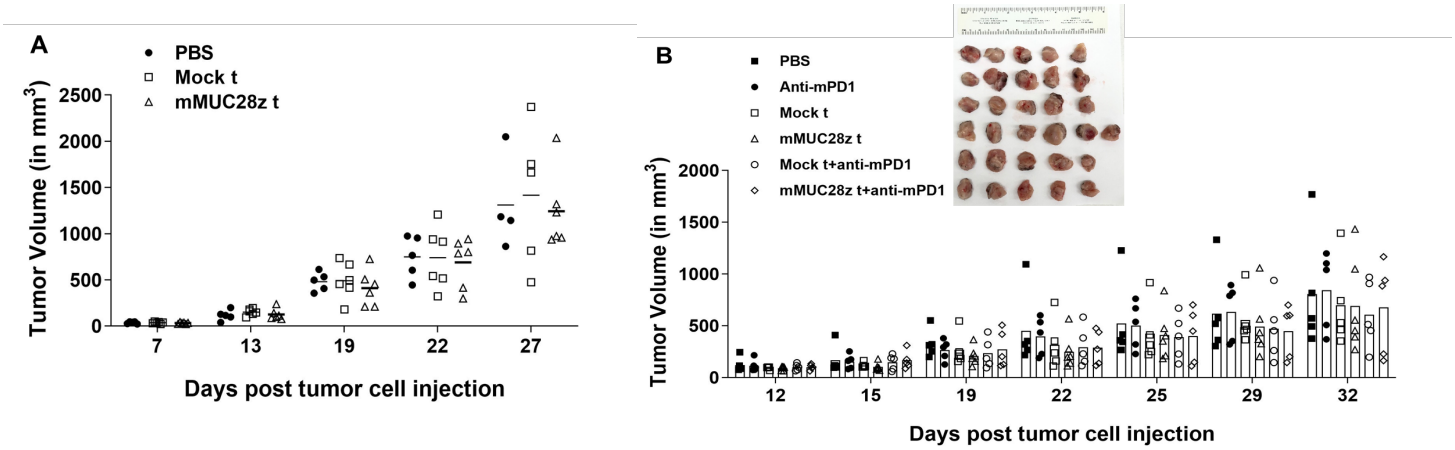


Figure 7. Mouse MUC28z CAR t cells against mouse Mtag.MUC1 breast tumor in human MUC1.Tg syngeneic immune competent mice. Mtag.MUC1 cells were orthotopically injected into the mammary fat pad of female MUC1.Tg mice. When tumors were palpable, (A) mice were randomized and received a single i.v. injection 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.

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.

