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NY-ESO-1-specific TCR-engineered T cell immunotherapy for triple negative breast cancer

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14. ABSTRACT

The purpose of this award wwill be to address the overarching challenge of eliminating mortality associated wwith metastatic breast cancer, specifically, the subtype, TNBC.
We hypothesize that treatment of TNBC patients wwith NY-ESO-1 TCR-transduced T cells is safe and wwill result in tumor regression and clinical benefits. WWe further hypothesize that maximal therapeutic immunity could be achieved by improving T cell persistence and trafficking of A2-ESO-1 TCR engineered T cells, as wwell as critical help and cytotopic function of DP4-ESO-1 TCR-transduced CD4+ T cells. Importantly, such an antitumor immunity could be further amplified in vivo by blocking immune suppression.

15. SUBJECT TERMS
Triple Negative Breast Cancer

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TABLE OF CONTENTS

1. INTRODUCTION:

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, and 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 found that Jmjd3-deficient CD4+ T cells had significantly increased proliferation and persistence than WT 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, we 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-T cells. Consistently, local injection of specific chemokines, or the combination of multiple chemokines, effectively induced the trafficking of tumor-specific T cells to the target. 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 enhance 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. For clinical study, we have generated the GMP-grade A2-ESO-1 TCR virus at Indiana University Vector Production Facility stored the stock at -80℃, and certified the stock annually. We also have conducted the validation runs at Ann Kimball W. Johnson Center for Cellular Therapy (KJCCT) facility. The KJCCT is a cGMP facility located at Houston Methodist's Outpatient Center and under the Office of Translational Production and Quality (OTPQ) at the Houston Methodist Academic Institute, where the A2-ESO-1 TCR-T products will be manufactured for future clinical trials. The GMP grade A2-ESO-1 TCR-T cells have been generated and their transduction efficiency, function, specificity, and sterility have been validated. The IND application for a clinical trial was submitted and approved by FDA.

2. KEYWORDS:

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, FDA approval

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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 PD-1 signaling and to assess the potential toxicity in preclinical studies	**Timeline (months) 13-36**	**Site 1 Dr. Wang**
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?

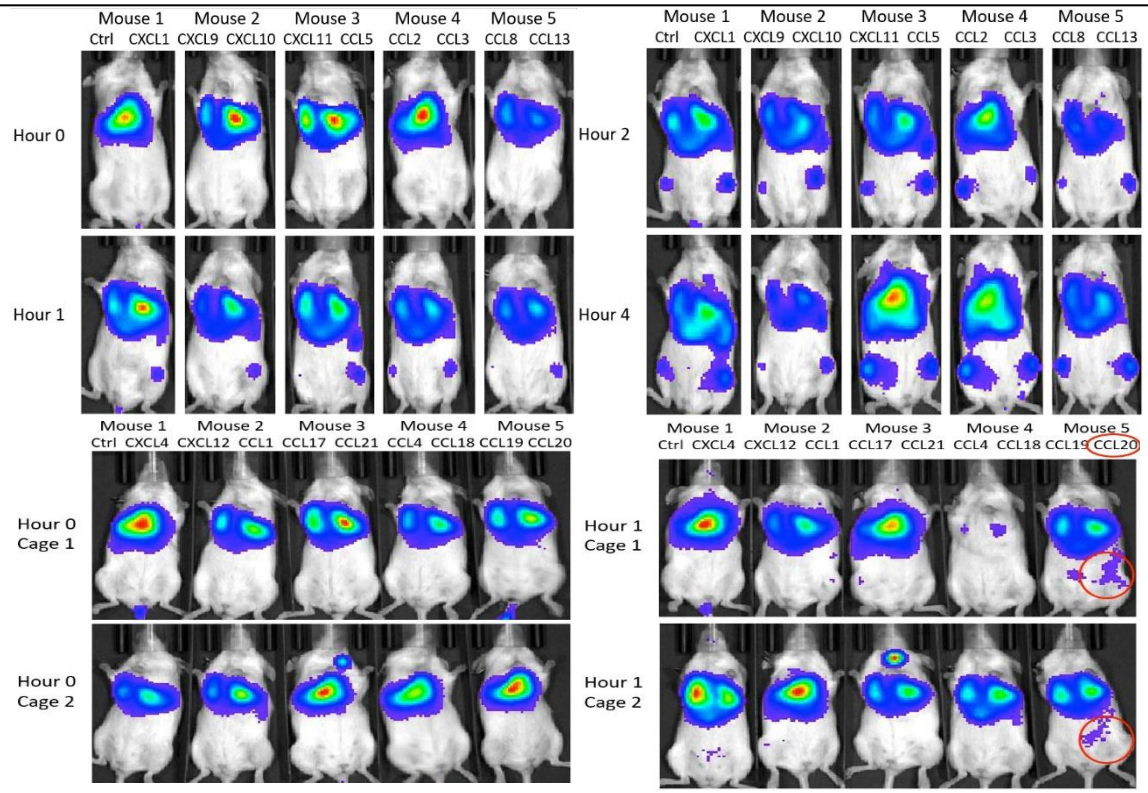
Specific Aim 1 (Months 1-18): To develop novel strategies for NY-ESO-1 TCR-engineered T cell immunotherapy.

Major Task 1 (Months 1-18): 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 3 (Months 8-12): *To enhance trafficking of NY-ESO-1-specific T cells to tumor sites.* Assess the *in vivo* trafficking, survival, and proliferation of A2-ESO-1 TCR $CD8^+$ T cells in peripheral blood, tumors, and lymph nodes. Monitor tumor growth.

In our previous work, we have successfully proved that the overexpression of chemokine receptors could significantly enhanced the trafficking of A2-ESO-1 TCR engineered T cells, and the consequential *in vivo* tumor killing efficacy. Furthermore, we have identified the optimum chemokine receptor that has the best performance. In this quarter, we expanded our strategies by injecting chemokines into the mammary fat pad (tumor site), and successfully enhanced the local T cell trafficking.

We selected 18 candidate chemokines based on their functions to attract T cells, and diluted them to an optimized concentration. Luciferase-labeled A2-ESO-1 TCR engineered T cells were next prepared, and intravenously injected into the NSG mice at 5 million cells per mouse. One hour after T cell injection, the chemokines were individually injected into the mammary fat pad of each NSG mouse, with the mixture of proper dose of Matrigel to maintain their localization. PBS mixed Matrigel solution was injected into the mammary fat pad as control group. Then the mice were observed with *in vivo* luciferase-imaging system at different time points for the distribution of A2-ESO-1 T cells.



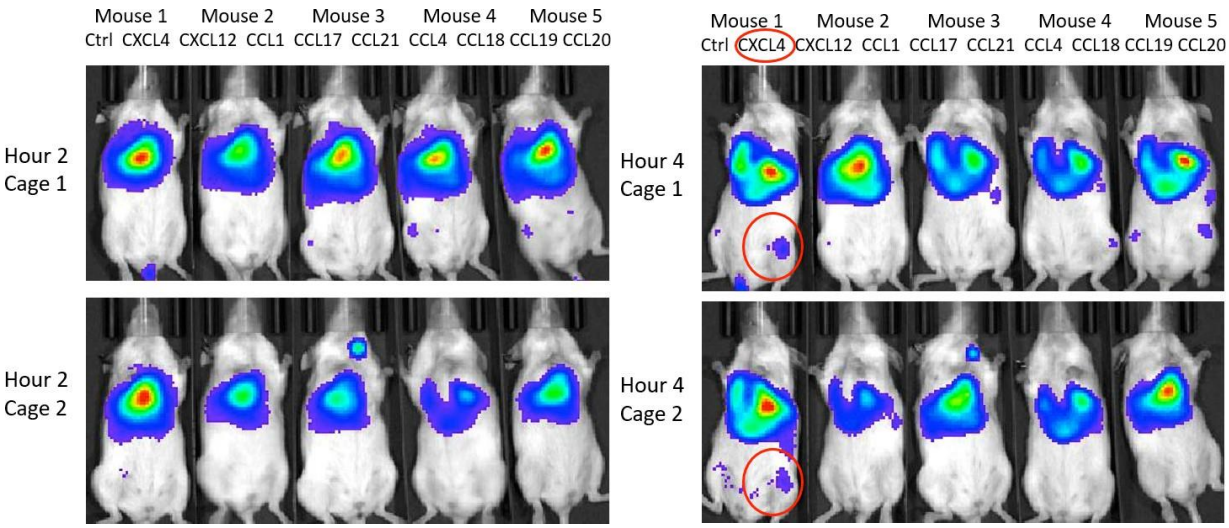


Figure 1. Luciferase assay to test the human T cell trafficking with chemokine attraction. Different chemokines (500 ng per mouse) were injected into the mammary fat pad of NSG mice, with the mixture of Matrigel to limit their location. Human A2-ESO-1 TCR engineered T cells with luciferase activity (5 million per mouse) were i.v. injected at the same time. The *in vivo* T cell attraction was observed by luciferase imaging.

At early time point (Hour 1), the injections of human CXCL1, CXCL10, CCL5, CCL3, CCL13, and CCL20 were observed to induce fast trafficking of A2-ESO-1 engineered T cell to the mammary fat pad area. Other chemokines did not have much effect at this time point.

At later time points (Hour 2 and Hour 4), the T cells induced by CCL20 disappeared, indicating that CCL20 did not have strong effect to maintain long-term T cell trafficking. CXCL11 and CCL2 had postponed but stronger attraction of T cell migration. CXCL1 still had strong effect, while CXCL10, CCL5, CCL3, and CCL13 became milder than others. Meanwhile, CXCL4 could induce the late trafficking of certain amount of T cells to the mammary fat pad. Other chemokines did not have much effect.

In summary, we found that CCR5 related chemokines (CCL5, CCL3, CCL13, labeled in green) and CXCR3 related chemokines (CXCL10, CXCL11, CXCL4, labeled in blue) had better T cell trafficking efficacy than other candidates, consistent with our previous flow cytometry data of chemokine receptor expression. CCR2-mediated CCL2 and CCR6-mediated CCL20 also showed certain importance. These chemokines may be used to enhance the trafficking of A2-ESO-1 TCR engineered T cells to the tumor sites.

Table 1. Summarization of key chemokines to induced T cell trafficking.

Chemokines	Other name	Induced T cell type	Receptor	Fast trafficking	Late trafficking
CXCL1	MGSA	Mainly neutrophils	CXCR2	D	D
CXCL9	MIG	Th1, CD8, NK	CXCR3		
CXCL10	Crg2	Th1, CD8, NK	CXCR3	D	

CXCL11	I-TAC	Th1, CD8, NK	CXCR3		D
CCL5	RANTES	T cells	CCR1/3/5	D	D
CCL2	MCP1	CD8	CCR2		D
CCL3	MIP1A	T cells	CCR1/5	D	D
CCL8	MCP2	Th2	CCR1/2/3/5		
CCL13	MCP4	Th2	CCR2/3/5	D	D
CXCL4	PF4	Th1, CD8,NK	CXCR3		D
CXCL12	SDF-1	Th17	CXCR4		
CCL1	I-309	Th2, Treg	CCR8		
CCL17	TARC	Th2, Treg	CCR4		
CCL21	6Ckine	T cells	CCR6/7		
CCL4	MIP1B	T cells	CCR5		
CCL18	MIP4	Th2	CCR8		
CCL19	MIP-3b	T cells	CCR7	D	
CCL20	MIP-3a	Th17	CCR6		

2. Combination of multiple chemokines to promote T cell trafficking to tumor sites.

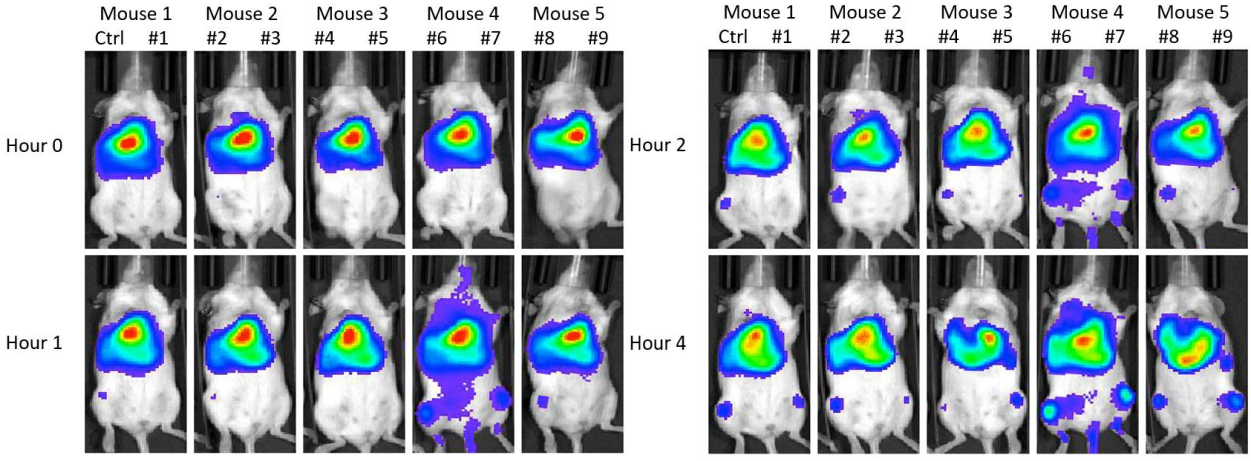


Figure 2. Luciferase assay to test the human T cell trafficking with combined chemokines. Combined chemokines (400 ng per chemokine per mouse) were injected into the mammary fat pad of NSG mice, with the mixture of Matrigel to limit their location. Human A2-ESO-1 TCR engineered T cells with luciferase activity (5 million per mouse) were i.v. injected at the same time. The *in vivo* T cell attraction was observed by luciferase imaging.

