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14. ABSTRACT

The purpose of this a ard ill be to address the overarching challenge of eliminating mortality associated ith metastatic breast cancer specifically the subtype TN C

e hypothesi e that treatment of TN C patients ith NY-ESO-1 TCR-transduced T cells is safe and ill result in tumor regression and clinical benefits e further hypothesi e that ma imal therapeutic immunity could be achieved by improving T cell persistence and traffic ing of -ESO-1 TCR engineered T cells as ell as critical help and cytoto ic function of -ESO-1 TCR-transduced C T cells mportantly such an antitumor immunity could be further amplified in vivo by bloc ing immune suppression

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Triple negative breast cancer (TNBC) is the most aggressive and difficultly treated subtype of metastatic breast cancer, with very limited treatment option due to lack of expression of estrogen receptors (ERs), progesterone receptors (PRs), or human epidermal growth factor receptor 2 (HER2). Cancer immunotherapy is becoming a promising approach for cancer treatment with little or controllable side effect. We hypothesize that treatment of TNBC patients with NY-ESO-1 TCR-transduced T cells is safe and will result in tumor regression and clinical benefits. We developed A2-ESO-1 TCR for CD8+ cells and DP4-ESO-1 TCR for CD4+ cells. We showed that A2-ESO-1 TCR-engineered T cells exhibited strong cytotoxicity against MDA-MB-231 cells in both *in vitro* and *in vivo* studies. To enhance T cell persistence, we generated CD4-specific Jmjd3 knockout mice and found that Jmjd3-deficient CD4+ T cells had significantly increased proliferation and persistence than WT T cells. Ectopic overexpression of key cytokines also enhanced the persistence and proliferation of A2-ESO-1-TCR-engineered T cells. To enhance the T cell trafficking to tumor sites, we have identified several chemokine receptors in T cells required for enhanced trafficking to breast tumor sites. Specifically, overexpression of CCR5 and CXCR3 enhanced the T cell trafficking to tumor sites and subsequent tumor-killing efficiency of A2-ESO-1 TCR engineered T cells. Consistently, local injection of specific chemokines for CCR5 and CXCR3 could effectively induce the trafficking of tumor-specific T cells to the target, and the combination of multiple chemokines had better outcome than single chemokines. We also showed that chemokine receptor expression and T cell survival could be regulated by epigenetic and metabolic inhibitors. And the pretreatment of tumor cells with cytokines and epigenetic inhibitors promoted the chemokine expression and *in vivo* A2-ESO-1 TCR T cell trafficking. DP4-ESO-1 CD4+ T cells were generated and exhibited excellent tumor recognition against breast cancer cells. *In vivo* data further showed that DP4-ESO-1 CD4+ T cells could significantly enhanced the tumor-killing efficacy of A2-ESO-1 CD8+ T cells against breast cancer. And the T cell reprogramming by ThPOK knockdown could significantly enhance the tumor killing ability of CD4+ T cells. ThPOK can interact with LSD1, and be regulated by LSD1 signaling. The treatment of LSD1 inhibitors could further enhance the anti-tumor immunity, by regulating cell metabolism and enhancing the proliferation potential and self-renewal ability. Knockdown of PD-1 and PPP2R2D enhanced the *in vivo* tumor-killing efficacy of A2-ESO-1 T cells against breast cancer. Through toxicity assays, we did not find any obvious toxicity or apparent side effect of A2-ESO-1 TCR engineered T cells in preclinical tumor-bearing NSG mice. We have generated and tested the GMP-grade A2-ESO-1 TCR virus, and our GMP cell facility has been built in Children's Hospital Los Angeles. The GMP grade A2-ESO-1 TCR-T cells have been generated and their transduction efficiency, function, specificity, and sterility have been validated. We are currently finalizing the IND application to FDA for a clinical trial, under the guidance of a professional consulting company, KM Pharmaceutical Consulting LLC.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Triple negative breast cancer, NY-ESO-1, T cell receptor, A2-ESO-1, DP4-ESO-1, T cell immunotherapy, clinical trial, T cell trafficking, chemokine receptor, T cell persistence, cytokine, JMJD3, PD-1, T cell proliferation, PPP2R2D, ThPOK, LSD1

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: To develop novel strategies for NY-ESO-1 TCR-engineered T cell immunotherapy	**Timeline (months) 1-18**	**Site 1 Dr. Wang**
Major Task 1: To increase persistence and trafficking of A2-ESO-1-specific TCR-engineered T cells, to increase T cell cytolytic activity, and to combine CD4+ and CD8+ T cells	1-18	
• ACURO Approval	1-3	Completed
• Local IACUC Approval	1-3	Completed
• To identify the most promising and safest NY-ESO-1 TCR for cancer immunotherapy	3-11	Completed
• To determine the persistence (long-lived memory) of WT and Jmjd3-KD T cells	3-11	Completed
• To enhance trafficking of NY-ESO-1-specific T cells to tumor sites	8-12	Completed
• **Year 1 Major Task/Milestone(s) Achieved**: T cell trafficking and persistence of T cells	1-12	Achieved
• To determine whether DP4-ESO-1 TCR-engineered CD4+ T cells could enhance the potency and clinical efficacy of A2-ESO-1 TCR-engineered CD8+ T cells	13-18	Completed
• To reprogram DP4-ESO-1 TCR-engineered CD4+ naïve T cells to a cytotoxic phenotype	13-15	Completed
• **Year 2 Major Task/Milestone(s) Achieved**: The critical role and cytolytic activity of CD4 T cells and CD8 T cells	13-18	Achieved
Specific Aim 2: To enhance NY-ESO-1-specific TCR-mediated therapeutic immunity by blocking	**Timeline (months) 13-36**	**Site 1 Dr. Wang**

PD-1 signaling and to assess the potential toxicity in preclinical studies		
Major Task 2: To test DP4-ESO-1 TCR-engineered CD4+ T cells, A2-ESO-1 TCR-engineered CD8+ T cells, or both along with blocking immune suppression or negative regulators	13-36	
• To enhance NY-ESO-1 TCR-mediated immunity by knockdown of PD-1 signaling molecules	13-24	Completed
• To determine the potential toxicity of NY-ESO-1 TCR-engineered T cells	25-36	Completed
• **Year 3 Major Task/Milestone(s) Achieved:** Completion of toxicity evaluation of TCR-engineered T cells and combined efficacy of PD-1 knockdown in preclinical model	24-36	Achieved
Specific Aim 3: To determine the safety and efficacy of NY-ESO-1 TCR-engineered T cells in a phase I clinical trial for TNBC patients	**Timeline (months) 1-60**	**Site 1 Dr. Wang**
Major Task 3: To test safety and efficacy of GMP-grade NY-ESO-1-specific adoptive T cell therapy for TNBC patients	13-36	
• To prepare NY-ESO-TCR viral particle and obtain regulatory approval such as FDA IND application, HRPO Approval, and local IRB approval	1-12	In progress
• To screen pathological samples for TNBC, contact and recruit study subjects	13-36	In progress

