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TITLE: Adoptive Cell Therapy Against Triple-Negative Breast Cancer Using a Novel tMUC1 Antibody-Derived CAR

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have completed most of Tasks 1, 2, and 3 which are all part of aim 1. We have received approval for IACUC and IRB protocol. We have completed the human CAR T cell constructs for the 2nd and 3rd generation CARs and confirmed the sequences. We have successfully completed cytolysis assays for the 2nd generation CAR on human TNBC cell lines in an antigen-specific manner. These CAR T cells do not lyse normal epithelial or fibroblast cell lines. We have shown that the 3rd generation CARs are well expressed on human T cells. The cytolysis assays using the 3rd generation CAR are ongoing. We have also generated the murine CARs and have successfully expressed the 2nd generation of the murine CARs on murine T cells. We also have shown successful cytolysis of murine TNBC cell line in an antigen-specific manner. A summary of the data are presented in the report. We have started some of the in vivo experiments in specific aim 2 in vivo.

15. SUBJECT TERMS

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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. A 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 engineered the chimeric receptor MUC28z comprising of the scFv of TAB004 coupled to CD28-CD3ζ. MUC28z was efficiently expressed on activated human T cells. Those MUC28z CAR T cells expressed higher levels of CD25 and CD11c. MUC28z CAR T cells demonstrated significant target-specific cytotoxicity against TNBC cells that corresponded strongly to tumor surface tMUC1 level. Only after exposure to tMUC1 on TNBC cells, MUC28z CAR T cells increased granzyme B release and produced IFN-γ and several other Th1 type cytokines, including IL-2 and TNF-α. Murine MUC28z CAR t cells also showed successful lysis of murine TNBC cell line in an antigen dependent manner in vitro. Thus, we envision that MUC28z CAR T cell will be highly efficacious for the treatment of $tMUC1^{+}$ TNBC tumors. The data will confirm tMUC1 as a high-priority target for engineered T cell therapy.

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 TAB 004-CAR-T mediated killing *in vitro* of human and mouse TNBC cells (with and without anti-PD1 Ab).
Aim 2a. Demonstrate TAB 004-CAR-T + anti-PD1 Ab mediated killing *in vivo* in xenograft model of human metastatic TNBC.
Aim 2b. Demonstrate TAB 004-CAR-T + anti-PD1 Ab mediated killing *in vivo* in orthotopic model of mouse metastatic TNBC in human MUC1.Tg syngeneic – immune competent mice.
Aim 2c. Demonstrate TAB 004-CAR-T + anti-PD1 Ab mediated killing in spontaneous breast cancer model in the human MUC1-PyV MT bitransgenic (MMT) mice.
Aim 3. Demonstrate TAB 004-CAR-T + anti-PD1 Ab mediated killing in human tumor explant models of metastatic, treatment refractory TNBC.

What was accomplished under these goals?

We have only done Aim 1 so far. We have started some in vivo work from aim 2. Tasks 1, 2, and 3 are mostly complete.

Task 1: Amendments to the existing UNCC IRB/IACUC for approval **(1-2 months):**

We completed this task and received UNCC IRB number: #18-0227 and this study was also approved through HRPO.

UNCC IUCUC number was also received #16-014 and the study in animal subjects was approved through ACURO.

Task 2: TAB-CAR-T mediated TNBC killing in vitro **(1-6 months):**

We have completed majority of this task to best of our abilities. The results are provided below:

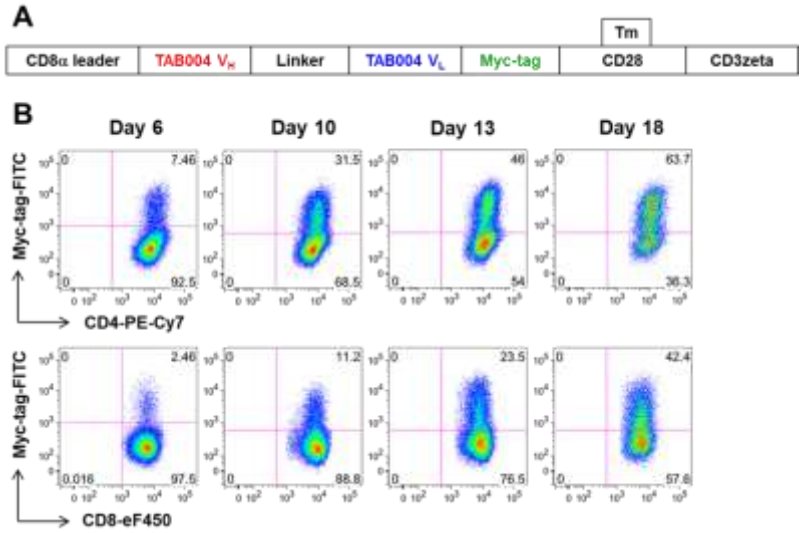

Figure 1. Increased MUC28z positivity on activated human PBMC*(A) Schematic diagram of the engineered receptor MUC28z. (B) MUC28z CAR expression in activated human T cells after retrovirus transduction, as determined by flow cytometry analysis of Myc-tag expression. Cells were gated for CD4 or CD8, and then analyzed for Myc-tag expression. Dead cells were excluded by 7-AAD staining.*

We show that MUC28z CAR is expressed on activated human T cells and binds with tMUC1^{high} TNBC tumor cells

We constructed a human CAR (MUC28z) that incorporated the scFv motif derived from TAB004, and the CD28/CD3ζ signaling domains. Figure 1A showed the schematic structure of the MUC28z CAR. MUC28z CAR expression on activated T cells was monitored by staining for Myc-tag and analyzed by flow cytometry. CD4$^+$ and CD8$^+$ T cells from human PBMC were effectively transduced. On average, the transduction efficiency was approximately 40% for CD8$^+$MUC28z T cells, and 60% for CD4$^+$MUC28z T cells (Figure 1B).

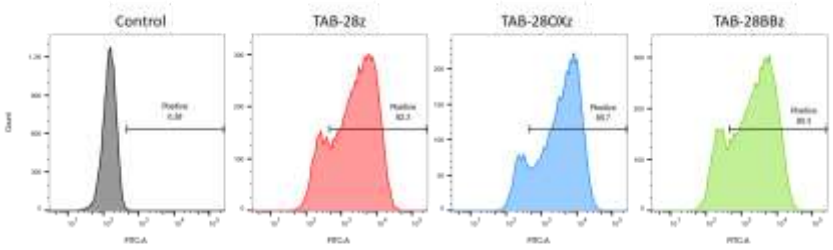

Figure 2. Percent positive tMUC1 expression of T cells 48 hours after infection.
Control – empty viral vector
FITC - Anti-Myc Tag antibody used to determine percent positive tMUC1 expression

CAR T constructs other than the original 2nd generation TAB-28z (TAB-28OXz and TAB-28BBz) are ready and the expression on T cells is successful (Figure 2). This shows that we get ~80% of human T cells express CAR construct on their surface.

MUC28z T cells are well activated

Next, we performed a detailed phenotyping of MUC28z CAR T cells. Since both CD4$^+$ and CD8$^+$ MUC28z T cells displayed similar phenotypical changes, we only showed data from CD8$^+$ population here.

A very recent publication by Eyquem *et al*. categorized CAR T cells into four different groups based on CD45RA versus CD62L expression [1]. CD45RA$^+$CD62L$^-$ cells were taken as terminal effectors, while CD62L$^+$ cells were naïve and central memory cells with less differentiation. The CD62L$^+$ cells have been associated with stronger anti-tumor activity [2,

3]. In Figure 3A, the MUC28z CAR T cells were grouped accordingly. MUC28z CAR T cells were more differentiated than the mock T control cells, but were similar to unstimulated normal PBMC (Figure 3A), which suggested that they could still persist and function well *in vivo* once transferred.

In Figure 3B, the expression of CD25 on total Myc-tag$^+$ CD8$^+$ MUC28z CAR T cells suggested their active functional status. It has been demonstrated that CXCR4 expression is down-regulated on human CD8$^+$ T cells during peripheral differentiation [4]. We found dramatically lower expression of CXCR4 on CD8$^+$MUC28z CAR T cells, suggesting their memory/effector or effector status corresponding with the up-regulation of CD25. We also found that a significant portion of Myc-tag$^+$CD8$^+$ MUC28z CAR T cells co-expressed CD11c. Even though CD8$^+$ MUC28z CAR T cells displayed strong expression of PD1 on the cell surface, it correlated more with their activation status (as evidenced by CD25 expression), rather than their exhaustion status. This was further evidenced by their superior tumor lysis function and cytokine release even at a low E:T ratio (data not shown). Together, Figure 3A and 3B showed that MUC28z CAR T cells are phenotypically heterogeneous.