Since we show that CCR2, CCR5, and CXCR3 related chemokines had better T cell trafficking efficacy than other candidates, we next selected the chemokines that had

shown strong performance in this screening, and combine them in a similar *in vivo* study to maximize the attraction of A2-ESO-1 TCR engineered T cells to the tumor sites. CCL2, CCL5, CCL13, CXCL10, CXCL11, and CXCL4 were selected based on their performance in Figure 1. 10 groups were included as listed below:

Group 0. PBS injection as control
Group 1. CCL5 (CCR1/3/5) alone (400 ng) Group 2. All 6 chemokines (200 ng each)
Group 3. CCL5 (CCR1/3/5) + CCL2 (CCR2) + CCL13 (CCR2/3/5) (400 ng each) Group 4. CCL5 (CCR1/3/5) + CCL2 (CCR2) + CXCL10 (CXCR3) (400 ng each) Group 5. CCL5 (CCR1/3/5) + CCL2 (CCR2) + CXCL11 (CXCR3) (400 ng each) Group 6. CCL5 (CCR1/3/5) + CCL2 (CCR2) + CXCL4 (CXCR3) (400 ng each) Group 7. CCL5 (CCR1/3/5) + CCL13 (CCR2/3/5) + CXCL10 (CXCR3) (400 ng each) Group 8. CCL5 (CCR1/3/5) + CCL13 (CCR2/3/5) + CXCL11 (CXCR3) (400 ng each) Group 9. CCL5 (CCR1/3/5) + CCL13 (CCR2/3/5) + CXCL4 (CXCR3) (400 ng each)

Our data in Figure 2 showed that the Groups 6, 7, and 8 had enhanced trafficking of A2- ESO-1 engineered T cell to the mammary fat pad area at early time point (Hour 1), compared with other groups.

At later time points (Hour 2 and Hour 4), other groups start to have certain amount of T cells attracted to the chemokine injecting sites, while Groups 6 and 7 still maintained higher levels of T cells in the mammary fat pad. These data indicate that the combination of CCL5/CCL2/CXCL4 and CCL5/CCL13/CXCL10 may have higher efficacy in attracting cancer specific T cells, and that the simultaneous activation of multiple chemokine receptors on T cells may achieve better trafficking outcome.

3. In our previous work, we observed the increased expression of chemokine receptors, especially CXCR3 and CCR5, in OKT3 (human CD3 antibody) activated human T cells purified from PBMC, and the gradual decrease of chemokine receptor expression in these T cells after long-term in vitro growth (over 2 weeks). To enhance the chemokine receptor expression and the eventual T cell trafficking to tumor sites, we used OKT3 to re-stimulate T cells after the transduction of A2-ESO-1 TCR, and tested the chemokine receptor expression by flow cytometry.

We used soluble OKT3 in growth medium, and found it could only slightly enhance the CCR8 expression (from 1.4% to 6.2%), but could not induce other chemokine receptors, such as CCR2, CCR3, CCR5, CXCR2, or CXCR3 (Figure 3A).

Next we pre-coated OKT3 in the culture plates to stimulate the T cells, and observed the simultaneous elevation of multiple chemokine receptors, including CCR2, CCR3, CCR5, CCR8, CXCR2, and CXCR3. In specific, CCR2 (18% → 24%), CCR8 (2% → 9%), and CXCR2 (3% → 14%) were rapidly increased on day 2 after OKT3 treatment. Furthermore, on day 4, CCR3 (0.1% → 9%), CCR5 (0.5% → 2.4%), CXCR2 (1% → 9%), and CXCR3 (0.3% → 13%) were all enhanced in OKT3 treated T cells (Figure 3B).

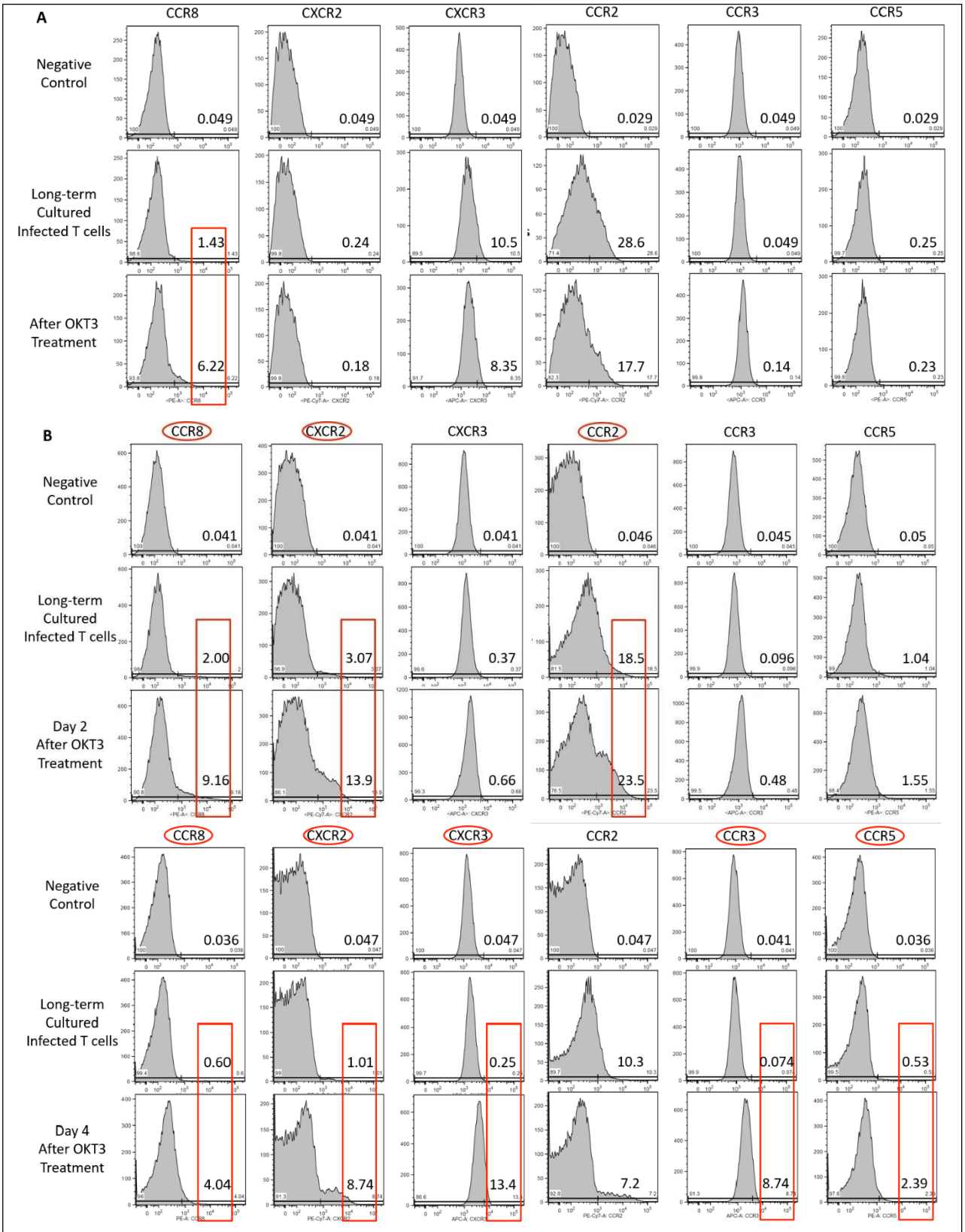


Figure 3. *In vitro* stimulation of T cells by coated OKT3 enhanced chemokine receptor expression. (A) Soluble OKT3 were used to simulate the long-term cultured A2-ESO-1

TCR engineered T cells for reactivation. (B) OKT3 was coated on plates for overnight, then the long-term cultured T cells were transferred to the coated plates for reactivation. Flow cytometry were performed to test the chemokine receptor expression on the surface of these T cells.

These data indicate that even if we could not transduce all the chemokine receptors into NY-ESO-1 T cells, we could administrate a short-term plate coated-OKT3 simulation before injection to widely enhance the chemokine receptors. In this way, the trafficking of NY-ESO-1 TCR engineered T cells can still be promoted to tumor sites.

Subtask 4 (Months 13-18): *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.* Assess the population of CD8$^+$ T cells, memory T cells by FACS analysis and monitor cytokines such as IFN-γ by ELISA. Monitor tumor volume.

4. Continued with our previous work, in which the *in vitro* assay of DP4-ESO-1 TCR for the engineering of cytotoxic CD4+ T cells has been finished, we further performed the in vivo function of DP4-ESO-1 CD4+ T cells, combined with A2-ESO-1 CD8+ T cells, in the antitumor immunity against breast cancer.

1) Preparation of MDA-MB-231/DP4/ESO cell line.
The engineered breast cancer cell line MDA-MB-231, which was originally HLA-A2 positive, was used for the animal model of *in vivo* functional test of A2-ESO-1 TCR and DP4-ESO-1 TCR and cultured in RPMI 1640 containing 10% FBS in 37°C, 5% CO_2. The original cell line was transduced by lentiviruses prepared from the constructs pLLEF-ESO-GFP and pFU3W-DP4 (α-P2A-β). Briefly, HEK-293T cells were seeded in poly-L-lysine coated 10-cm dish in DMEM containing 10% FBS (8×10^6 cells per dish). 2- 3 hours later, 10 ug of pLLEF-ESO-GFP or pFU3W-DP4 (α-P2A-β) were respectively mixed with 2.5 ug of pMD2.G and 7.5 ug of psPAX2 in 1 ml of filtered 0.25M $CaCl_2$ solution, then 1 ml of 2X BES buffered saline was added and mixed well in each tube.
15 min later, each mixture was transferred to a separate 293T-seeded dish. The transfected 293T cells were culture in 37°C, 5% CO_2. 16 hours after the plasmid transfection, the supernatant in each dish was replaced by 10 ml of fresh culture medium. 48 hrs after the plasmid transfection, the supernatant containing lentiviruses in each dish was collected and replaced by 10 ml of fresh culture medium. The lentiviral supernatant was filtered by 0.45 um filter. 5 ml of ESO lentiviral supernatant and 5 ml of DP4 lentiviral supernatant was added together to the original MDA-MB-231 cell line (0.2×10^6 cells in T25 flask) and mixed with 10 ml of fresh culture medium. 72 hrs after the transfection, the second batch of lentiviral supernatant was collected, filtered and applied in the same way. After the transduced MDA-MB-231 was changed to fresh culture medium, the cells were cultured in 37°C, 5% CO_2 for 48 hrs. The expression of NY-ESO-1, HLA-A2 and HLA-DP4 in transduced MDA-MB-231 cells was verified by GFP expression, antibody staining and FACS. The cells which were ESO positive, A2 positive and DP4 positive were sorted by flow cytometer and the sorted cells were expanded in RPMI 1640 containing 10% FBS in 37°C, 5% CO_2.

2) Injection of MDA-MB-231/DP4/NY-ESO-1 in NSG mice.
Each female NSG fat pad was injected with 1 million of DP4+/NY-ESO-1+ MDA-MB- 231 cells, which were suspended in 150 ul volume with 50% Matrigel. 5 mice were used for each group.

3) Transduction of naïve CD4+ and CD8+ T cells.