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Specific Aim 3: To determine the safety and efficacy of NY-ESO-1 TCR-engineered T cells in a phase I clinical trial for TNBC patients

Major Task 3: To test safety and efficacy of GMP-grade NY-ESO-1-specific adoptive T cell therapy for TNBC patients

Subtask 8: *To prepare NY-ESO-TCR viral particle and to prepare and obtain regulatory approval, such as FDA IND application, HRPO Approval, and local IRB approval.*

1. IND application.

We worked with KM Pharmaceutical Consulting LLC to finalize our IND submission regarding the phase I clinical trial of A2-ESO-1 TCR-T cells against triple negative breast cancer.

For the paperwork of IND submission, we mainly worked on the module 2, module 3, module 4 and module 5 of Common Technical Document (CTD).

Module 2 provides the summaries of module 3, module 4 and module 5. In section 2.3, we summarized the properties and the quality studies of the substances (A2-ESO-1 TCR constructs, PG13 cell lines producing A2-ESO-1 TCR and GMP-grade retroviral particles of A2-ESO-1 TCR produced by Indiana University Vector Production Facility) and the final products (GMP-grade A2-ESO-1 TCR transduced human T cells) of the project. In section 2.4 and 2.6, all the non-clinical studies of A2-ESO-1 TCR were summarized corresponding to module 4. We mainly performed three pre-clinical studies with A2-ESO-1 TCR including pharmacology study, distribution study and toxicology study. All the figures and tables of non-clinical studies were included in section 2.6. In section 2.5 and 2.7, we summarized the clinical studies reported in other trials against sarcoma and melanoma with the same A2-ESO-1 TCR as we currently used (Paul F. Robbins, 2011 and 2015).

Module 3 provides the detailed quality information regarding the substances and products as mentioned above. In this section, we described the sequence, the structure and the general properties of A2-ESO-1 TCR, the process of producing PG13 cell lines, clones and master cell bank of A2-ESO-1 TCR, the process of manufacturing retroviral particles of A2-ESO-1 TCR and the process of manufacturing clinical grade A2-ESO-1 TCR-T cells in a GMP facility. The quality control of substances (e.g., viral particles) and products (e.g., TCR-T cells) was supported by Indiana University Vector Production Facility and GMP facility at CHLA/USC. The authorized letter for access to the control documents of the Indiana University Vector Production Facility and the Certificate of Authenticity documents regarding master cell bank and viral particles were also included in this section.

Module 4 provides the detailed nonclinical study information of our product. There standalone study reports were included in this section. The biological potency study report described the T cell response against tumors, the antitumor activity and the specificity of A2-ESO-1 TCR-T cells in vitro and in vivo. The biodistribution study report described the in-vivo dynamic distribution of A2-ESO-1 TCR-T cells in murine models and the distribution of A2-ESO-1 TCR-T cells in multiple murine organs after injection. The toxicity study report described the in-vivo tests of body weight, major organ weight, histopathology, hematology and serum chemistry after A2-ESO-1 TCR-T cells were injected in murine models. All study reports were stand-alone respectively and concluded A2-ESO-1 TCR-T cells were safe for tumor treatment in murine models.

Module 5 provides the study information of clinical trials with the same A2-ESO-1 TCR against sarcoma and melanoma previously reported ([NCI] 08-C-0121 and NCT00670748). The information of clinical study group was also included.

We are actively working with KM Pharmaceutical Consulting LLC for the editing of Common Technical Document for the submission of our IND application.

2. Pre-test of cell products for clinical immunotherapy of triple negative breast cancer.

The pre-test of clinical grade cell products for cancer immunotherapy was prepared and performed at GMP facility at Children's Hospital Los Angeles (CHLA). The pre-test with the procedures of PBMC isolation, T cell isolation and activation, T cell transduction and T cell expansion completely followed the standard operating procedures (SOPs) of the project and the GMP facility. Several conditions were tested to optimize the production of NY-ESO-1 TCR-transduced T cells for clinical therapy.

2.1. Methods

2.1.1. PBMCs isolation

Healthy donors' anti-coagulated blood was shipped from Gulf Coast Regional Blood Center within one day and released upon arrival, then was diluted with equal volume of 1 x CTS DPBS. Every 30ml of diluted blood was mixed with 20ml of Ficoll-Paque PREMIUM. The mixture was centrifuged at 800 x g for 30 min at room temperature for stratification. After the centrifugation, the upper layer containing plasma and platelets was discarded and the mononuclear cell layer was transferred to a new tube. The transferred cells, which were washed by three volumes of 1 x CTS DPBS more than once and then centrifuged and resuspended, were ready for fresh use or cryopreservation.

2.1.2. T cell activation

Every 3 × 10^6 fresh or thawed PBMCs were mixed with 25ul of Dynabeads® Human T-Activator CD3/CD28 in a well of 24-well plate to reach a bead-to-cell ratio = 1:1. The mixture was incubated at 37°C for 48h. After the incubation, the bead/cell suspension was resuspended thoroughly by pipetting to increase cell recovery and the beads were removed on a magnet. The supernatant containing the activated T cells were ready for viral transduction.

2.1.3. T cell transduction

The 24-well suspension culture plate was pre-coated with 10 μg/ml RetroNectin and then blocked with 2% BSA in 1 x CTS DPBS (w/v). Every 2.5ml of clinical-grade retroviral supernatant was added to one well. The supernatant of PG-13 Master Cell Bank encoding NY-ESO-1 TCR was manufactured from Indiana University. The plate with retroviral supernatant was centrifuged at 2000 x g for 2h at 32C. After the centrifugation, the supernatant was discarded and 0.5 × 10^6 activated T cells were added to the same well, followed by incubation at 37°C. The same procedure was repeated the next day, which stood for a standard two-round transduction for the clinical-grade T cell products.