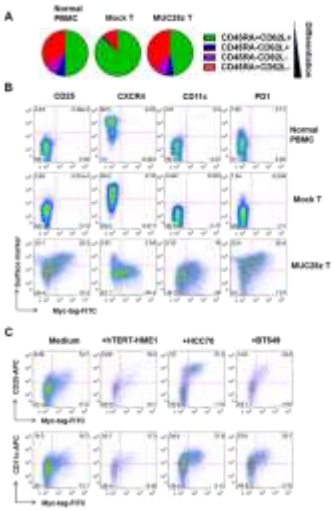

Figure 3. Differential activation/differentiation status between mock T cells and MUC28z CAR T cells. (A) The differentiation status of MUC28z CAR T cells was similar to untreated PBMCs. Cells were analyzed for CD62L and CD45RA on CD8$^+$MUC28z CAR T cells. The PBMCs from normal healthy donor were cultured overnight and used as normal PBMC control. Data are presented from a representative experiment of n=3. (B) The expression of surface markers on CD8$^+$ MUC28z CAR T cells on day 14 after retrovirus transduction. Data are presented from a representative experiment of n=4. (C) The expression of CD25 and CD11c on CD8$^+$ MUC28z CAR T cells after co-culture with TNBC cell lines for 24hr. Data are presented from a representative experiment of n=2.

To further validate that the increase in CD25 and CD11c expression on CAR T cells was associated with specific activation status, CD8$^+$ MUC28z T cells were co-cultured with tMUC1^{high} HCC70 and BT549 tumor cells as well as with normal mammary epithelial cells (hTERT-HME1). Data in Figure 3C showed that when co-cultured with tumor cells, the vast majority of CD8$^+$ MUC28z CAR T cells expressed increased levels of CD25 and CD11c on their surface, whereas, when co-cultured with hTERT-HME1, there was no change in CD25 and CD11c expression on the same CAR T cells.

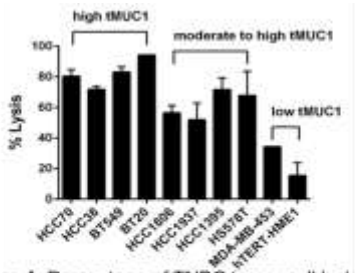

Figure 4: Percentage of TNBC tumor cell lysis by MUC28z CAR T cells at E:T ratio of 5:1 after co-culture for 3 days, as determined by apopotosis assay. Data are presented as the mean ± SEM of 4 individual experiments.

MUC28z CAR T cells induce tMUC1-dependent cytolysis of TNBC tumor cell *in vitro*

For testing lysis efficacy, TNBC cells were co-cultured with MUC28z CAR T cells at a E:T ratio of 5:1 (Figure 4). MUC28z CAR T cells demonstrated significant tMUC1-specific TNBC cell lysis *in vitro*. The intensity of tumor cell lysis by MUC28z CAR T cells highly corresponded with the cell surface expression of tMUC1 on TNBC cells. hTERT-HME1 was then used as a tMUC1 low control cells. We have completed the cytolytic assay using the 2nd generation CAR

T cells but the cytolytic assays with the 3rd generation CAR T cells are ongoing and results will become available in the next month.

MUC28z CAR T cells release antigen-specific cytokines after co-culture with MUC1^{+} TNBC cells

Next, MUC28z CAR T cells were compared with mock T cells for cytokine release. With or without the presence of tMUC1^{high} HCC70 cells, mock T cells were "quiescent," since there was no stimulatory signal between tumor cells and mock T cells (Figure 5A, top). However, after co-culture with HCC70 cells for 24hr, CD8^{+} MUC28z CAR T cells showed significant increases in IFN-γ and Granzyme B production (Figure 5A, bottom). Further, we measured the production of IFN-γ from MUC28z CAR T cells by ELISA in the supernatants post co-culture with HCC70, BT549, HCC1806 tumor cells and hTERT-HME1 cells. Data in Figure 5B clearly demonstrated that the amount of IFN-γ production by MUC28z CAR T cells was significantly higher and antigen dependent when co-cultured with tumor cells versus when cultured with normal cells. Mock T cells co-cultured with the same TNBC cell lines showed no release of IFN-γ. Taken together, Figure 5A and Figure 5B demonstrated that the release of IFN-γ and Granzyme B required the scFv of TAB004 on the cell surface of CD8^{+} MUC28z CAR T cells to bind the tMUC1 tumor antigen. To determine if cytolytic activity of the CAR T cells was IFN-γ-dependent, we neutralized IFN-γ with anti-IFN-γ antibody during co-culture, and found that IFN-γ neutralization significantly compromised the tumor lysis ability of MUC28z CAR T cells at E:T ratio of 0.5:1 and 1:1 but not 2:1 (Figure 5C). The target tumor cell in this experiment was HCC70 cells.