Naïve PBMCs from healthy donor were used for isolation of CD4+ and CD8+ T cells. 20×10^6 CD4+ or CD8+ T cells were isolated from 200×10^6 PBMCs respectively by magnetic beads. Then the isolated CD4+ or CD8+ T cells were activated in a 24-well plate respectively. For T cell activation, 24-well tissue culture plates were coated with 1ml of PBS containing 0.5 µg/ml OKT3 per well and incubated at 4°C overnight. The isolated CD4+ or CD8+ T cells were added in (0.5×10^6 per well) and cultured in TCM containing 300 IU/ml IL-2 at 37°C, 5% CO_2 for 48 hrs. 48hrs later, the activated CD4+ or CD8+ T cells were transduced by retroviruses produced by high quality PG-13 cell clones. 24-well non-tissue culture plates were pre-coated with 0.5 ml of human recombinant RetroNectin per well (Takara, 10 µg/ml in PBS) one day prior to the transduction and incubated at 4°C overnight. After RetroNectin was removed, the plates were blocked with 2% (w/v) BSA in PBS at room temperature for 30 min. The plates were then washed twice with PBS containing 2.5% HEPES. Retroviral supernatants encoding DP4-ESO-1 TCR or A2-ESO-1 TCR were added to the RetroNectin-coated plates respectively (2.5 ml per well). The plates were centrifuged at 2000×g, 32°C for 2 hours. After the viral supernatant was discarded, activated naïve CD4+ T cells were added to the DP4-ESO-1 TCR plate (1×10^6/well) while CD8+ T cells in A2-ESO-1 TCR plate. Transduced CD4+ or CD8+ T cells were cultured in 37°C, 5% CO_2 overnight. A second round of transduction was carried on the next day completely the same as described above. 48hrs after the transduction, the TCR expression in transduced CD4+ or CD8+ T cells were verified by FACS with TRBV6-5 and TRBV30-specific antibody staining respectively (showing >50% of TCR expression).

4) Injection of effective CD4+ and CD8+ T cells in tumor-bearing mice.
Effective CD4+ and CD8+ T cells were mixed in PBS by 1:1 ratio. Mixed T cells were injected to tumor-bearing mice at Day 3 by tail vein I.V. injection.
Group 1: 2 million mock CD4+ T cells + 2 million mock CD8+ T cells
Group 2: 2 million DP4-ESO-1 TCR CD4+ T cells + 2 million mock CD8+ T cells
Group 3: 2 million mock CD4+ T cells + 2 million A2-ESO-1 TCR CD8+ T cells
Group 4: 2 million DP4-ESO-1 TCR CD4+ T + 2 million A2-ESO-1 TCR CD8+ T cells
Each mouse was injected with 50K Unit IL-2 by I.P. at Day 3, 4, 5. Tumor volume was measured by calipers from Day 7. Mice were sacrificed when the tumor diameter in the control group reached 2 cm.

5) Results.
Our data showed that DP4-ESO-1 CD4+ T cells could dramatically enhanced the tumor-killing efficacy of A2-ESO-1 CD8+ T cells. By comparing Group 1 and Group 2, we found that DP4-ESO-1 CD4+ T cells alone could lead to a 15% decrease of tumor size and weight at the end point, indicating that DP4-ESO-1 CD4+ T cells alone had certain but limited tumor killing function. When combined with CD8+ T cells, we found that DP4-ESO-1 CD4+ T cell injection

(Group 4) could induced a 35%-50% shrink of tumor size (varied in repeated independent experiments), compared with A2-ESO-1 CD8+ T cells alone (Group 3). These data suggest that the combined therapy with NY- ESO-1 TCR engineered CD4+ and CD8+ T cells could be used to better treat the triple negative breast cancer.

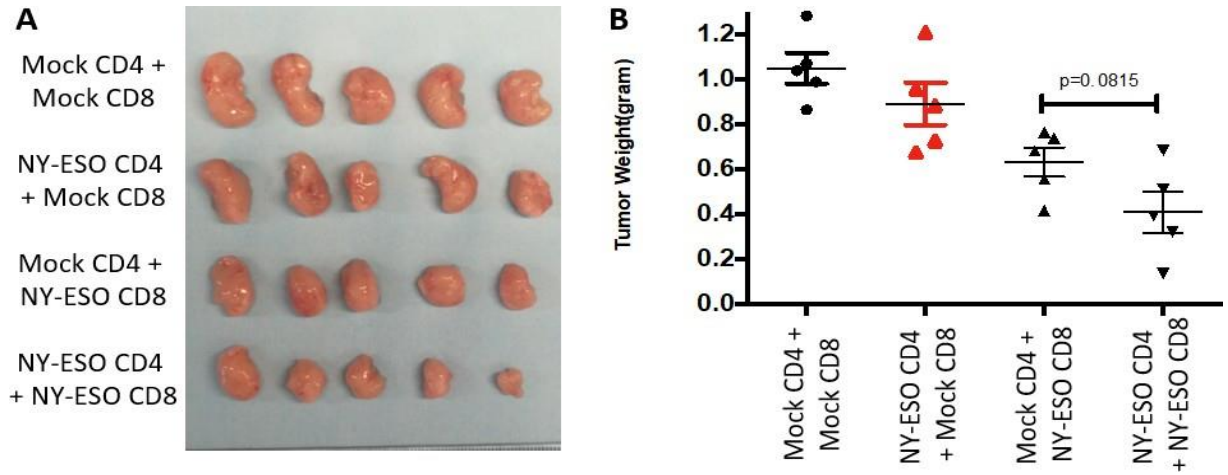


Figure 4. DP4-ESO-1 CD4+ T cells could dramatically enhanced the tumor killing efficacy of A2-ESO-1 CD8+ T cells. (A) Tumor development on the termination day. (B) Analysis of tumor weight.

5. After we confirmed the *in vivo* function of DP4-ESO-1 CD4+ T cells, combined with A2-ESO-1 CD8+ T cells, in the antitumor immunity against breast cancer, we optimized the condition by using humanized NSG mice.

The humanized mice were used to assess the potency and safety of NY-ESO-1 TCRs due to the limitation of transgenic mice. Humanized NSG (NOD;SCID;IL2γ-/-) mice were obtained and used for testing human cancer cell growth. MDA-MB-231/DP4/ESO tumor cells in 50 µl growth medium/matrigel (50%) were prepared and orthotopically injected into the 4th fat pad of female NSG mice (n=6 per group) on day 1. We observed that this human tumor line could grow in NSG mice. On day 5, we started to treat tumor- bearing NSG mice with 4 groups of human T cells (1. untransduced CD8+ and CD4+; 2. A2-ESO-TCR-CD8+ and untransduced CD4+; 3. untransduced CD8+ and DP4-ESO- TCR-CD4+; 4. A2-ESO-TCR-CD8+ and DP4-ESO-TCR-CD4+) at 2×10^6 cells per
mouse by i.v. injection, followed with 3 doses of IL-2 by i.p. injection to boost T cells proliferation. The tumor growth in each group was monitored every 3 days and the T- cell migration was tracked by Luciferase transduced in CD8+ T cells.

Our result firstly showed that injected A2-ESO-TCR-engineered CD8+ T cells gradually but significantly migrated to the tumor sites after injection, indicating the specificity and safety of engineered T cells *in vivo*. At later time points (Day 12 to Day 19), all of the transferred T cells were localized at the tumor sites, but not other organs, in NSG mice (Figure 5A).

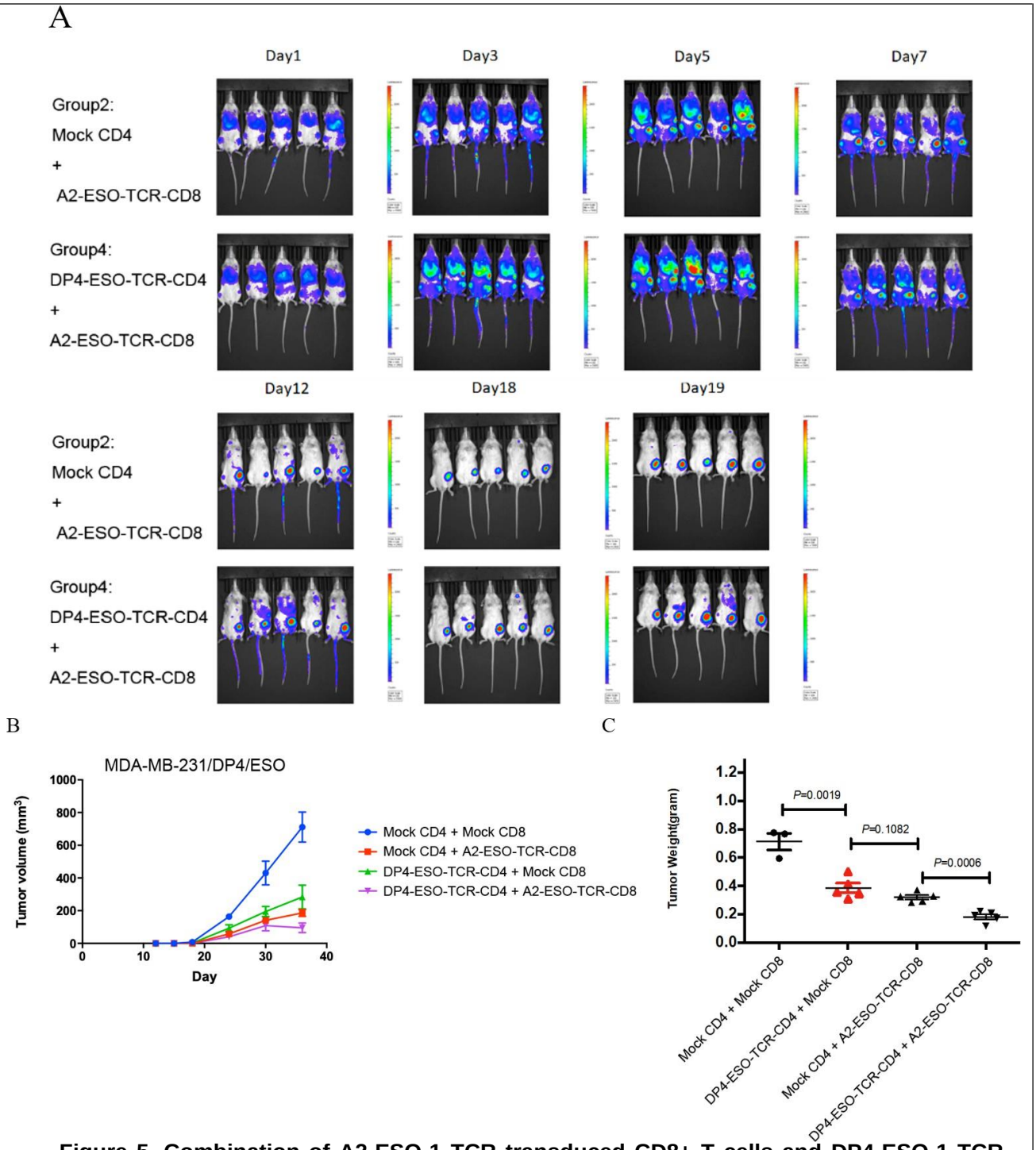


Figure 5. Combination of A2-ESO-1 TCR transduced CD8+ T cells and DP4-ESO-1 TCR transduced CD4+ T cells against MDA-MB-231/DP4/ESO *in vivo*. (A) The migration of injected A2-ESO-1 TCR transduced CD8+ T cells tracked *in vivo* by luciferase.
(B) The growth of MDA-MB-231/DP4/ESO *in vivo* treated by different groups of T cells. (C) The comparison of tumor sizes treated by different groups of T cells when mice were sacrificed.

When comparing the tumor growth in different groups, we observed that A2-ESO-TCR-

with ThPOK. We further validated the endogenous interaction of LSD1 and ThPOK through immunoprecipitation. By using ThPOK antibody, endogenous LSD1 was successively pulled down in primary human CD4+ T cells (Figure 6C).