2.1.4. T cell expansion
2.5 × 10^6 NY-ESO-1 TCR-transduced T cells were added to one well of sterile G-Rex 6M bioreactor in 100ml of T cell medium. Human anti-CD3 and human anti-CD28 antibodies were added to the final concentration of 35 ng/ml. The G-Rex 6M was placed in a humidified 37°C, 5% CO2 incubator and incubated. 1 ml of 300 IU/μl recombinant human Interleukin-2 was added to the G-Rex 6M without disturbing the cells on day 1, 4, and 7 respectively. On day 9 after expansion initiation, 900ml of medium in the G-Rex 6M was discarded without disturbing the cells on the gas permeable membrane. Expanded T cells were resuspended in the remaining medium and collected for analysis.

2.1.5. The pre-test was scheduled to perform in three batches, two of which have been completed. The first batch was conducted to compare several conditions: 1) Initiation with PBMCs or bead-isolated CD8+ T cells; 2) T cell activation with anti-CD3 antibody (OKT3) or anti-CD3/CD28 Dynabeads; 3) T cell expansion under non-feeder condition. Thus the pre-test was initiated with three groups (Group 1: OKT3-PBMC, Group 2: OKT3-CD8, Group 3: Bead-PBMC) from the same donor. The PBMCs or T cells in the three groups were thawed from cryopreservation, isolated and activated under different conditions respectively and then transduced with the equal amount of retroviral supernatant.

2.2. Results

As we previously reported, the transduction efficiency of T cells with A2-ESO TCR viral supernatants was compared among the three groups within two days after the transduction by staining with anti-CD3, anti-CD8, anti-CD4 and anti-TCR antibodies and was at 70-80%. The viability of transduced T cells was also compared by staining with Propidium Iodide (PI) and showed to be 98%. The A2-ESO TCR transduced T cells could specifically recognize HLA-A2/NY-ESO-1 cancer cells. These results indicate that T cell activation by Dynabeads is optimizing for T cell transduction in GMP facility and there is no special need to isolate CD8+ T cells which helps to simplify the operation. We also tested the anti-tumor activity with the expanded TCR-T cells and showed that the expanded TCR-T cells specifically killed breast cancer cells expressing NY-ESO-1.

During this funding period, we continued working on the biological potency of A2-ESO TCR transduced T cells. Briefly, in the 3rd round of pre-test, the transduced T cells were composed of 79% of CD8+ T cells and 13.2% of CD4+ T cells after 10-day expansion (**Figure 1A**). Similar as the previous two rounds, FACS results with A2-ESO-1 TCR-T cells stained by TCR-specific antibody (anti-Vbeta13.1-FITC) showed high efficiency of A2-ESO-1 TCR transduction in human T cells (87.1% in CD8+ T cells and 88.7% in CD4+ T cells) (**Figure 1B**). The Propidium Iodide staining also showed the high viability of transduced T cells (>90%) (**Figure 1C**). The in-vitro T cell response of A2-ESO-1 TCR-T cells was assayed with NY-ESO-1 plasmid-transfected HEK293T cells, A2ESO epitope peptide-pulsed HEK293T cells, and NY-ESO-1-expressing triple negative

breast cancer lines. An irrelevant antigen (CT83) was added as a negative control for antigen specificity. After overnight incubation, the cytokine release (Interferon-γ, IFN-γ) in the supernatant was measured by sandwich ELISA with a capture antibody and a biotin-conjugated detection antibody against different epitopes of IFN-γ. Our results confirmed the T cell response and specificity of A2-ESO-1 TCR-T cells (**Figure 2**). The cytolytic activity of A2-ESO-1 TCR-T cells was also determined by incubating TCR-T cells with NY-ESO-1-expressing triple negative breast cancer lines at different Effector/Target (T cell/Tumor) ratio in vitro (20:1, 10:1, 5:1). After at least 4-hour incubation, the lactate dehydrogenase (LDH) level in the supernatant of each well was measured. We found that A2-ESO-1 TCR-T cells specifically killed 70% of triple negative breast cancer cells in a dose-dependent manner, while autologous untransduced T cells had no cytolysis as a negative control (**Figure 3**).