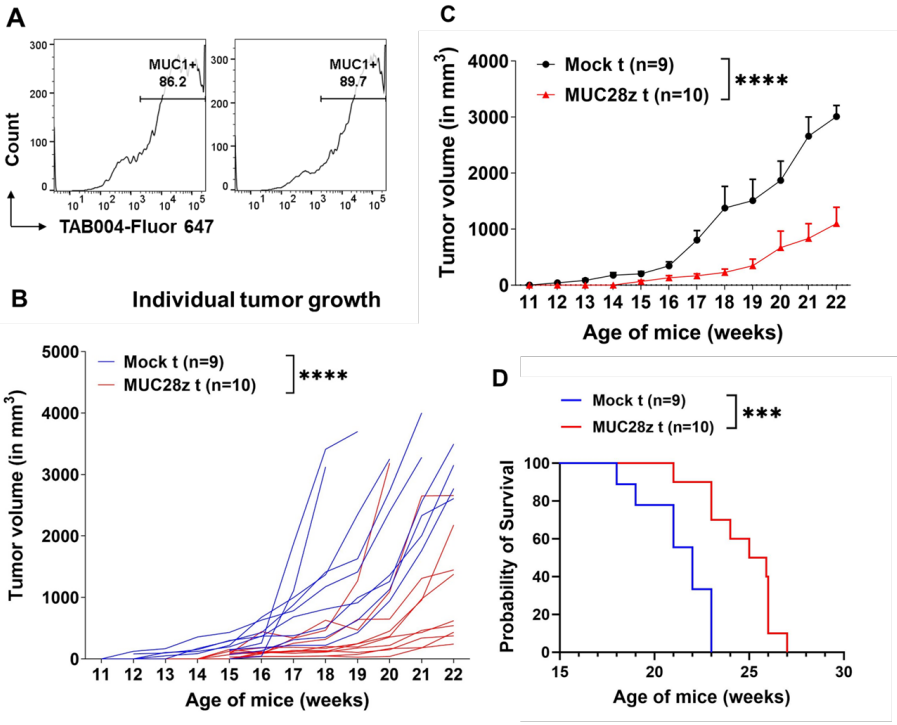


Figure 8. MUC28z CAR t cells control spontaneous MMT tumor growth. (A) Tumors from two female MMT mice (~20 weeks of age) were resected and single cell suspensions were prepared. Cells were cultured for 2 days. The attached cells were then stained with TAB004-Fluor 647 and analyzed for MUC1 level by 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 highlight 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 9: Determine tMUC1-CAR-T mediated killing of TNBC explant cells in vitro (Months 26-28).
Task 10: Determine tMUC1-CAR-T mediated killing of TNBC explant cells in vivo (Months 28-36).

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. 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.

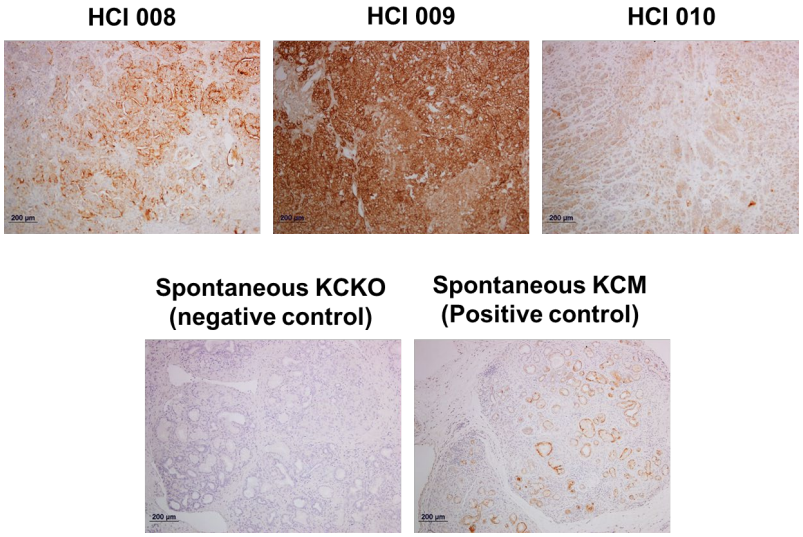


Figure 9. Tumor MUC1 expression in three primary human metastatic breast cancer PDX samples. Immunohistochemistry staining of tMUC1 was performed with TAB004 antibody. Spontaneous KCKO sample was used as negative control since KCKO mice are lack of both human and mouse MUC1. Spontaneous KCM samples was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

After confirming tumor MUC1 level in those PDX 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 passages. 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 in vivo, we now have sufficient PDX explant stocks and are ready to begin CAR T cell treatment.

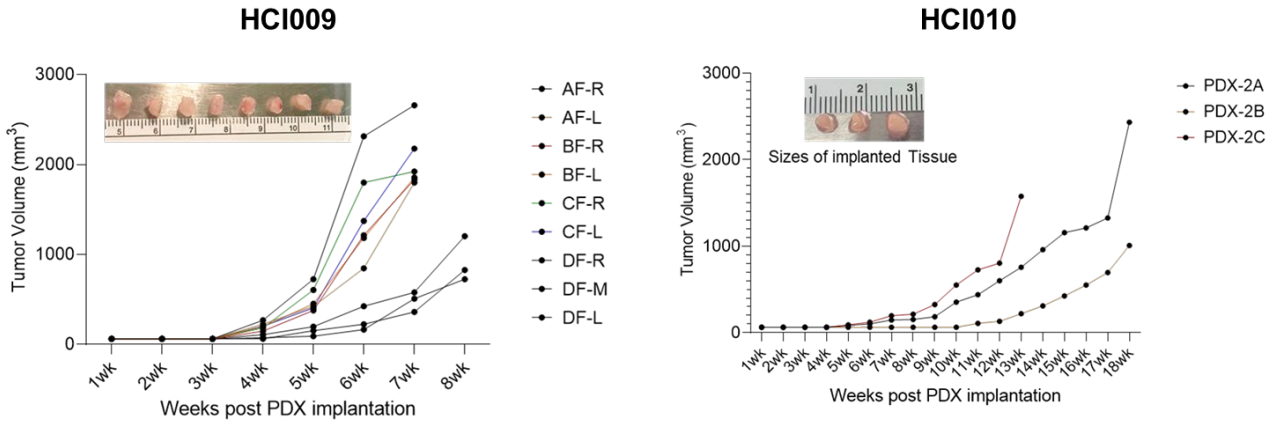


Figure 10. Human breast PDX tumor progression in NSG mice. The thawed human breast PDX tumor explants were surgically implanted into mammary fat pads of female NSG mice (two explants for two fat pads in same mouse). Tumor growths were measured by caliper. The inserts show the sizes of explants right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

During this report period (10/15/2021-10/14/2022), we have achieved the following:

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 2 Progress: tMUC1-CAR-T mediated TNBC killing in vitro.