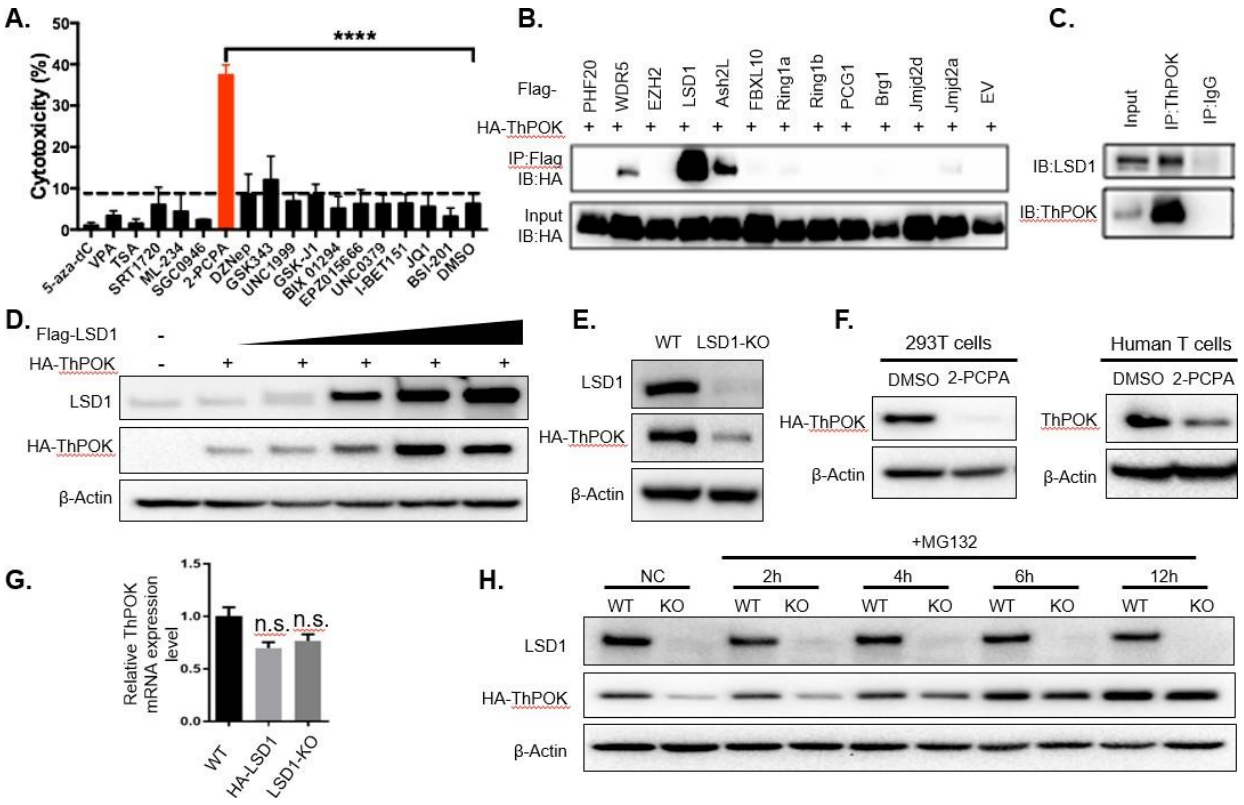


Figure 6. LSD1 stabilizes ThPOK protein through direct protein-protein interaction.
(A) Epigenetic inhibitor screening identified LSD1 inhibition increases cytotoxicity of antigen specific CD4+ T cells. (B) ThPOK interacting epigenetic gene screening identified that LSD1 could directly interact with ThPOK. (C) Endogenous immunoprecipitation in human primary CD4+ T cells validated ThPOK could interact with LSD1 in T cells. (D) ThPOK protein level was up regulated by LSD1 in a dose dependent manner. (E) LSD1 knockout in 293T cells inhibited the expression of ectopically expressed ThPOK. (F) LSD1 inhibitor 2- PCPA could inhibit ThPOK protein in both 293T cells and primary human T cells. (G) Quantitative PCR detected the RNA level of ThPOK after LSD1 overexpression or knockout in 293T cells. (H) Knockout of LSD1 reduced the protein level of ThPOK and can be rescued by proteasome inhibitor MG132.

During the experiment, we found ThPOK protein level had positive correlation with LSD1 protein level. Therefore, we co-transfected gradient amount of LSD1 expression plasmid with the same amount of HA-ThPOK expression plasmid in 293T cells. The results showed that ThPOK protein level was up-regulated by LSD1 (Figure 6D). Correspondingly, ThPOK protein level was significantly down-regulated in LSD1 knockout 293T cells (Figure 6E). Constantly, we used LSD1-specific inhibitor 2-PCPA to treat ThPOK expression 293T cells and human primary T cells, the protein level of ThPOK was repressed in both cell types (Figure 6F). There was no significant change of ThPOK RNA level in LSD1 overexpressing or knockout 293T cells compared with

wild type 293T cells (Figure 6G). We further found the LSD1-deletion-induced ThPOK protein down-regulation could be rescued by proteasome inhibitor MG-132 (Figure 6H). In summary, those results show that direct interaction of LSD1 may stabilize the ThPOK protein and further inhibit the cytotoxic function of tumor specific CD4+ T cells.

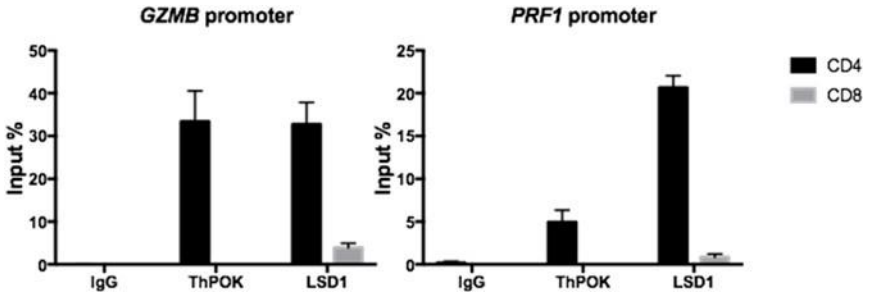


Figure 7. The relative occupancy of ThPOK and LSD1 in GZMB and PRF1 gene promoters. Q-PCR analysis of recovered DNA from the anti-ThPOK and LSD1 Chromatin immunoprecipitation assay.

Since LSD1 has also been reported to regulate target gene expression by modifying their promoter histone methylation status. To test other possible mechanisms of how LSD1 and ThPOK regulates CD4+ T cell cytotoxicity, we performed Chromatin Immunoprecipitation (ChIP) coupled with quantitative PCR to detect the occupancy of ThPOK and LSD1 in key cytotoxic gene promoters. CHIP-qPCR was performed with Pierce Magnetic ChIP Kit (Thermo Fisher Scientific) according to the manufacturer's instructions in CD4+ T cells and CD8+ T cells. We identified the co-occupancy of ThPOK and LSD1 at the promoter regions of GZMB and PRF1, both of which are key cytotoxic genes, in CD4+ but not CD8+ T cells (Figure 7). These data illustrate that ThPOK and LSD1 collaborate and regulate the cytotoxicity of CD4+ T cells through GZMB and PRF1 signaling.

6. Constant sequence modification enhances A2-ESO-1 TCR T cell antitumor efficiency

A2-ESO-1 TCR transduced T cell is an attractive strategy to endow patient autologous T cells tumor cell recognize and target breast tumor. By introducing an A2-ESO-1 specific TCR, large number of T cells with defined specificity can be produced and used for adoptive transfer therapy. However, since autologous T cells contain endogenous expressed TRA and TRB genes, which coding for TCR alpha (α) chain and (β) chain respectively, exogenously and endogenously expressed TCR-α and TCR-β could form mixed TCR heterodimers. Heterodimeric TCRs are non-functional and could harbor potentially harmful specificities. To avoid mismatch pairing, we modified the constant sequence of A2-ESO-1 targeting TCR by replacing with mouse constant sequence, which will not affect its specificity and only can be paired with transduced exogenous TCR. In this part of research, we test the breast cancer targeting function and specificity of modified TCR T (mTCR-T) cells both *in vitro* and *in vivo* model.

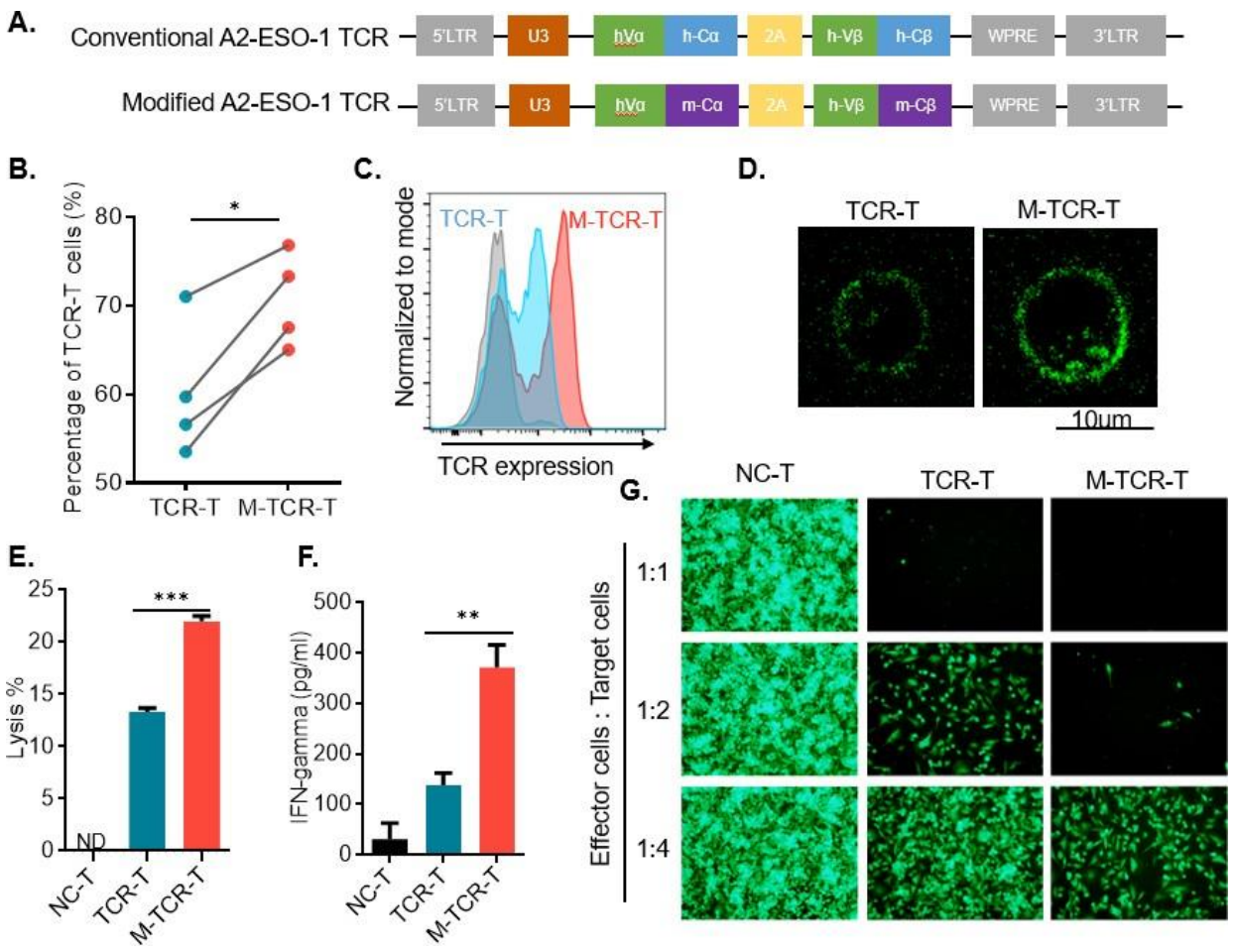


Figure 8. Mouse constant sequence enhances A2-ESO-1 specific TCR cell surface expression and function in human T cells. (A) Schematic diagram of conventional (TCR) and modified A2-ESO-1 TCR (M-TCR) construct. (B) Modified TCR has higher transduction efficiency than conventional TCR construct (Paired t-test was used for statistics analysis, *: $p<0.05$). (C, D) TCR cell surface expression was detected by FACS and confocal. (E) LDH assay was performed to detect the target cell killing ability of conventional and modified TCR-T cells (T-test was used for statistics analysis, ***: $p<0.001$). (F) Cytokine secretion was detected by ELISA after co-cultured with NY-ESO positive breast cancer cells (T-test was used for statistics analysis, **: $p<0.01$). (G) Long term tumor cell killing ability detected by *in vitro* co-culture at different effector cell and target cell ratios. Image was captured at day 4 after co-culture (target cell: MDA-MB-231 with NY-ESO and GFP expression).

Firstly, we constructed A2-ESO-1 mTCR expression lentivirus plasmid and transduced human T cells using an optimized method (Figure 8A). TCR gene transduction efficiency was detected two days after the last transduction (Figure 8B). Compared with conventional A2-ESO-1 TCR, mTCR has a higher transduction efficiency (70.6% vs 60.2%, n=4, $p<0.05$). Moreover, the mTCR-T cells have a significantly enhanced mean fluorescence intensity (MFI) by flow cytometry analysis (Figure 8C). The higher surface expression of mTCR further confirmed by confocal microscopy (Figure 8D). This part of results indicated that modified constant sequence could enhance A2-ESO-1 specific

TCR-T cell transduction and surface expression.