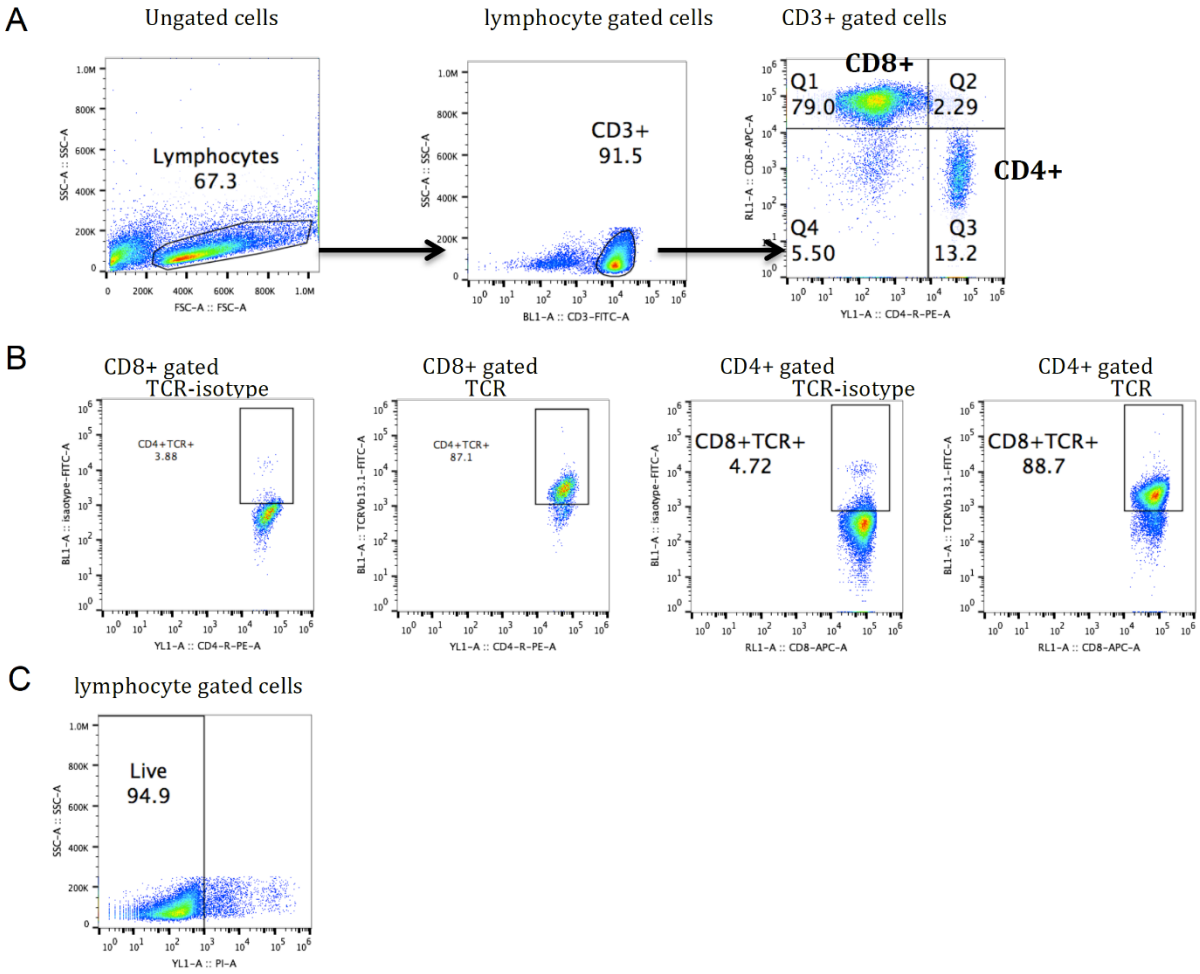

Figure 1. **Transduction efficiency and viability of A2-ESO-1 TCR-T cells**. After 10-day in vitro expansion, A2-ESO-1 TCR-transduced T cells of ND5 were stained with (A) anti-CD3-FITC, anti-CD4-PE, anti-CD8-APC antibodies, (B) A2-ESO-1 TCR-specific antibody (anti-Vβ13.1-FITC) and (C) Propidium Iodide for flow cytometry analysis.

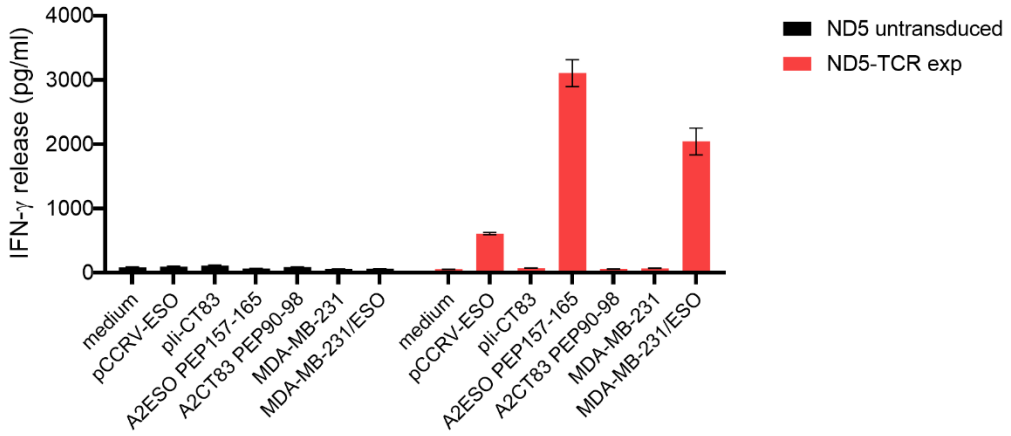

Figure 2. **T cell response and specificity assay of A2-ESO-1 TCR-T cells.** A2-ESO-1 TCR-T cells of ND5 after 10-day in vitro expansion incubated with ESO-transfected HEK293T (HLA-A2+) (red column 2), A2-ESO peptide-pulsed HEK293T (HLA A2+) (red column 4), and A2+ ESO+ triple negative breast cancer cell line MDA-MB-231 (red column 7). Negative control: T cell only (red column 1), irrelevant antigen-transfected HEK293T (red column 3), irrelevant peptide-pulsed HKE293T (red column 5) and ESO-negative tumor cells (red column 6). Autologous untransduced T cells were used as a negative control (see black columns).

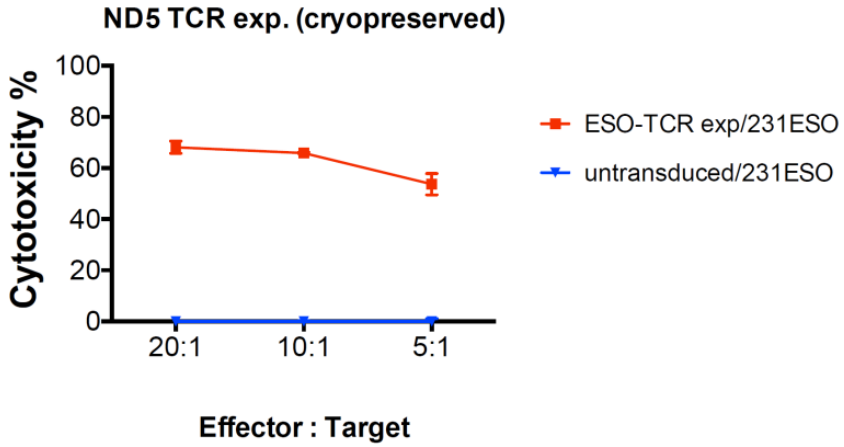

Figure 3. **Cytolytic activity of A2-ESO-1 TCR-T cells.** A2-ESO-TCR T cells of ND5 after 10-day in vitro expansion incubated with A2+ ESO+ triple negative breast cancer cell line MDA-MB-231 overnight to test the cytolytic ability of TCR-T cells by LDH assay. Autologous untransduced T cells were used as a negative control.

All three rounds of TCR-T products from the GMP facility were sent for sterility tests at CHLA Pathology & Laboratory Medicine after the 10-day expansion post transduction. Meanwhile, mycoplasma test was also performed. All TCR-T samples were confirmed to be sterile for aerobic and anaerobic bacteria, fungus and mycoplasma (**Table 1**). The test reports were included in CTD module 3.

The stability of A2-ESO-1 TCR-T cells as final products was tested when parallel vials of GMP-grade TCR-T cells (5×10^5 cells/vial) in 1 ml of either culture medium (before formulation) or PBS (after formulation) stood for different hours. The cell viability was examined by FACS after Propidium Iodide staining (**Figure 4**). The data suggests that A2-ESO-1 TCR-T cells are stable in both culture medium and PBS within 6 hours at room temperature or 4℃ with little loss of cell viability.