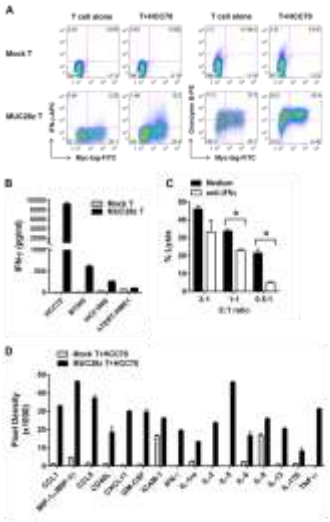

Figure 5. Antigen-specific cytokine release by MUC28z CAR T cells. (A) CD8^{+} MUC28z CAR T cells produced IFN-γ and released Granzyme B in response to tMUC1^{high} HCC70 cells *in vitro*. MUC28z CAR T cells were co-cultured with HCC70 tumor cells for 24hr, and cells were stained intracellularly for IFN-γ and Granzyme B in addition to Myc-tag, gated on CD8^{+} T cells, followed by flow cytometry analysis. Data are presented from a representative experiment of n=3. (B) MUC28z CAR T cells produced IFN-γ in response to tMUC1-expressing TNBC *in vitro*. T cells were co-cultured with the selected tumor cell lines (E:T = 2:1) for 24hr, and then the culture supernatants were assayed for IFN-γ by ELISA. Data are presented as mean ± SD of replicates from a representative experiment of n=4. (C) The lysis of HCC70 cells by MUC28z CAR T cells was partially reversed by IFN-γ neutralization. HCC70 cells were co-cultured with MUC28z CAR T cells for 24hr in the absence or presence of IFN-γ neutralizing antibody. The HCC70 cell viability was determined by MTT assay. Data are presented as the mean ± SEM of 2 individual experiments. *, $p<0.05$ (student *t*-test). (D) A large panel of cytokines released by MUC28z CAR T cells after tMUC1-specific cell activation. T cells were co-cultured with HCC70 cells (E:T = 2:1) for 24hr, and then the culture supernatants were assayed for cytokine concentration by human cytokine array. Data are presented as mean ± SD of replicates.

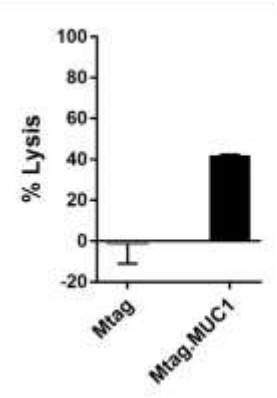

Figure 5:

- Splenocytes from MUC1.Tg mice were first enriched for T cells, followed by CD8+ T cell selection.
- CD8+ T cells were transduced with mouse CAR virus and cultured for 4 days.
- Mtag or Mtag.MUC1 cells were plated overnight. Then mouse CAR t cells were co-cultured with those tumor cells at E:T=20:1 for 48hr. The lysis of tumor cells were determined by cytolytic assay. Mock T cell lysis data were used for calculating % lysis of CAR t. The calculation formula was: (mock T – CAR T)/mock T × 100.

Using a human cytokine array, we found that in addition to IFN-γ, there was a dramatic increase of other cytokines and chemokines, particularly IL-2, TNF-α, and IL-5 by MUC28z CAR T cells (Figure 5D), which were totally absent in the mock T control group, further confirmed that only MUC28z CAR T cells recognized the tMUC1 antigen and got activated.

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

We were able to generate the mouse CAR t cells and have data using 1 of the cell lines. The rest of the cell lines will be conducted within the next 2 months. Although we hoped to complete this task fully, it took us longer to generate the murine CAR t cells. It was not as easy to infect the murine primary T cells compared to human T cells. This is now recognized by most labs working with murine T cells. Nevertheless, we are excited to report that the CAR t cells were effective in lysing the Mtag.MUC1 cells but not the Mtag cells. We are currently doing the cytolytic assay using the other murine cell lines.