Next, we performed an *in vitro* TCR-T functional assay. To test the mTCR-T cells' cytotoxicity, we performed cytotoxicity analysis using non-radioactive cytotoxicity assay (Pierce LDH Cytotoxicity Assay Kit, Promega) following the manufacturer's instruction. NY-ESO-1 and HLA-A2 positive breast cancer cell line MDA-MB-231-ESO cells were seeded at 5000/well in a 96-U bottom plate. Conventional A2-ESO-1 specific TCR-T or mTCR-T cells were co-cultured at an effector to target ratio of 10:1 for 5 hours. The results showed that mTCR-T cell cytotoxicity was much higher than conventional TCR-T cells ($p<0.001$) (Figure 8E). We next analyzed the IFN-γ level in overnight-cultured cell supernatant by ELISA assay, and found that mTCR-T cells secreted more cytokines ($p<0.01$) (Figure 8F). In the long time target cell killing assay, we co-cultured TCR-T cells or mTCR-T cells with different ratios of effector and target cells (MDA-MB-231- ESO) E:T = 1:1, 1:2, 1:4, and 1:8. Fresh T cell medium (without IL-2) was changed every day and GFP+ target cells were counted at day 4 after co-culture. The results showed that mTCR-T cells were more efficiently to kill tumor cells (Figure 8G). Furthermore, we showed that mTCR-T could be activated by a much lower concentration of the NY-ESO-1 peptide than conventional TCR. These results indicate that the modification of A2-ESO-1 TCR-T cells did not affect their specificity, but enhanced the ability to recognize the target cells and the subsequent killing activity.

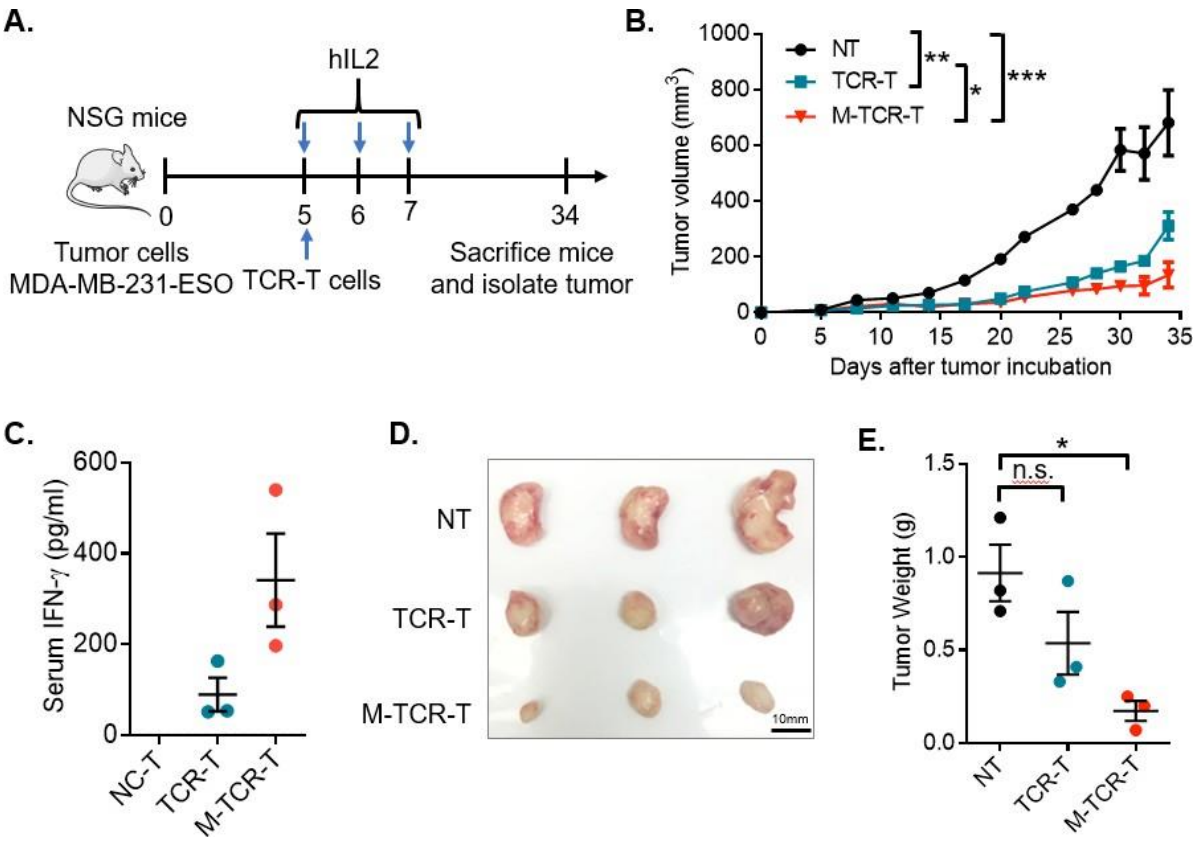


Figure 9. Modified A2-ESO specific TCR-T cells have better therapeutic efficiency in pre-clinical breast cancer model. (A) Schematic diagram of animal experimental design. $1x10^6$ MDA-MB-231-ESO cells were subcutaneously injected to the fat pad of NSG mice.

TCR-T cells were intravenously injected to tumor bearing mice, followed by 3 doses of IL-2. (B) Tumor growth among different groups (Mean ± SEM, n=3, two-way ANOVA test was used for statistics analysis. * $p<0.05$, ** $p<0.01$, *** $p<0.001$). (C) Serum IFN-gamma was determined by ELISA at day 8 after TCR-T cell injection (Mean ± SEM, n=3). (D) Tumor sizes and weights were measured after mouse euthanasia (Mean ± SEM, n=3, T test was used for statistics analysis. * $p<0.05$).

Furthermore, to investigate the mTCR-T cell therapy efficiency in breast tumor model, we employed the breast tumor xenograft model in NSG mice (Figure 9A). 1×10^6 MDA- MB-231-ESO cells were subcutaneously injected to the fat pad of NSG mice. $3x10^6$ TCR-T or mTCR-T cells were adoptively transferred into tumor-bearing mice on Day 5 through i.v. injection. And human recombinant IL-2 was administrated for the first 3 days after T cell transfer through i.p. injection. The tumor growth was monitored every 2-3 days with an electronic caliper, and serum IFN-gamma secretion was determined by ELISA at day 8 after T cell transfer. Our data showed that mTCR-T treated mice had a higher IFN-gamma level in serum, and their tumor growth was much slower than conventional TCR-T treated mice (n=3, $p<0.05$, Wilcoxon rank sum test) (Figure 9B, C). Mice were sacrificed one month after the T cell transfer, with tumors isolated and weighed. Consistently, tumors from mTCR-T treated mice were significantly smaller than those from the conventional TCR-T treated mice ($p<0.05$) (Figure 9D, E). These data indicate that mTCR-T cells have enhanced *in vivo* antitumor efficiency.

In summary, to further enhance NY-ESO specific TCR-T cell function and avoid mismatch pairing, we modified conventional TCR construct by replacing the human constant sequence with murine sequence. Modified TCR-T cells show significantly higher cell surface expression, enhanced target cell recognition and killing ability both *in vitro* and *in vivo* models.

Specific Aim 2 (Months 13-36): To enhance NY-ESO-1-specific TCR-mediated therapeutic immunity by blocking PD-1 signaling and to assess the potential toxicity in preclinical studies

Major Task 2 (Months 13-36): 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

Subtask 6 (Months 13-24): *To enhance NY-ESO-1 TCR-mediated immunity by knockdown of PD-1 signaling molecules*

7. Combination of NY-ESO-1 TCR T cell therapy and PD-1 inhibitory therapy could achieve better outcome than either alone.

Programmed cell death protein 1 (PD-1) is a highly expressed immune checkpoint molecule in activated T cells. PD-1 ligands induced PD-1 single pathway activation promotes tumor specific T cell apoptosis in lymph nodes and reduces apoptosis in regulatory T cells. PD-1 ligands, such as PD-L1, are highly expressed in human breast

tumor cells, which may limit the anti-tumor function of adoptive transferred A2-ESO-1 specific TCR T cells. PD-1 antibodies as immune checkpoint blockade therapies have been approved by FDA to treat several types of solid cancer, with impressive and durable clinical response. Lack of tumor-infiltrating T cells is one of the major issues in non-responding patients to PD-1 blockade therapy. Therefore, the combination of A2- ESO-1 TCR T cell adoptive transfer and anti-PD-1 antibody blockade therapy may greatly improve the anti-tumor efficacy and extend the breast cancer patient survival.

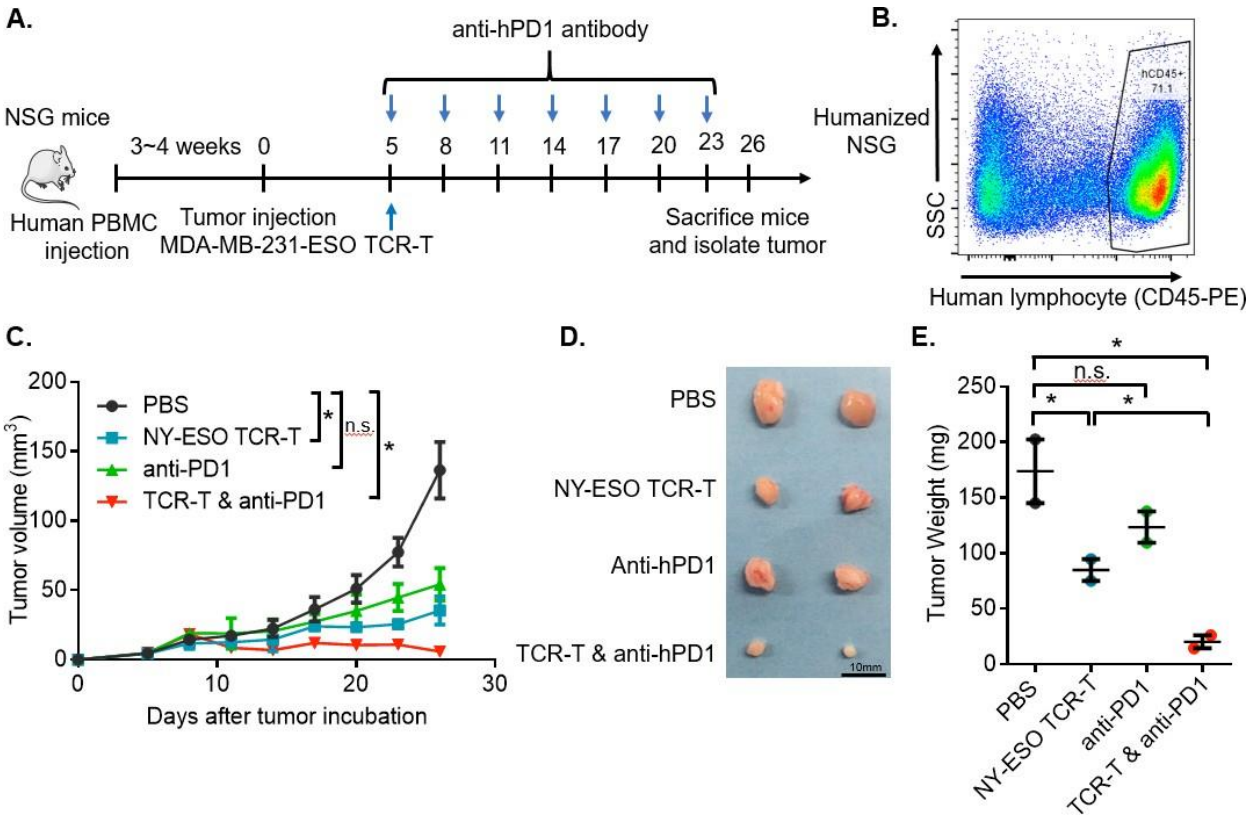


Figure 10. Combined therapy of A2-ESO-1 TCR T cells and anti-human PD-1 antibody in humanized NSG mice. (A) Schematic diagram of animal experimental design. (B) Human immune cells were analyzed by flow cytometry in NSG mice after 3-weeks human PBMC reconstitution. (C) Tumor growth was monitored after treatment (Mean ± SEM, n=2, two-way ANOVA test was used for statistics analysis. *: $p<0.05$). (D, E) Tumor size images and weights were determined after mouse sacrifice (Mean ± SE, n=2, T test was used for statistics analysis. * $p<0.05$).