We have successfully made 3rd generation human CAR T cells which express similar level of CAR as the 2nd generation CAR T cells. We have now completed this task with both generations of CAR T cells using a 3D cell death assay/Spheroid formation assay. Representative data for spheroid formation is shown as Figure 11. Both 2nd and 3rd generation CAR T cells significantly reduced the size of spheroids with similar efficacy (Figure 11A). Data were simultaneously confirmed using MTT assay (Figure 11B). A total of 7 TNBC cell lines were tested (HCC1937, HCC70, BT-20, HCC1395, BT-549, HCC38 and HCC1806) in spheroid assay and their sensitivity to CAR T cell cytotoxicity was summarized in Table 1.

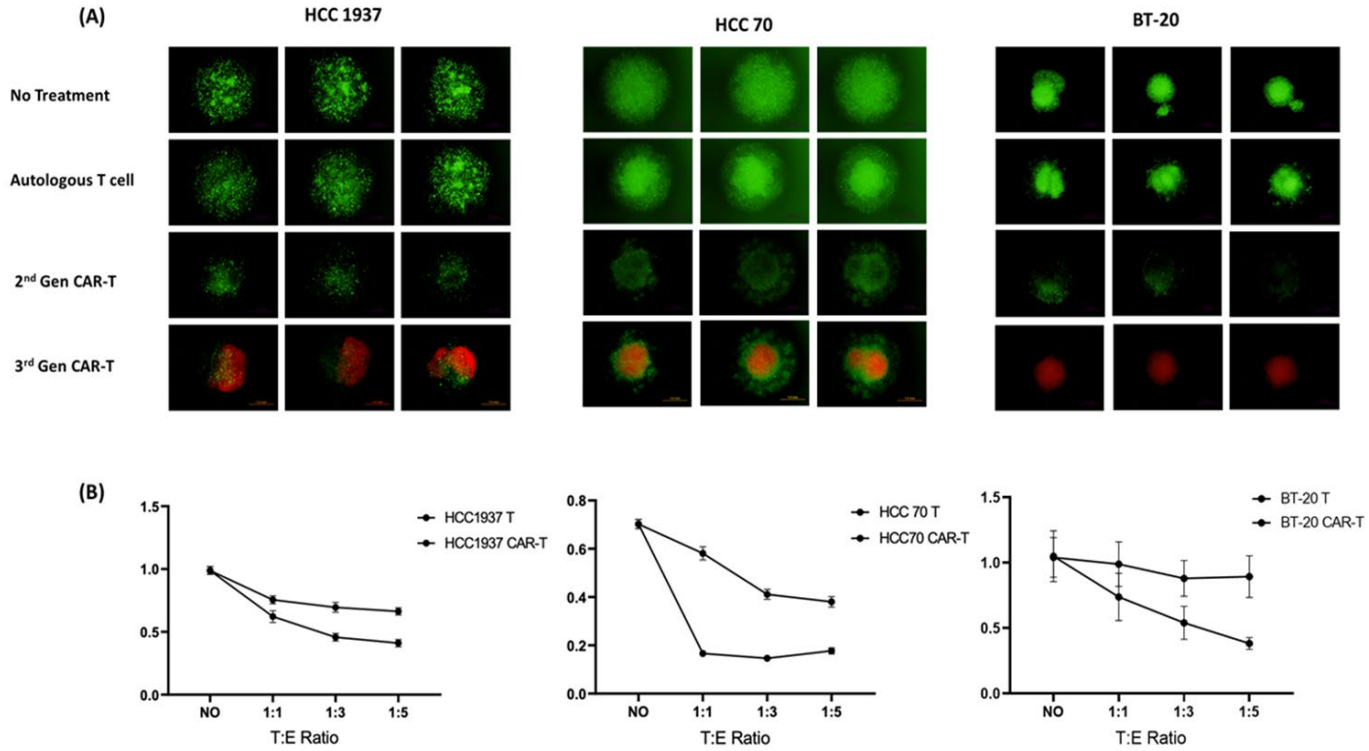


Figure 11. The 3rd generation CAR T mediated the killing in TNBC cell lines. (A) Representative images showing cytolytic activities of 2nd and 3rd generation CAR-T cells in reducing the growth of spheroids following 48hr of co-culture. No treatment, media only; Autologous T cell, mock T cell control. Green fluorescence, tumor spheroid; Red fluorescence, 3rd generation CAR T cells. (B) The reduction of spheroids was compared and confirmed using MTT cell viability assay (2D assay). Y axis represents the absolute MTT OD value.