Table 1. Sterility tests of GMP-grade A2-ESO-1 TCR-T cells

Samples	Methods	Limits	Results
Round 1 (ND2)	Aerobic and Anaerobic for bacteria and fungus	No growth within 14 days	No growth within 14 days
	Mycoplasma test kit	Negative for the presence of mycoplasma	Negative for the presence of mycoplasma
Round 2 (ND4)	Aerobic and Anaerobic for bacteria and fungus	No growth within 14 days	No growth within 14 days
	Mycoplasma test kit	Negative for the presence of mycoplasma	Negative for the presence of mycoplasma
Round 3 (ND5)	Aerobic and Anaerobic for bacteria and fungus	No growth within 14 days	No growth within 14 days
	Mycoplasma test kit	Negative for the presence of mycoplasma	Negative for the presence of mycoplasma

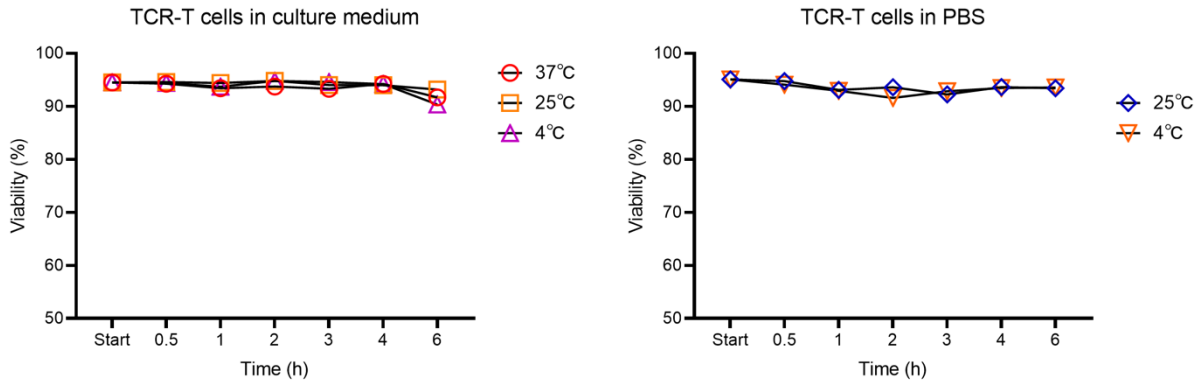

Figure 4. Results of stability tests of GMP-grade A2-ESO-1 TCR-T cells. Left: Stability of TCR-T cells in culture medium within 6 hrs. Right: Stability of TCR-T cells in PBS within 6 hrs.

To further determine the in-vivo antitumor activity of human A2-ESO TCR-T cells against triple negative breast cancer cells (ESO+/A2+), we performed experiments by injection of A2-ESO-1 TCR-T cells into a tumor-bearing NSG mouse model. Briefly, 3×10^6 MDA-MB-231/ESO cells were injected on day 0 per mouse. On day 3, the A2-ESO-1 TCR-T cells or control T cells were intravenously injected at 1×10^7 T cells per mouse, followed by intraperitoneal injection of 5×10^4 units of interleukin-2 per mouse on days 3, 4 and 5. The tumor growth in each tumor-bearing mouse monitored

Compared with the control T cells, the A2-ESO-1 TCR-T cells showed significant tumor-suppressive activity in an NSG mouse model (**Figure 5**). Notably, the tumor growth in vivo was efficiently inhibited, but was not eliminated by A2-ESO TCR-T cells. We are investigating how to optimize TCR-T cell therapy by modulating the trafficking and persistence of T cells in the tumor microenvironment.

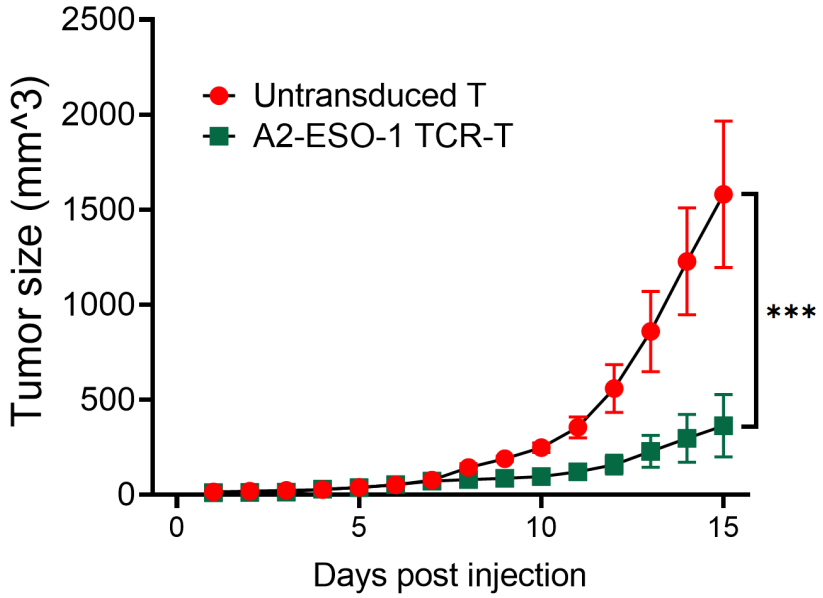

Figure 5. **In vivo antitumor activity of GMP-grade A2-ESO-1 TCR-T cells.** Tumor growth (HLA-A2+, NY-ESO-1+) in NSG mice with treatment by human T cells transduced by GMP-grade retroviral particles compared to autologous untransduced T cells.

To exclude the possibility of the potential cross-reactivity of A2-ESO TCR-T cells, we tested T cell recognition against a panel of tumor cells derived from different human tissues (**Table 2**).