We tested the combination of TCR T cell and PD-1 antibody therapies in two preclinical mouse models. Firstly, we partially reconstituted human immune system in immunodeficient NSG mice by intravenous injection of human PBMCs (obtained from HLA-A2.1+ healthy donor) (Figure 10A). Three-week old NSG mice were intravenously injected with 10 million human PBMC (HLA-A2.1+). Human immune cells were detected by flow cytometry in the spleen after 3 weeks of humanization (Figure 10B). After 4 weeks, these reconstituted mice were used for experiments. MDA-MB-231-ESO cells were subcutaneously injected into mammary fat pad of humanized NSG mice (1 million cells/mouse). Tumor-bearing mice were treated by A2-ESO-1 TCR T cells at day 5 after

tumor injection. Three doses of human IL-2 (50000 IU) and 7 doses of anti-human PD-1 antibody (150 μg) were administrated through intraperitoneal injection. A2-ESO-1 TCR-T cells were generated using the T cells from the same healthy human donor. Tumor growth was monitored every 2 days. We found that the combined treatment resulted in a marked tumor regression, compared with either T cell alone or PD-1 antibody alone (Figure 10C). Tumor tissues were then dissected from the mice after euthanasia, and measured for their weights. Consistently, the tumor volume in the group treated by A2-ESO-1 TCR-T cells plus anti-PD-1 was much smaller than those from A2-ESO-1 TCR-T cells or anti-PD-1 alone (Figure 10D, E).

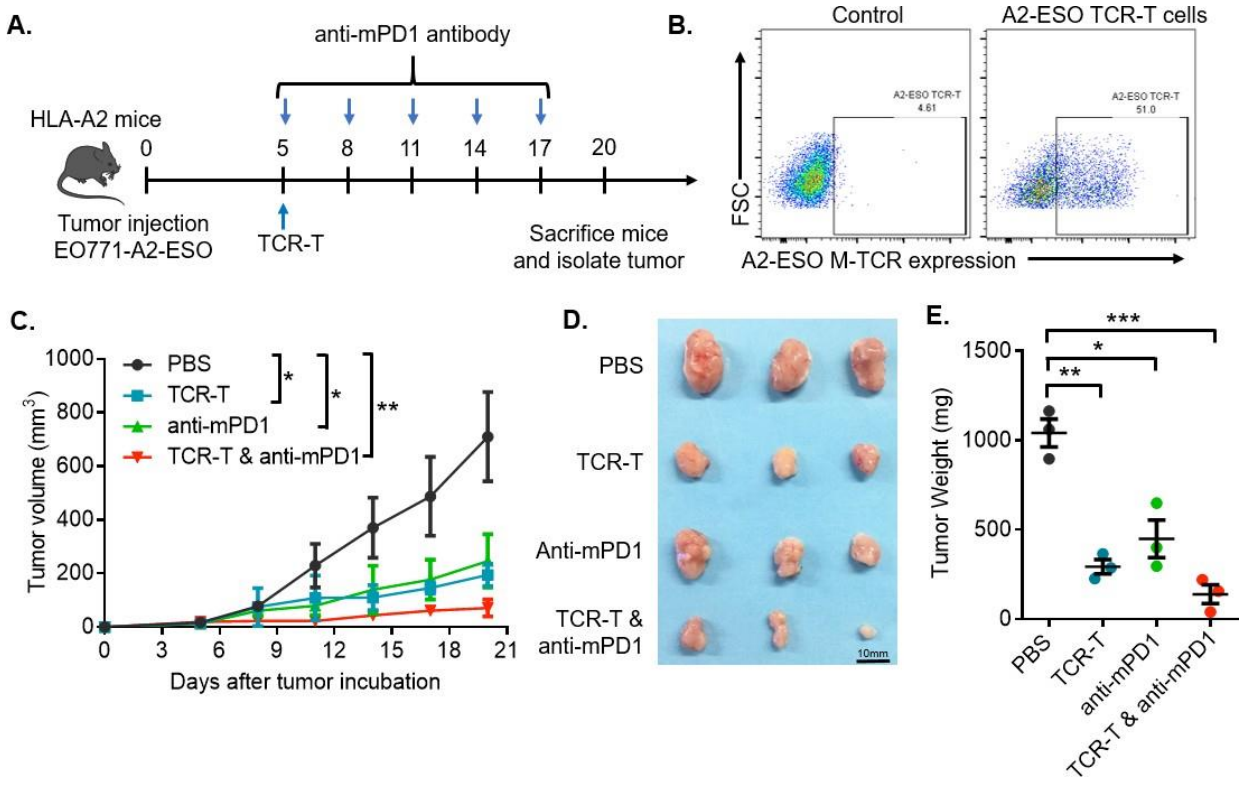


Figure 11. Combined therapy of mouse A2-ESO-1 TCR T cells and anti PD-1 antibody in HLA-A2.1 transgenic mouse model. (A) Schematic diagram of animal experimental design. (B) A2-ESO-1 TCR-T cells transduction efficiency was determined by flow cytometry. (C) Tumor growth was monitored after treatment (Mean ± SEM, n=3, two-way ANOVA test was used for statistics analysis. * $p<0.05$, ** $p<0.01$). (D, E) Tumor weight was measured after sacrifice (Mean ± SEM, n=3, T test was used for statistics analysis. * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

We also tested the combined therapy using HLA-A2 transgenic mice. HLA-A2 and NY-ESO-1 antigen exogenous overexpressing mouse breast cancer cells were subcutaneously injected to the fat pad of HLA-A2.1 transgenic mice at 1 million cells per mouse (Figure 11A). Splenocytes from HLA-A2.1 transgenic mice were isolated, and the mouse T cells were activated by anti-CD3/CD28 activator beads, and then transduced with A2-ESO-1 specific mouse TCR by lentiviral infection. And the expression of A2-ESO-1 TCR was validated by flow cytometry (Figure 11B). Tumor

bearing mice were treated with mouse A2-ESO-1 T cells at day 5. Anti-mouse PD-1 antibody (150 ug/injection) was administrated through intraperitoneal injection every three days. The tumor growth was timely monitored and the results showed that tumors under combined treatment grew much slower than non-treated group or single treated group (Figure 11C). Mice were sacrificed at day 20 for tumor analysis, and tumor samples were isolated and weighted after euthanasia. Consistently, the tumors with combined treatment were much smaller than other groups (Figure 11D, E).

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 (Months 1-12): 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. After relocation of our laboratory from Houston Methodist to University of Southern California and Children's Hospital Los Angeles, we obtained IACUC approval from USC IACUC (protocol number: 21097) to perform according animal experiments in this project. This animal protocol has been reviewed and approved by DoD ACURO. The IBC protocol has been approved by USC EH&S (Environmental Health & Safety) for the use of hazardous substances (protocol number: BUA-19-00022). The IRB protocol for using human PBMCs (protocol number: HS-19-00957) has also been approved by Institutional Board Review.
2. The GMP facility is secured for us to use at Children's Hospital Los Angeles/USC. Next, we have prepared the detailed GMP documents, including all SOPs for our A2-ESO1-TCR pipeline.
3. Pre-testing of T cell products for clinical immunotherapy of triple negative breast cancer.

Before we submit our IRB and IND application to DoD HRPO and FDA, we need to demonstrate that our GMP facility, TCR-T viral particles and SOPs (standard of procedures) are working properly for transducing human T cells (isolated from discarded bloods without personal identification information) and meeting DoD and FDA requirement. The pre-testing of clinical grade T cell products for cancer immunotherapy was prepared and performed at GMP facility at CHLA/USC. 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 studies.

3.1 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.

3.2 T cell activation

Every 3×10^6 fresh or thawed PBMCs were mixed with 25 ul 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.

3.3 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.

3.4 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. 1ml 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.

3.5 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 and then transduced with the equal amount of retroviral supernatant.

The transduction efficiency, which determined the effectiveness of the TCR-T product, 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 (**Figure 12A**). The viability of transduced T cells was also compared by staining with Propidium Iodide (PI) (**Figure 12B**). The potency of transduced T cells was assayed in vitro as well (Figure 1C). All three groups showed extremely high TCR transduction efficiency (>80%). Among them, the Bead-PBMC group showed the highest transduction efficiency in both CD8+ and CD4+ T cells and the lowest death rate (**Figure 12A, 12B**). In vitro assay also showed this group of transduced T cells had high activity against HLA-A2/NY-ESO-1 cell models with good specificity that did not recognize non-ESO antigens (**Figure 12C**). 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.

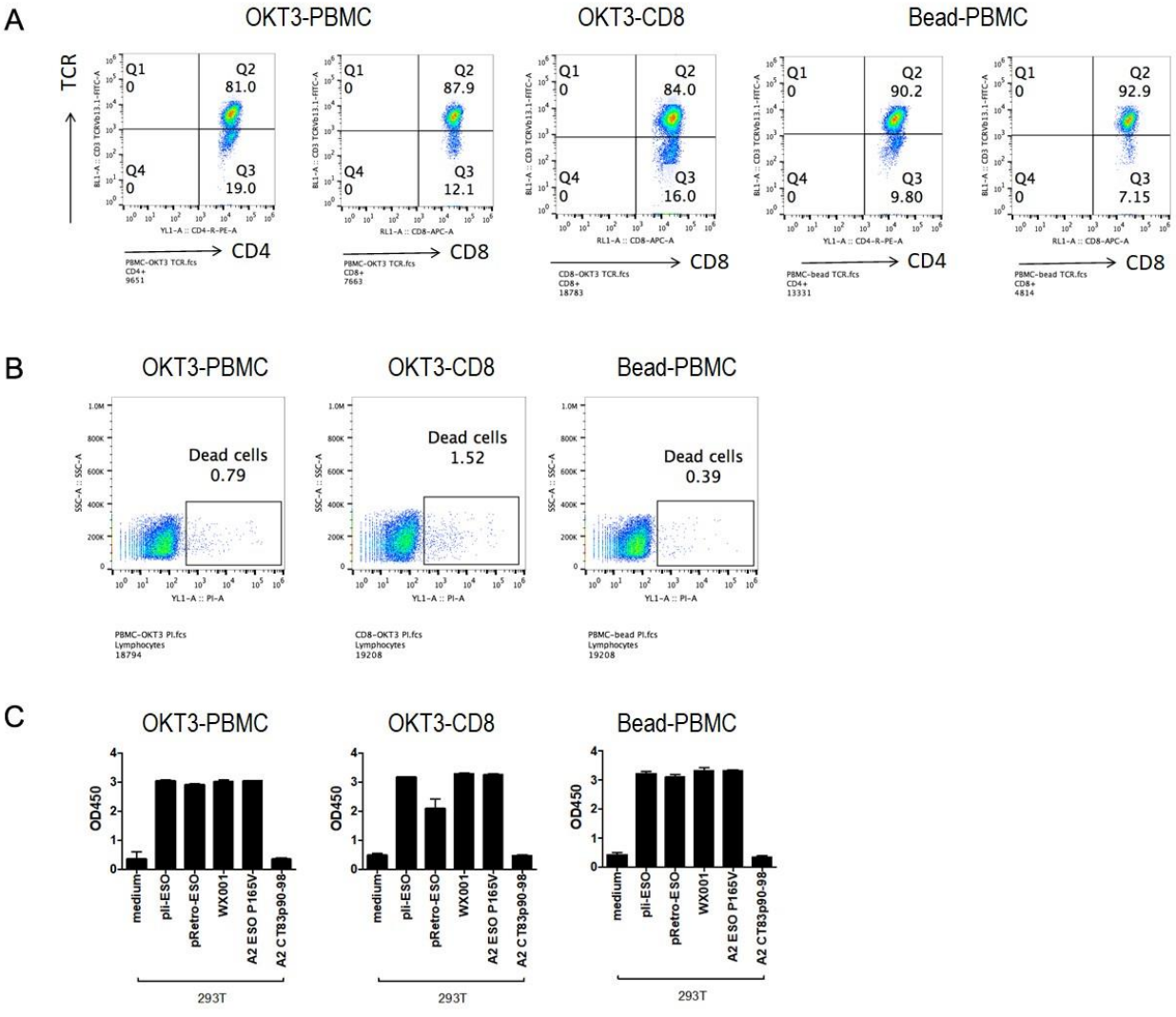


Figure 12. Analysis after transduction among three groups in the 1st batch of pre-test

The test was continued with the expansion of Bead-PBMC group. After 9 days, expanded T cells were tested again with TCR transduction efficiency and expansion folds. Our data showed that the expansion did not cause much loss of TCR transduction efficiency. The expanded T cell population remained high percentage of TCR- transduced T cells, which was higher than our expectation (50%) (**Figure 13A**). However, the number of expanded T cells did not meet our expectation (100 × 10^6 cells when starting from 2.5 × 10^6 cells) (**Figure 13B**), which indicates more efforts are needed for optimizing the production of clinical-grade NY-ESO-1 TCR-T cells.