Table 1. Summary of CAR T cell cytotoxicity against TNBC

TBNC Cell lines	No treatment	Autologous T cells	2rd Gen CAR-T cells	3rd Gen CAR-T cells
HCC1937	-	-	++	++
HCC70	-	-	++	++
BT-20	-	-	+++	+++
HCC1395	-	-	+	+
BT-549	-	-	+++	+++
HCC38	-	-	+	+
HCC1806	-	-	+	+

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 Aim 1 is now accomplished. We have compared human 2nd and 3rd generation CAR T cells in TNBC cell lysis and a similar killing efficacy was observed. We have completed the 2nd generation murine CAR t cells in killing 4 murine breast tumor cell lines. 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.

We were trouble shooting with making 3rd generation CAR T cells to express its CAR to a level that was comparable to 2nd generation CAR T cells for in vivo evaluation and comparation. We achieved this goal and both CARs were highly expressed in human T cells (Figure 12). We now have made sufficient stock of both CAR T cells for in vitro and in vivo use. In the past two years, there has been delays in vendor supply of adult female NSG mice. Currently we already obtained enough female NSG mice. Once their ages become appropriate for experiments in 2-3 weeks, tumor inoculation and CAR T cell treatments (2nd and 3rd) will start immediately. Experiments will be completed in about 3 months.

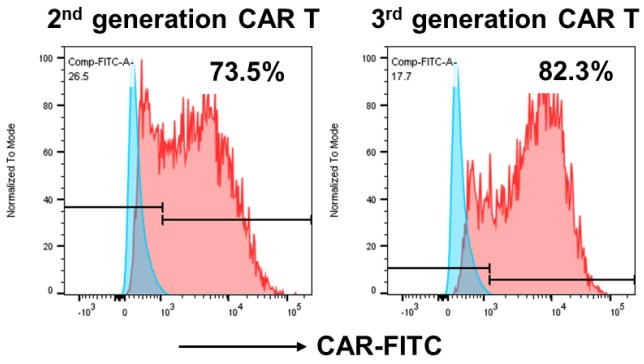


Figure 12. Successful expression of 3rd generation CAR. Activated human T cells were transduced with retrovirus expressing 2nd or 3rd generation CAR. The level of CAR expression was detected on 21 days post virus transduction by flow cytometry.

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

Task 5 has been simultaneously planned to be completed as Task 4. CAR T cells and other reagents are ready in stock. Once female NSG mice reach appropriate ages, we will start tumor inoculation, CAR T cell treatment, and PD-1 antibody treatment.

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. The experiments with 3rd generation CAR T cells in comparison with 2nd generation CAR T cells have begun and will be accomplished.

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.

We have achieved highly encouraging efficacy and survival data using murine CAR t cells in spontaneous MMT mouse model as we reported in last cycle. We continued the observation and the endpoint studies using the blood and tissue samples we collected from Figure 8 experiment. We randomly selected 3 lungs and 3 tumors for histology staining. Figure 13A shows significantly reduced tumor distant metastasis into lung after CAR t cell interference. Figure 13B shows that the tumor tissue morphology was less advanced after CAR t cell treatment even at the humane endpoint (top panels), and still remained tumor MUC1 expression (middle