Table 2. HLA-A2 positive tumor lines

Tumor type	Tumor line	HLA-A2 expression	NY-ESO-1 expression
Lung cancer	NCI-H2023	positive	negative
Breast cancer	MCF7	positive	negative
	CAMA1	positive	negative
	MDA-MB-231/ESO	positive	engineered positive
Ovarian cancer	OVCAR3	positive	negative
Lymphoma	HDLM2	positive	negative
Prostate cancer	LNCaP	positive	negative
	PC3/A2	engineered positive	negative
Gastric cancer	AGS	positive	negative
Head and Neck cancer	SCC-25	positive	negative
Cervix cancer	Caski	positive	negative

Colon cancer	Colo205	positive	negative
Melanoma	624mel	positive	positive
Neuroblastoma	SK-N-AS/A2	engineered positive	positive
Glioblastoma	U-87 MG	positive	negative

1×10^4 of tumor cells were seeded in a 96-round-well plate. Control T cells and A2-ESO-1 TCR-T cells were then added at 1×10^5 per well. Cells were co-cultured at 37°C, 5% CO_2 overnight, Next day, the supernatant of each well was harvested for IFN-γ release assay. We showed that A2-ESO-1 TCR-T cells specifically recognized tumor cells lines expressing both HLA-A2 and NY-ESO-1 positive, while control T cells had no activity against any tumor lines (**Figure 6**). Because all tumor lines were HLA-A2 positive, these results suggest that A2-ESO-1 TCR-T cells do not have cross-reactivity against any other antigens presented by HLA-A2 in those NY-ESO-1 negative tumor lines.

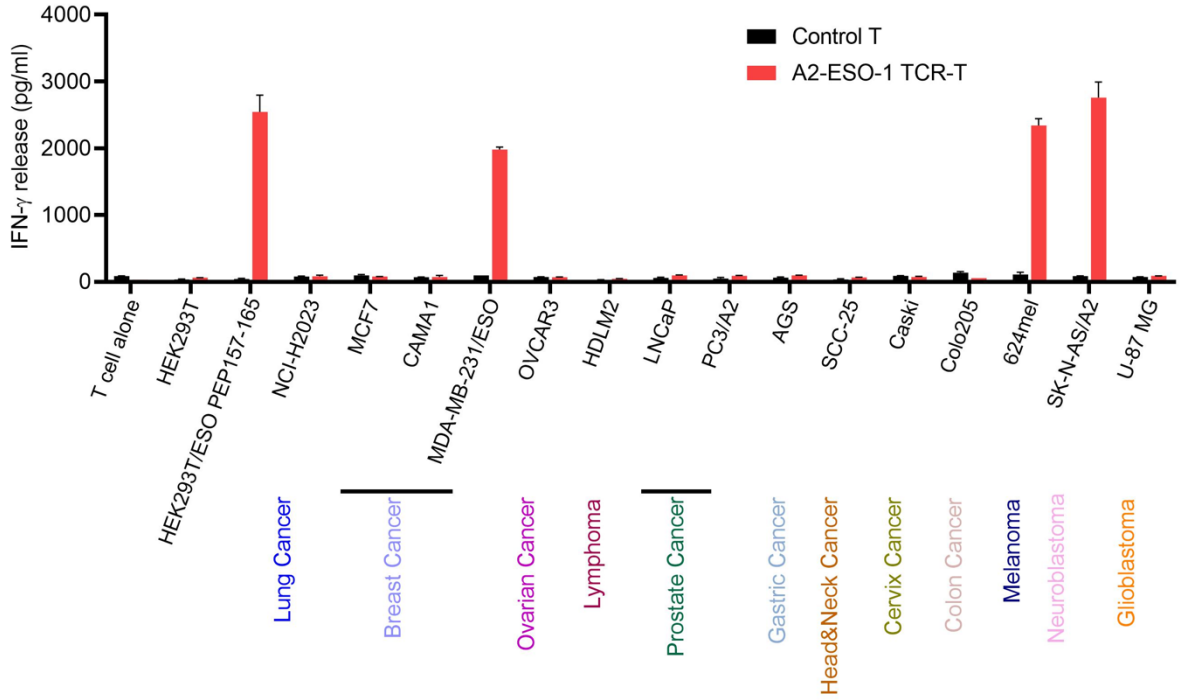

Figure 6. In vitro assay of A2-ESO-1 TCR-T cells against HLA-A2 positive tumor lines. A2-ESO-1 TCR-T cells specifically recognized tumor lines expressing HLA-A2 and NY-ESO-1, but not HLA-A2-positive and NY-ESO-1$^-$ tumor cells.

All test results above provided the validation of our quality control for the final product release for the future clinical trials.

Specific Aim 1: To develop novel strategies for NY-ESO-1 TCR-engineered T cell immunotherapy.

Major Task 1: To increase persistence and trafficking of A2-ESO-1-specific TCR-

engineered T cells, to increase T cell cytolytic activity, and to combine $CD4^+$ and $CD8^+$ T cells.

Subtask 5: *To reprogram DP4-ESO-1 TCR-engineered CD4+ naïve T cells to a cytotoxic phenotype.* LDH release assay for cytolytic T cells activity.

3. In our previous study, we have identified that LSD1, also known as KDM1A (lysine-specific demethylase 1A) gene expression is negatively associated with memory potential of ex-vivo prepared Chimeric antigen receptor (CAR) T cells. LSD1 inhibitor (2-PCPA) treatment promoted both in vitro and in vivo function of CAR-T cells, next we wanted to determine whether it is also effective on our NY-ESO-1-TCR-T cell model.