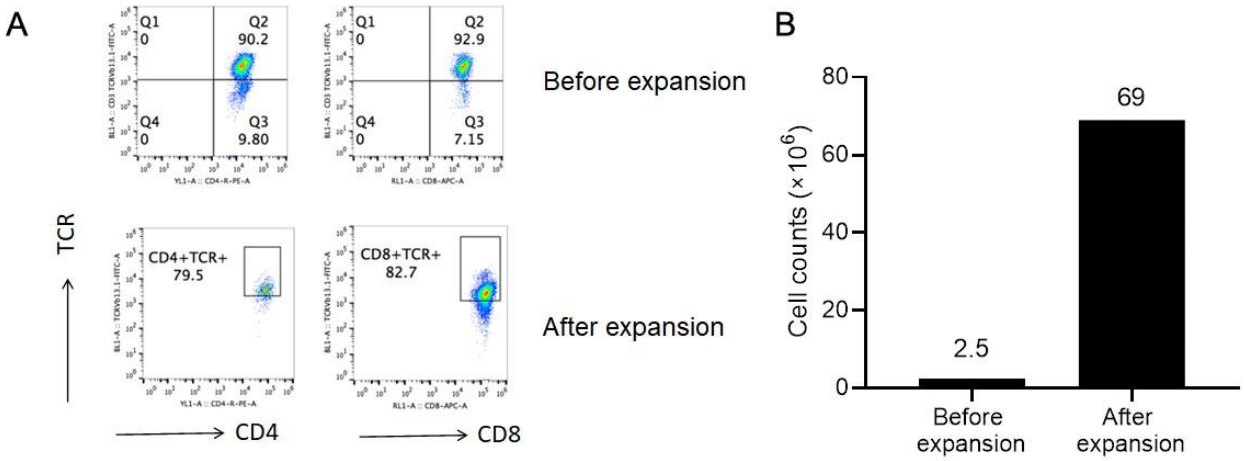


Figure 13. Analysis after T cell expansion in the 1st batch of pre-test

The second batch of pre-test focused on the optimization of T cell expansion. To obtain enough expanded functional T cells, we started with freshly-isolated PBMCs instead of frozen stock. The T cells from healthy donor were activated, transduced and expanded under the SOPs and conditions determined during the first batch of pre-test. The TCR-T product maintained high transduction efficiency before and after cell expansion, which was even increased after expansion (**Figure 14A**). The CD8+/CD4+ T cell ratio and the cell viability did not change much before and after the expansion, which confirmed the stability and reliability of the expansion procedure (**Figure 14B, 14C**). As we expected, the number of expanded cells we harvested significantly increased when we initiated with fresh PBMCs (**Figure 14D**). We also tested the anti-tumor activity with the expanded TCR-T cells which mimic the clinical-grade products in vitro. The result showed the expanded TCR-T cells specifically killed breast cancer cells expressing NY-ESO-1 with high activity (**Figure 14E**).

The third batch of pre-test is ongoing with PBMCs from three different donors simultaneously. The purpose of this batch is to optimize the T cell expansion with cryopreserved PBMCs and aiming to harvest 100 × 10^6 cells TCR-transduced T cells just like fresh PBMCs whenever starting from fresh or cryopreserved PBMCs, considering patients' PBMCs from the clinic are probably in the frozen status in the future.

The final products of each batch of pre-test have been or will be sent for sterility test

at Department of Pathology & Laboratory Medicine, CHLA. Such test represents the quality control of the final products, which are qualified to be released to the clinic or not. Once the third batch of pre-test is completed and all test results are collected, we will organize the data and prepare to submit an IND application to FDA for the approval.

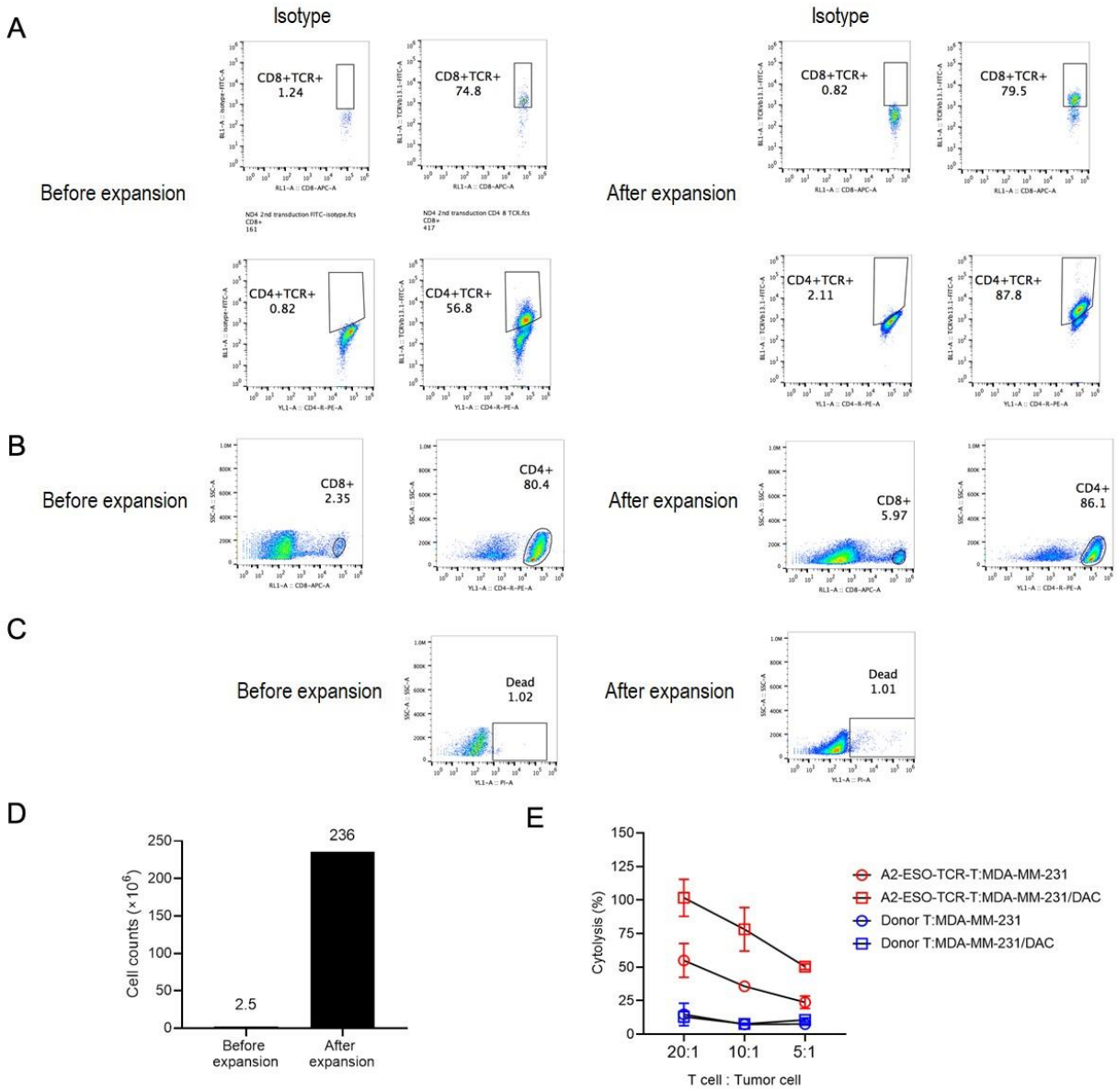


Figure 14. Analysis after T cell expansion in the 2nd batch of pre-test

Key findings and results:

1) A GMP facility for T cell production has been fully set up for the proposed work.
2) Successfully validated procedures of PBMC isolation, T cell isolation and activation, T cell transduction and T cell expansion in GMP facility.

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 15A**). 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 15B**). The Propidium Iodide staining also showed the high viability of transduced T cells (>90%) (**Figure 15C**). 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 16**). 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 17**).

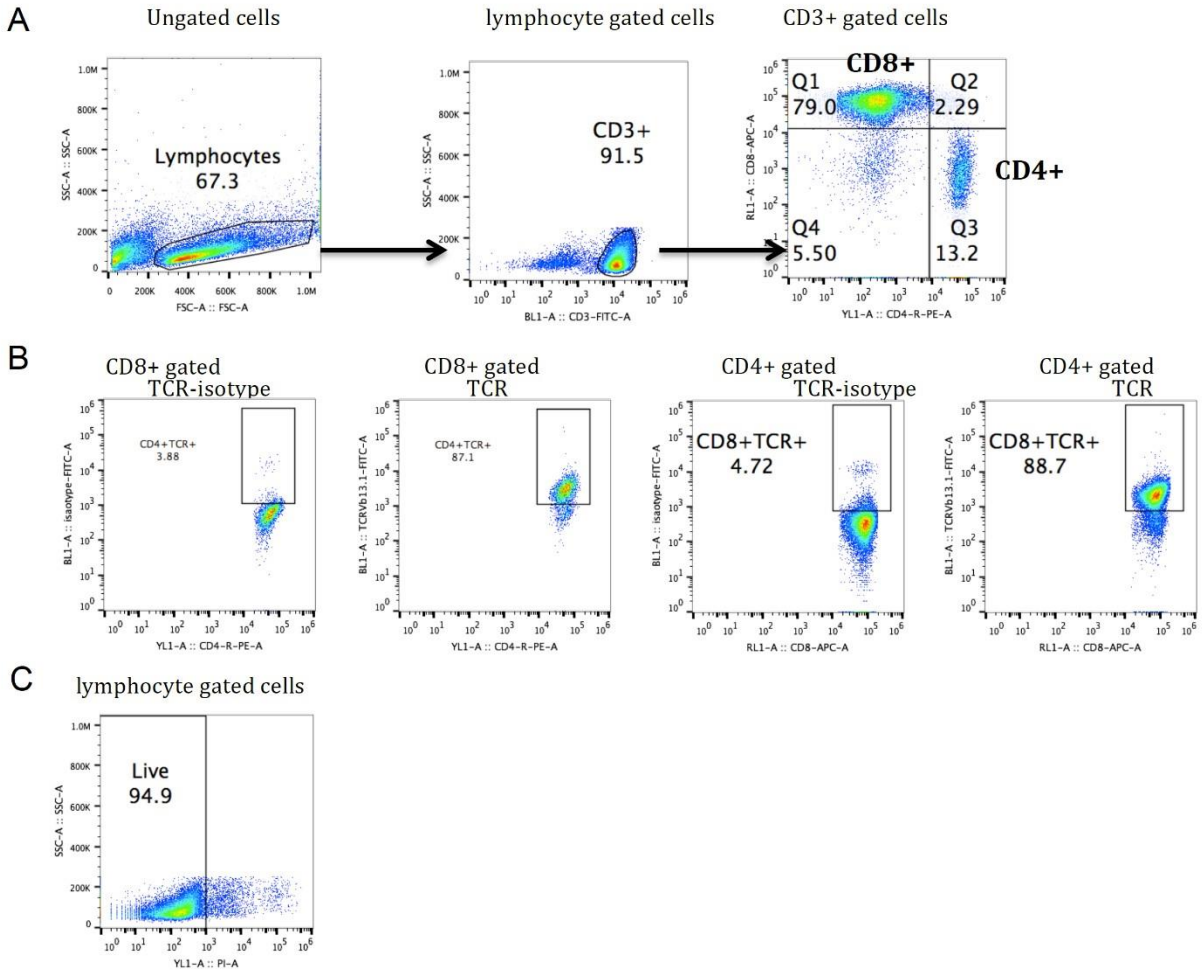


Figure 15. **Transduction efficiency and viability of A2-ESO-1 TCR-T cells**. After 10-day in vitro expansion, A2-ESO-1 TCR-transduced T cells of ND5 were stained with (A) anti-CD3- FITC, anti-CD4-PE, anti-CD8-APC antibodies, (B) A2-ESO-1 TCR-specific antibody (anti- Vβ13.1-FITC) and (C) Propidium Iodide for flow cytometry analysis.