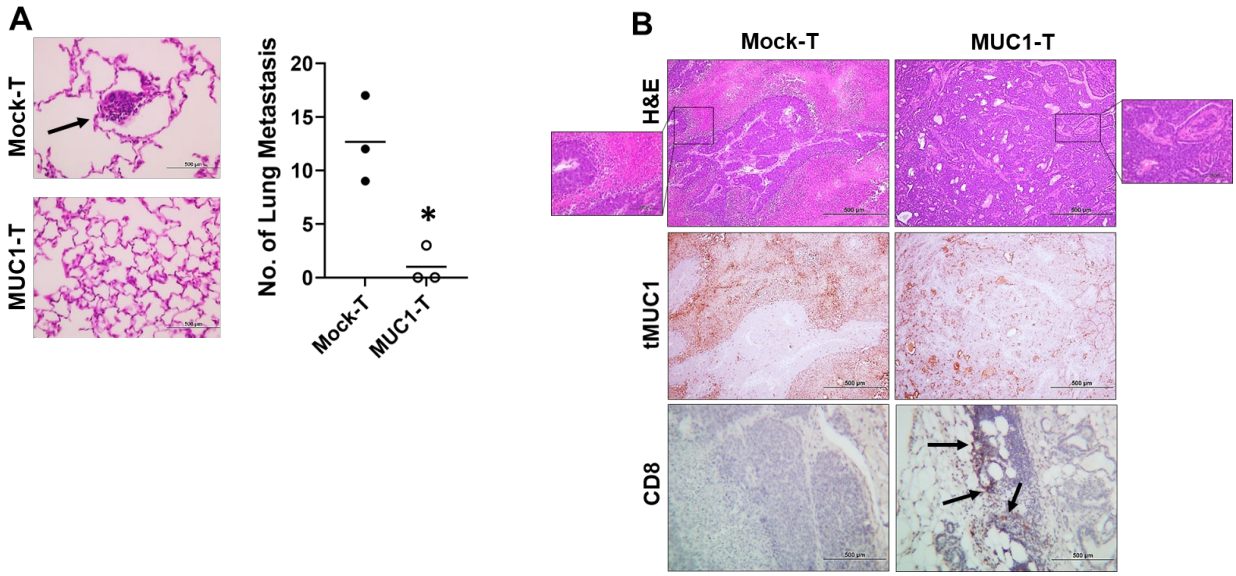


Figure 13. Reduced tumor metastasis and less advanced tumor tissue morphology. Lungs (A) and tumors (B) were harvested at the endpoint from Figure 8 experiment. n=3 mice from each group were used for sectioning and staining. (A) Dramatic decrease of tumor distant metastasis into lung. Left, H&E staining of lung sections. The black arrow points to the metastasis tumor. Magnification=400x. Right, the number of tumor metastasis in lung. *, $p<0.05$ (Unpaired t test). (B) Top panels, tumor tissue morphology by H&E staining (100x). The boxed areas are shown in 400x magnification. Middle panels, IHC staining for tumor MUC1 with TAB004 (100x, same area as for H&E). Brown staining shows tMUC1 positivity. Bottom panels, IHC staining for mouse CD8 T cells (250x). Brown staining shows CD8 positivity, as pointed by the black arrows. MUC1-T, MUC1 CAR t cells.

panels). At the humane endpoint which was 4-5 weeks post last CAR t cell injection, the T cell infiltration into tumor was still detectable (bottom panels).

One of the endpoint studies was to test blood panels for tumor lysis syndrome. We had withdrawn blood and made serum as stock from efficacy/survival study as presented in Figure 8. We considered this CAR t cell treatment schedule for late phase toxicity evaluation (4 injections of CAR t cells at 2 weeks interval). We have also collected blood 6 days after the 1st dose of CAR t cells to serve as acute toxicity samples. Sera from healthy untreated MUC1.Tg mice were used as control. Within the liver panel, the only significance noticed was in Creatine kinase (Figure 14A). The level of Creatine kinase was lower in mock t cell group from the survival study (Mock-T, Late) when compared to normal MUC1.Tg group and to its respective CAR t cell group. Within the renal panel, the changes with significance were marked (Figure 14B). Since those significant changes happened only between T cell treatment groups versus normal healthy control, this was very likely due to disease progression in MMT mice, not due to interference by T cells. Most importantly, there was no