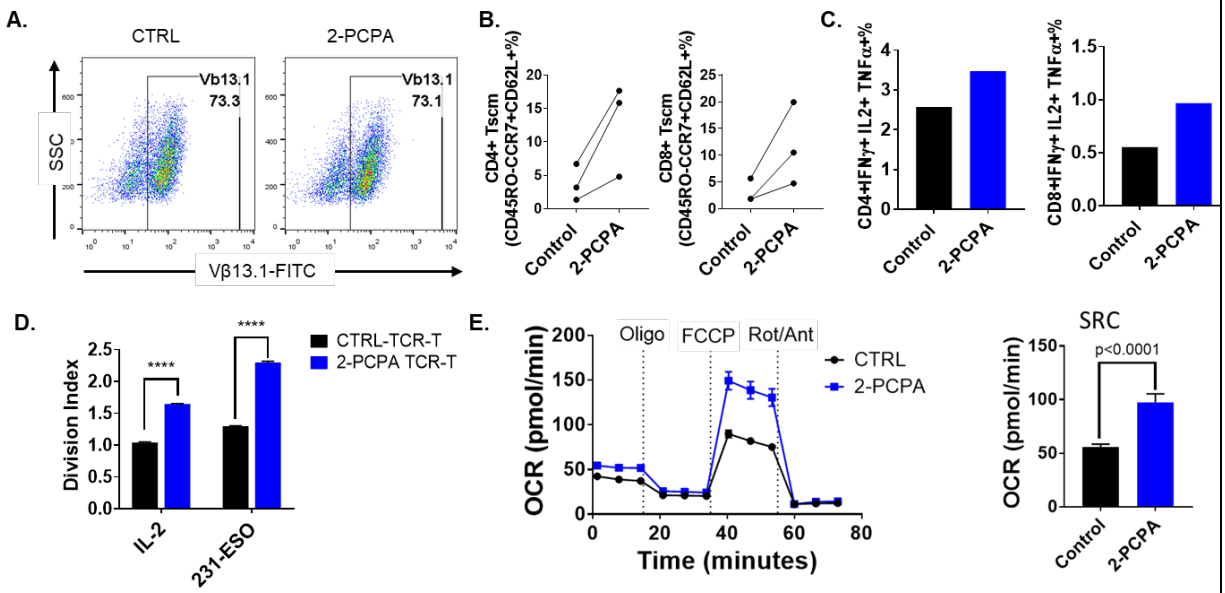

Figure 7. Treatment of LSD1 inhibitor 2-PCPA during ex-vivo culture promotes NY-ESO-1-TCR-T cells memory potential and function. A. NY-ESO-1-TCR expression detected by flow cytometry with or without 2-PCPA treatment. B. LSD1 inhibitor treatment increased Tscm percentages of NY-ESO-1-TCR-T cells. C. DMSO or 2-PCPA treated NY-ESO-1-TCR-T cells were stimulated by PMA and inonmycin for 5 hours, fixed, permeabilized and IFN-γ, IL-2 and TNFα expression in CD4 and CD8 T cells were checked. D. 2-PCPA treatment render ESO-TCR-T cells with increased OXPHOS and spare respiratory capacity (SRC). OCR was detected by Seahorse XFe96 Analyzer. E. DMSO or 2-PCPA treated NY-ESO-1-TCR-T cells were labeled with CFSE and cultured under IL-2 or stimulated by MDA-MB-231-ESO cells for 3 days. Division Index was calculated by FlowJo software.

To test this hypothesis, we directly administrated 2-PCPA at the beginning of NY-ESO-1-TCR-T cells generation. Activated human T cells were transduced at day 2 and day 3 with HLA-A2-restricted NY-ESO-1 specific TCR virus that was packaged by PG13 cell clone. Results showed that 2-PCPA treatment dose no impair TCR transduction (**Figure 7A**) but increased Tscm percentages in TCR-T cells, indicating a less-

differentiated phenotype (**Figure 7B**). To investigate the functional change of NY-ESO-1-TCR-T cells after 2-PCPA treatment, we performed intracellular staining (ICS) to check the expression of effector cytokines, IL-2, IFN-γ and TNF-α. Interestingly, although 2-PCPA treatment maintained ESO-TCR-T cells in a less-differentiated phenotype, it didn't reduce but slightly increased the frequency of NY-ESO-1-TCR-T cells with polyfunctional cytokine secretion (**Figure 7C**). High proliferation potential is a key characteristic of Tscm cells. Our in vitro proliferation assay using CFSE staining and stimulating NY-ESO-1-TCR-T cells with IL-2 or MDA-MB-231-ESO cells confirmed this feature of 2-PCPA treated NY-ESO-1-TCR-T cells (**Figure 7D**). Another key feature of less-differentiated T cell is increased oxidative metabolism and mitochondrial respiratory capacity. It was also reported that LSD1-inhibition upregulated oxidative metabolism genes in myogenic cells. Our resulted also showed that 2-PCPA treatment rendered NY-ESO-1-TCR-T cells with increased oxidative metabolism and elevated spare respiratory capacity (SRC) (**Figure 7E**).

Our in vitro results of 2-PCPA treated NY-ESO-1-TCR-T cells showed less-differentiated memory phenotype and increased function and proliferation, we attempted to test to in vivo effect of 2-PCPA-treated NY-ESO-1-TCR-T cells. We established breast cancer model using MDA-MB-231 cells expressing HLA-A2 and NY-ESO-1 antigen in 8 weeks old NOD-scid IL2Rγ^{null} (NSG) mice (**Figure 8A**). The in vivo anti-tumor function of NY-ESO-1-TCR-T cells was significantly enhanced by 2-PCPA treatment (**Figure 8B**). Through determining the memory phenotype of T cells after adoptive transfer, we found that Tscm population was barely detectable in control NY-ESO-1-TCR-T cells, while 2-PCPA treated NY-ESO-1-TCR-T cells maintained higher frequency of Tscm cells (**Figure 8C**). These results indicated that 2-PCPA treatment promotes Tscm generation in NY-ESO-1-TCR-T cells with higher proliferation potential and self-renewal ability.