Few experiments remaining in tasks 2 and 3 will be completed in a 2-3 months. The reason for the delay was some challenge with the hiring a new postdoctoral fellow and getting them up to speed as well as some of the cell lines are very hard to grow.

References

1. Eyquem, J., et al., *Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection.* Nature, 2017. **543**(7643): p. 113-117.
2. Gattinoni, L., et al., *A human memory T cell subset with stem cell-like properties.* Nat Med, 2011. **17**(10): p. 1290-7.
3. Sommermeyer, D., et al., *Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4+ subsets confer superior antitumor reactivity in vivo.* Leukemia, 2016. **30**(2): p. 492-500.
4. Kobayashi, N., et al., *Down-regulation of CXCR4 expression on human CD8+ T cells during peripheral differentiation.* Eur J Immunol, 2004. **34**(12): p. 3370-8.

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

Nothing to report

How were the results disseminated to communities of interest?

We had an AACR poster presentation in Atlanta Georgia 2019 annual meeting (abstract attached in appendix)-section 9

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?

Nothing to report

What was the impact on other disciplines?

Nothing to report

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:

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

We are having a problem growing the HCC1428 and ZR-75-1. Therefore, we propose to remove these cell lines from the experiments. We will replace 184A1 with two other normal breast epithelial cells, AG11132 and AG11134. These grow better than the 184A1 cells. These revisions will not change in any way the overall impact. We are still conducting experiments with 11 TNBC cell lines and 3-4 normal epithelial cell lines as controls.

Changes that had a significant impact on expenditures

Nothing to report

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: Nothing to report

Presented a poster in April 2019 Annual Meeting AACR- April 2019– poster number 2305

7. Participants & Other Collaborating Organizations: Nothing to report

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

Nothing to report

What other organizations were involved as partners?
Nothing to report

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

9. **Appendices:** Abstract attached.

AACR March 29-April 3 2019 : Atlanta, Georgia. Session PO.IM02.03 - Adoptive Cell Therapy 2

2305 / 4 - Tumor MUC1 glycoprotein-highly specific CAR T cells control triple-negative breast cancer

April 1, 2019, 1:00 PM - 5:00 PM	Section 22

Presenter/Authors

Ru Zhou[1], Mahboubeh Yazdanifar[1], Lopamudra Das Roy[1], John Maher[2], Pinku Mukherjee[1]. [1]Univ. of North Carolina at Charlotte, Charlotte, NC; [2]King's College London, London, United Kingdom

Disclosures

R. Zhou: None. **M. Yazdanifar:** None. **L. Roy:** None. **J. Maher:** None. **P. Mukherjee:** None.

Abstract

Antibody-derived Chimeric Antigen Receptor (CAR) T cells have great success in reduction of liquid form of tumor, but not solid tumor. Our novel antibody TAB004 can specifically recognize the tumor form of MUC1 (tMUC1) while sparing the normal MUC1 in several subtypes of breast cancers including >95% of triple negative breast cancer (TNBC). In vivo delivery of TAB004 specifically accumulates in the xenograft TNBC tumor only. Therefore, we hypothesize that TNBC can be specifically targeted with TAB004-derived tMUC1-specific CAR T cells. A panel of 45 human breast cancer cell lines were tested for tMUC1 level, and 11 out of 13 TNBC cell lines showed higher frequency of tMUC1 expression compared to that on normal cells. We engineered a 2nd generation human CAR using TAB004 that was coupled to the CD28-CD3zeta, named as MUC28z CAR. MUC28z was well expressed on activated human T cells. Compared to their mock control, MUC28z CAR T cells retained higher CD25 and CD11c, particularly on CD8 T cells. The susceptibility of TNBC cells to MUC28z CAR T cell cytolysis was dramatic while still corresponding to their tMUC1 level. Only the tMUC1-specific MUC28z CAR T cells expressed and released large amount of IFN-gamma after antigen recognition on TNBC cells. However, IFN-gamma was only involved in the tumor killing by MUC28z CAR T cells at lower E:T ratio. The in vivo treatment of TNBC tumor by MUC28z CAR T cells dramatically controlled HCC70 TNBC tumor growth in a xenograft model. Taken together, our TAB004-derived MUC28z CAR T cells are very potent at killing TNBC tumor cells both in vitro and in vivo, which is very promising to provide a safe, effective and tumor antigen-specific novel immunotherapeutic intervention.