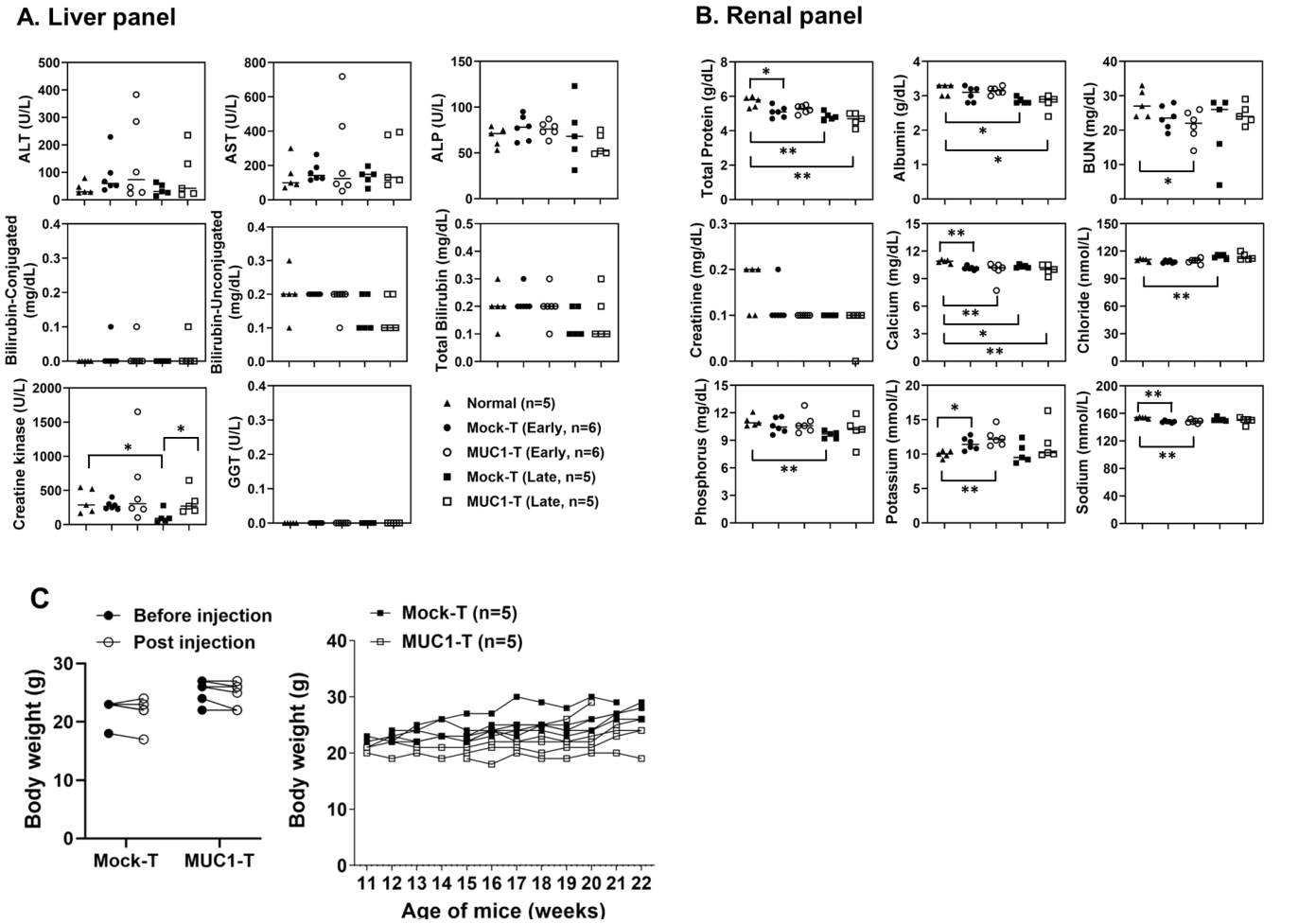


Figure 14. MUC1 CAR T cells are well tolerated in spontaneous MMT mice. (A and B) The mouse sera were collected to evaluate CAR T cell toxicity for liver panel (A) and renal panel (B). Normal, sera from normal MUC1.Tg mice; n=5. Early, sera from spontaneous MMT mice six days after receiving one dose of T cells; n=6 for each group. Late, sera from spontaneous MMT mice at the survival endpoint as in Figure 5B; n=5 picked from each group. The statistical comparison was performed between normal and each T cell treatment group, and between Mock-T and MUC1-T at early stage or at late stage. *, $p< 0.05$, **, $p< 0.01$ (Unpaired t tests). (C) No significant body weight changes post T cell treatment. Mice were the same as for (A and B) and no statistical difference was achieved. Left: mice were injected with mock T cells or MUC1 CAR T cells. Body weights were recorded right before T cell injection (Before injection) and on day6 endpoint (Post injection). Right: mice were from Figure 8B and body weights were recorded during the T cell treatment course.

significant difference between CAR t cell group and their respective mock t cell control, despite of T cell treatment being acute or prolonged. The body weights were monitored and no statistical significance was noted for each individual MMT mouse over the T cell treatment period (Figure 14C). Therefore, our CAR t cells are well tolerated in immunocompetent host and likely safe for in vivo application.