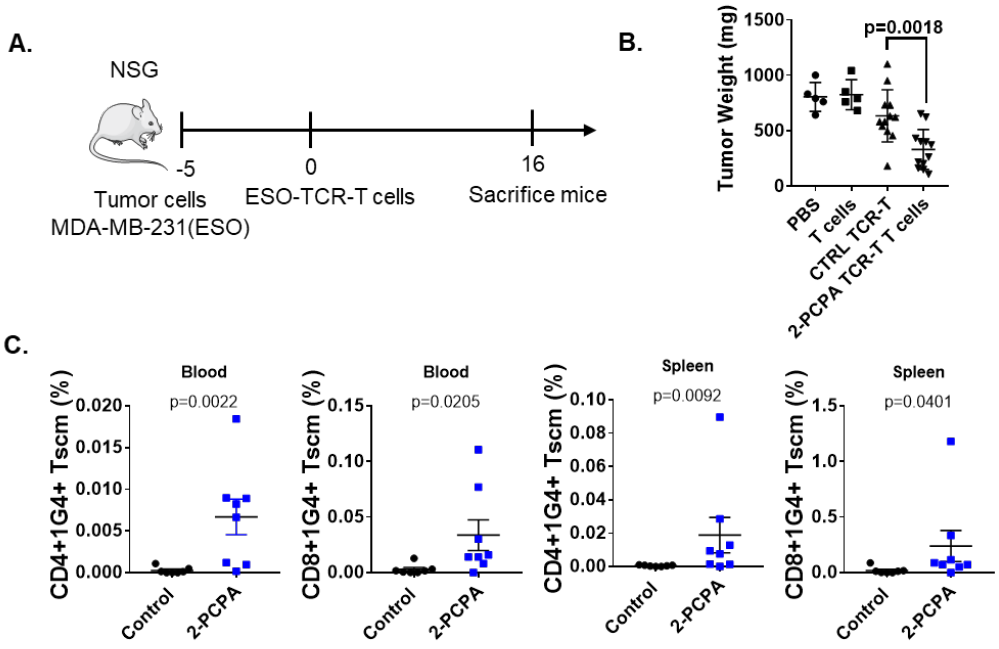

Figure 8. LSD1 inhibitor 2-PCPA treatment promotes survival and anti-tumor effect of NY-ESO-1-TCR-T cells. A. Schematic diagram of animal experiment. B. After euthanizing mice, tumor tissues were isolated and weighted. C. CD4+ and CD8+ NY-ESO-1-TCR-T Tscm cells percentage within peripheral blood and spleen were detected by flow cytometry.

Key findings and results:

1) Finalize the IND application files under the guidance of KM Pharmaceutical Consulting LLC.
2) Successfully validated procedures of PBMC isolation, T cell isolation and activation, T cell transduction and T cell expansion in GMP facility.
3) The A2-ESO-1 TCR-T cells from our GMP facility were confirmed to be sterile for aerobic and anaerobic bacteria, fungus, and mycoplasma.
4) A2-ESO-1 TCR-T cells are stable in both culture medium and PBS within 6 hours at room temperature or 4℃ with little loss of cell viability.
5) A2-ESO-1 TCR-T cells from GMP facility had specific T cell response against tumor cells only expressing NY-ESO-1 and HLA-A2 and had no cross-activity against other potential antigens in HLA-A2 positive tumor lines.
6) LSD1 inhibitor (2-PCPA) rendered A2-ESO-1 TCR-T cells with increased oxidative metabolism and elevated spare respiratory capacity (SRC).
7) LSD1 inhibitor (2-PCPA) treatment promotes Tscm generation in A2-ESO-1 TCR-T cells with higher proliferation potential and self-renewal ability.

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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

In the next reporting period, we plan to continue our proposed work on the following aspects:

1. In GMP facility at CHLA/USC, transduce human T cells with A2-ESO-TCR following the SOPs for clinical trial purpose.

2. Submit an IND for FDA approval, and initiate a clinical trial for TNBC immunotherapy using A2-ESO-TCR T cells.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to Report

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. CHANGES/PROBLEMS: The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Our project has been severely delayed due to the outbreak of COVID-19 pandemic in 2020-2021. After the partial reopening of our institutes, our lab members in University of Southern California (USC) and Children's Hospital Los Angeles (CHLA) managed to complete all the required trainings, and produced the NY-ESO-1 TCR-T cells in GMP facility, following a serial of SOPs designed for clinical trials. The transduction efficiency, function, specificity, and sterility of these T cells have been validated. We have worked on the IND application (modules 1-5) under the guidance of KM Pharmaceutical Consulting LLC. We are in the final stage and will submit to FDA before the end of 2021 for the approval of IND required for clinical trial. Once the approval of IND, we will submit IND and IRB protocol to the USAMRDC ORP Human Research Protection Office (HRPO) for review and approval before we start our clinical study. As the PI, I will continue to serve as adjunct professor at HMRI and closely work with the partner PI Dr. Chang to initiate our clinical trial using engineered T cells.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Zhu M, Deng G, Xing C, Nie G, Wang RF. BECN2 (beclin 2)-mediated non-canonical autophagy in innate immune signaling and tumor development. ***Autophagy***. 2020 Dec;16(12):2310-2312. doi: 10.1080/15548627.2020.1839277. Epub 2020 Oct 29. PMID: 33121356

2. Xing C, Wang M, Ajibade AA, Tan P, Fu C, Chen L, Zhu M, Hao ZZ, Chu J, Yu X, Yin B, Zhu J, Shen WJ, Duan T, Wang HY, Wang RF. Microbiota regulate innate immune signaling and protective immunity against cancer. ***Cell Host Microbe***. 2021 Jun 9;29(6):959-974.e7. doi: 10.1016/j.chom.2021.03.016. Epub 2021 Apr 23. PMID: 33894128

3. Deng G, Li C, Chen L, Xing C, Fu C, Qian C, Liu X, Wang HY, Zhu M, Wang RF. BECN2 (beclin 2) Negatively Regulates Inflammasome Sensors Through ATG9A-Dependent but ATG16L1- and LC3-Independent Non-Canonical Autophagy. ***Autophagy***. 2021 Jun 21:1-17. doi: 10.1080/15548627.2021.1934270. PMID: 34152938

4. Chu J, Xing C, Du Y, Duan T, Liu S, Zhang P, Cheng C, Henley J, Liu X, Qian C, Yin B, Wang HY, Wang RF. Pharmacological inhibition of fatty acid synthesis blocks SARS-CoV-2 replication. ***Nature Metabolism***. 2021 Sep 27:1-10. doi: 10.1038/s42255-021-00479-4. PMID: 34580494

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: Rongfu Wang, PhD
Project Role: PI, Professor
Contribution to Project: Dr. Wang continues to supervise the whole project, plan experiments, and analyze and interpret data.

Name: Changsheng Xing, PhD
Project Role: Study Coordinator, Assistant Professor of Research

Contribution to Project: Dr. Xing coordinates the study, conducts bench and animal experiments, collects and analyzes data, and plans future experiments.

Name: Helen Yicheng Wang
Project Role: Associate Professor of Research
Contribution to Project: Ms. Wang performs work by assisting in planning experiments, analyzing data, and interpreting results.

Name: Chen Qian, PhD
Project Role: Assistant Professor of Research
Contribution to Project: Dr. Qian works on the T cell infections, generation of the constructs, and in vitro assays.

Name: Junjun Chu, PhD
Project Role: Postdoctoral Fellow
Contribution to Project: Dr. Chu assists in the T cell infections, and both in vitro and in vivo functional assays.

Name: Yang Du
Project Role: Postdoctoral Fellow
Contribution to Project: Dr. Du assists in the animal preparation and data collection from the in vivo experiments.

Name: Xin Liu
Project Role: Postdoctoral Fellow
Contribution to Project: Dr. Liu performs T cell infections and assists in the generation of constructs.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.