To reveal the underlying mechanism for MMT tumor reduction by MUC1 CAR t cells in vivo, gene expression profiling was conducted using mouse Clariom S array which was based on all known well-annotated genes. The differential expression analysis identified 725 genes with significance in CAR t cell treated tumors when compared with mock t cell treated tumors (Figure 15). Those differentially expressed genes in CAR t cell group were input into Ingenuity Pathway Analysis and then those genes-associated top pathways were profiled as Figure 15A. Very interestingly, multiple biosynthesis pathways were on the list, as detailed in Figure 15A, which suggested the metabolism changes within the tumor microenvironment. Another noticeable change was the pathways involved in cell/tissue structure, including Actin Cytoskeleton Signaling pathway and Remodeling of Epithelial Adherens Junctions, which suggested their role in tumor tissue morphology change or tumor metastasis. Out of the 725 genes, selected genes were plotted as heatmap (Figure 15B). Those genes were involved in tumor cell signaling (Areg, Cadm1, Bmp8b), tumor metabolisms (Glul), tissue structure (Dsg3), matrix protein regulation (Mmp8, Slpi), tumor metastasis (Il20ra, Cd302), and cell infiltration and function

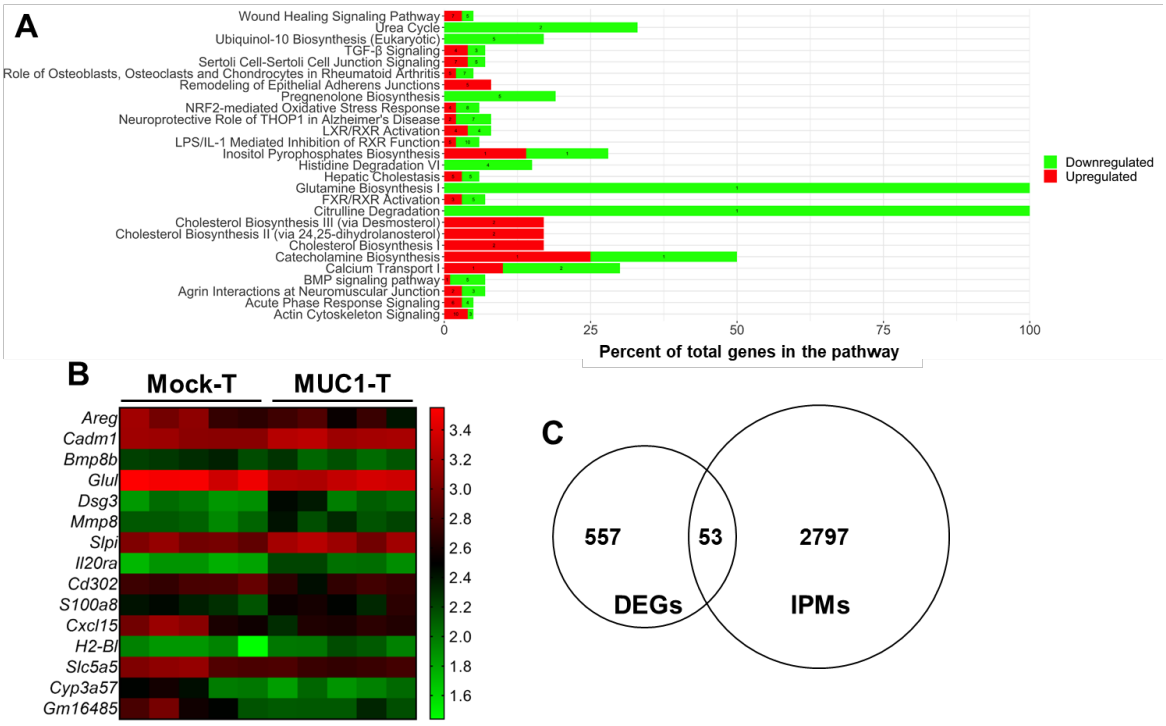


Figure 15. MUC1 CAR T cells regulate differential gene expression in spontaneous MMT mice. The tumors were collected from spontaneous MMT mice six days after receiving only one dose of T cells; n=5 tumor RNA samples for each group. The RNAs were isolated from whole tumor tissues for Clariom S Array. (A) Top pathways affected by MUC1 CAR T cells. Differential expression analysis was conducted using limma between CAR T cell group and mock T cell group. Genes with adjusted p value <0.05 were input into Ingenuity Pathway Analysis (IPA) to identify top associated pathways. In each pathway, the green bar shows the downregulated genes while the red bar shows the upregulated genes. The numbers of affected genes are labeled on the bars. (B) Heatmap of selected genes of interest. (C) Comparison of MUC CAR T cell altered genes with known immunity pathway genes. DEGs, differential expression genes, which showed significant difference between the mock-T group and MUC1-T group; IPMs, immunity pathway molecules.

(S100a8, Cxcl15, H2-Bl). Furthermore, 610 out of 725 genes were able to be compared with immunity pathway genes to determine the immune responses altered by CAR T cells. In CAR T cell treated tumors, 53 from 610 of the differentially expressed genes were involved in immunity pathways (Figure 15C).

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.

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. We have completed the endpoint studies and have the efficacy and toxicity profiles for our murine CAR t cells in this model which highly resembles the human disease. We have also found that even with short period of treatment, our CAR t cells could start remodeling the tumor microenvironment as potential mechanism of action.

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 9: Determine tMUC1-CAR-T mediated killing of TNBC explant cells in vitro (Months 26-28).
Task 10: Determine tMUC1-CAR-T mediated killing of TNBC explant cells in vivo (Months 28-36).

Task 8 Progress: Receive cells generated from breast cancer specimens from patients with metastatic/treatment refractory breast cancer.
We have completed the in vivo passage of 2 previously confirmed PDX explants and made stocks for experiments proposed in Task 10.

Task 9 Progress: Determine tMUC1-CAR-T mediated killing of TNBC explant cells in vitro.
Instead of injection with explant-derived cells, TNBC explant (PDX tissues) will be implanted for in vivo treatment to maintain the heterogeneity of PDX and to have better PDX survival and growth in vivo. Thus, Task 9 (Aim 3) will not be continued. Instead the work will be done in vivo.

Task 10 Progress: Determine tMUC1-CAR-T mediated killing of TNBC explant cells in vivo.
Stocks for PDX tissues and CAR T cells have been completed for Tasks 10 (Aim3). This Task will start very shortly. Mice have been ordered for the experiment.

Milestones: Duke University (Dr. Lyerly's group) will collect at least 15 breast cancer samples. They will establish at least 10 breast cancer explants 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 MUC1 expression and in vivo tumor growth. We will soon start the in vivo 2nd and 3rd generation CAR T cell treatment in those two PDX orthotopic models.

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

We had PhD students and undergraduate students work on parts of this project. Dr. Chandrav De, a post-doc fellow gained experience in the field of tumor immunotherapy and has made cell stocks for 2nd and 3rd generation human CAR T cells. He has completed PDX tissue passage in NSG mice and made tissue stocks. Dr. Saleh Alabbas will continue the work as a post-doc fellow to gain experience in tumor biology and cancer immunotherapy. He is working on this project till its completion.

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.)

We presented the work recently at a Breast Cancer Conference in Barcelona Spain. GICBC-2022, Sept 20-21, 2022. Title: Immunological targeting of the oncogene tMUC1 leads to significant anti-tumor effects in breast cancer

The manuscript is ready for submission to Journal of Immunotherapy of Cancer titled: 'Anti-MUC1 CAR T cells are effective and safe in controlling solid tumor in immunocompetent host'

The 3rd manuscript using 3rd generation CAR T cells and PDX models is in draft form.

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 extended period of this project. All the new data will be published in peer-reviewed journals and also will be shared in the conferences.

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 immunocompetent* 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. However, because of our publication in Frontiers, there has been interest from pharmaceutical to develop CAR NK cells using the same scfv construct from TAB004.

What was the impact on society beyond science and technology?

Nothing to report

4. 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

We were and are still experiencing difficulty to obtain adult female NSG mice in time. We are aware of this unexpected situation and have been requesting those mice way ahead of time to make sure the rest of experiments will start as scheduled.

Changes that had a significant impact on expenditures

The pandemic

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

5. **Products:** murine CAR t cells were successfully generated and tested in vitro and in vivo in animal models. The 3rd generation human CAR T cells were successfully generated and tested in spheroid assay.

6. **Participants & Other Collaborating Organizations**

What individuals have worked on the project?

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

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

Source: GeoVax-Contract (pending-recommended for funding)
Role: Principal Investigator
Title: Preclinical Evaluation of MUC1 Combination Therapy for Pancreatic Tumors
Dates: 03/15/2022 – 03/14/2023
Amount:
No overlap

Source: NIH/NCI – 1 R01 CA263897-01A1 (Vivero)
Role: Co- Investigator
Title: Stimuli-responsive mucin 1 specific nanoparticles for efficacious combinatorial chemotherapy of pancreatic ductal adenocarcinoma.
Dates: 06/30/2022 – 06/29/2027
Amount:

What other organizations were involved as partners?

Nothing to report

7. **Special Reporting Requirements:** Nothing to report

8. **Appendices:** none