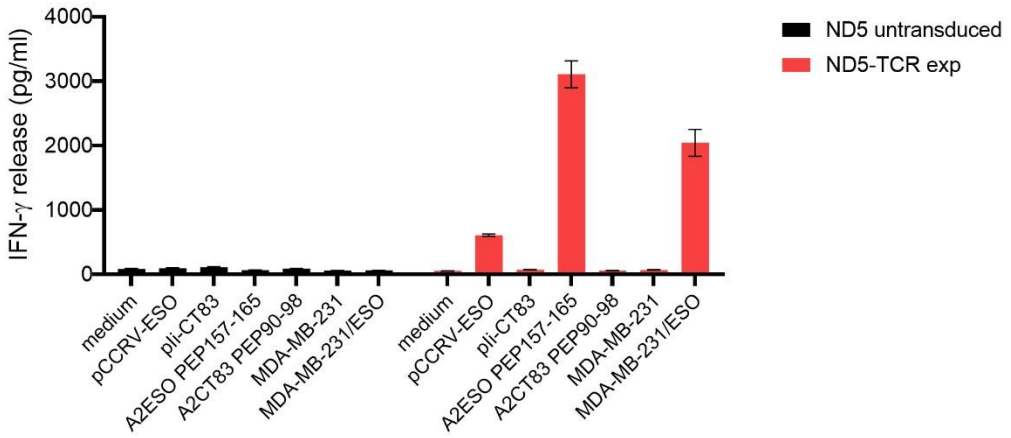


Figure 16. **T cell response and specificity assay of A2-ESO-1 TCR-T cells.** A2-ESO-1 TCR- T cells of ND5 after 10-day in vitro expansion incubated with ESO-transfected HEK293T (HLA- A2+) (red column 2), A2-ESO peptide-pulsed HEK293T (HLA A2+) (red column 4), and A2+ ESO+ triple negative breast cancer 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).

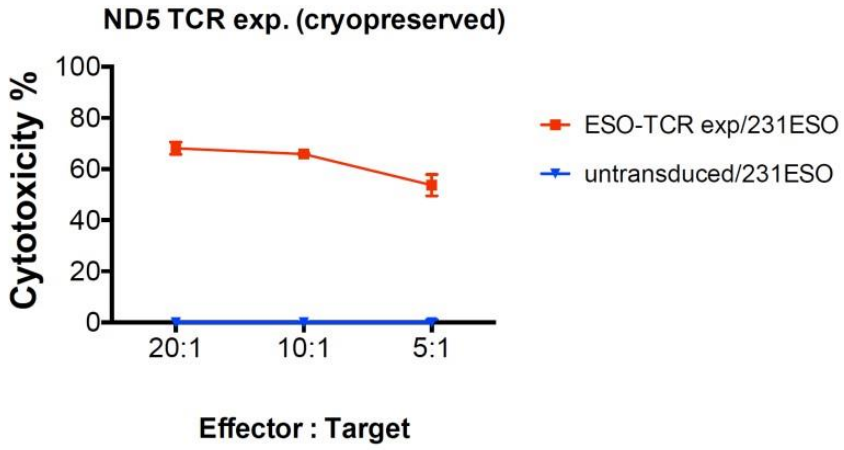


Figure 17. **Cytolytic activity of A2-ESO-1 TCR-T cells.** A2-ESO-TCR T cells of ND5 after 10- day in vitro expansion incubated with A2+ ESO+ triple negative breast cancer cell line MDA- MB-231 overnight to test the cytolytic ability of TCR-T cells by LDH assay. Autologous untransduced T cells were used 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 2**). 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 18**). 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 2. 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

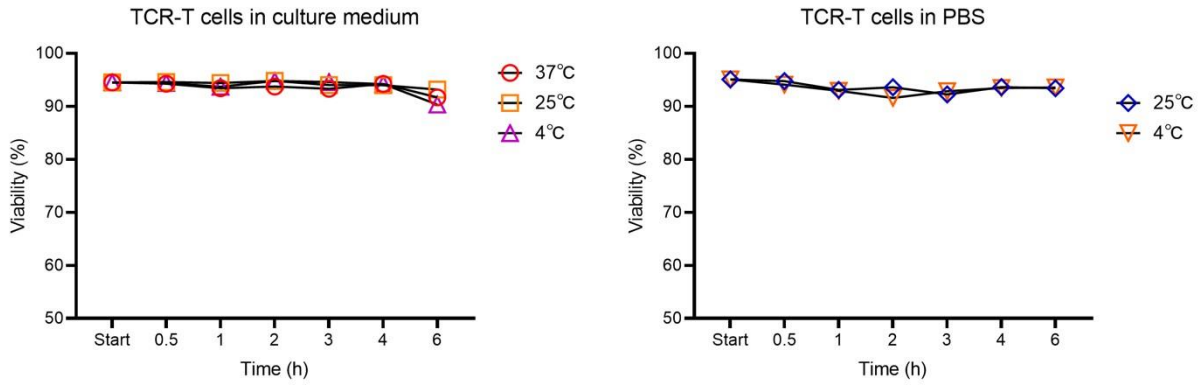


Figure 18. Results of stability tests of GMP-grade A2-ESO-1 TCR-T cells. Left: Stability of TCR-T cells in culture medium within 6 hrs. Right: Stability of TCR-T cells in PBS within 6 hrs.

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 19**). 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.

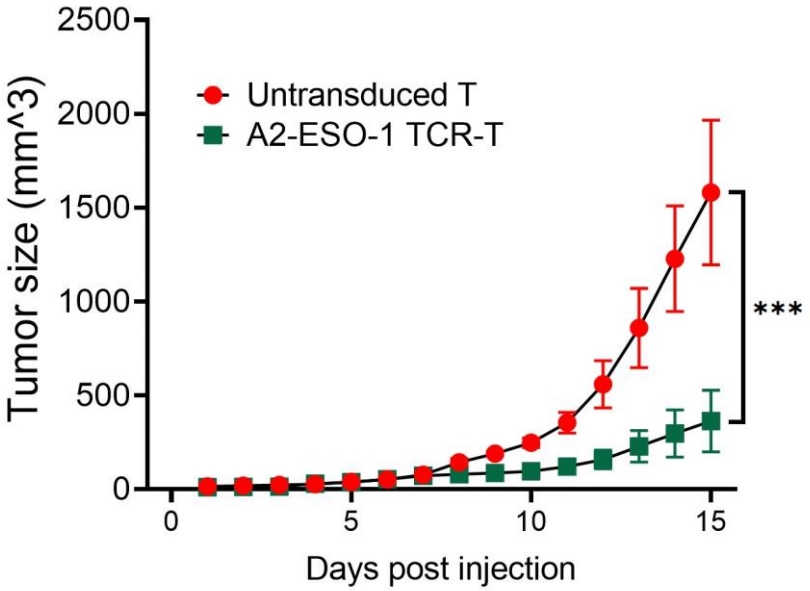


Figure 19. **In vivo antitumor activity of GMP-grade A2-ESO-1 TCR-T cells.** Tumor growth (HLA-A2+, NY-ESO-1+) in NSG mice with treatment by human T cells transduced by GMP- grade retroviral particles compared to autologous untransduced T cells.

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 3**).

Table 3. 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 20**). 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.

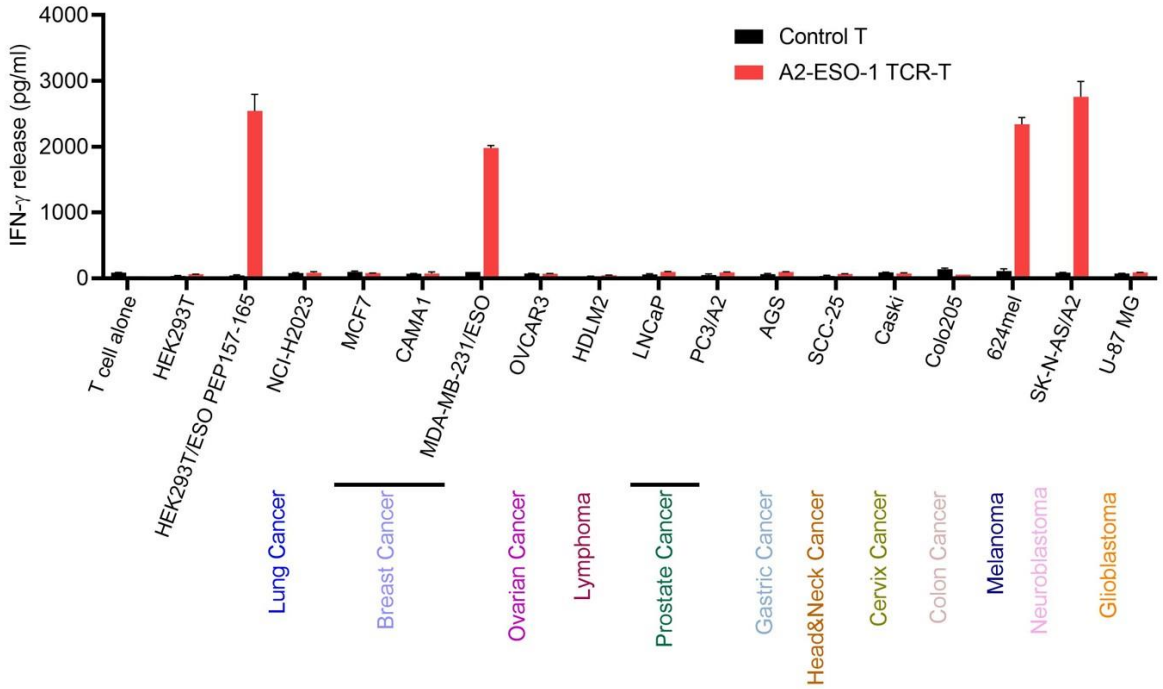


Figure 20. In vitro assay of A2-ESO-1 TCR-T cells against HLA-A2 positive tumor lines. A2-ESO-1 TCR-T cells specifically recognized tumor lines expressing HLA-A2 and NY-ESO- 1, but not HLA-A2-positive and NY-ESO-1$^-$ tumor cells.

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

Annual certification of the stability of GMP-grade retrovirus

In the current funding period, we finished several key modules for IND application. The Module 3 is the most important section of the application, which provides the Chemistry, Manufacturing, and Control (CMC) information of the substance, manufacturing and products of our A2-ESO-1 TCR-T cell product. The Module 2 is the summaries of the Modules 4 and 5. Module 4 is the nonclinical study information, while Module 5 describes the study information of clinical trials with the A2-ESO-1 TCR against human cancers.

The GMP-grade retroviral vector particles were produced at Indiana University Vector Production Facility and stored at -80℃ until use. The stability of this substance (viral particles) for producing A2-ESO-1 TCR-T cells was and tested and evaluated during the annual certification. Two frozen bags were randomly taken out from Harvest 1 and Harvest 7 in November 2021. T cell transduction was conducted with thawed viral particles in quadruplicates, in a BSL2 hood according to the previous protocol, following by the measurement of the transduction efficiency of transduced T cells via TCR-specific antibody staining (anti-Vbeta 13.1) and FACS analysis. As seen in the following Table 1, the transduction efficiency of the two retroviral particle samples in T cells were measured in quadruplicates remained as high as it was initially produced (>70%), indicating the A2-ESO-1 TCR retroviral particles have been stable at −80℃. The transduction efficiency of the stored retrovirus is shown in Table 4.

Table 4. Test result of retroviral transduction efficiency of TCR viral particles

	Harvest 1	Harvest 7
Transduction Efficiency (%)	79.0	78.3
	78.7	79.2
	79.5	80.1
	78.6	78.5

Qualification runs at KJCCT for IND application

To prepare the IND application of the immunotherapy on triple-negative breast cancer (TNBC) with A2-ESO-1 TCR-engineered T cells, we conducted the validation runs at Ann Kimball W. Johnson Center for Cellular Therapy (KJCCT) facility. The KJCCT is a cGMP facility located at Houston Methodist's Outpatient Center and under the Office of Translational Production and Quality (OTPQ) at the Houston Methodist Academic Institute, where the A2-ESO-1 TCR-T products are planned to be manufactured for a clinical trial in TNBC. To develop the manufacturing processes00 for our products that qualifies the clinical requirements, we first performed pre-tests in the GMP facility at CHLA in the previous year, which was important training and practice under a GMP facility. During the validation runs at KJCCT, we conducted TCR-T cell manufacturing and tests under a GMP condition, developed a procedure from frozen T cell product to patient infusion, and established release criteria with batch records.

To initiate the validation runs at KJCCT, our staffs were well trained during three rounds of training (Tier 1, Tier 2 and Tier 3 at KJCCT) and passed the test for access qualification. Batch records for recording the manufacturing process in detail were well developed. All materials and reagents required for the manufacturing were applied

before their expiration dates and certified by manufacturer's Certificate of Analysis (CoA) or Certificate of Conformance (CoC) to show their compliance with GMP-grade production and shipped to KJCCT. Particularly, the GMP-grade retroviral particles of A2-ESO-1, produced from Indiana University, were shipped in dry ice and monitored by Cryoguard™ M-40 indicator (Cryoguard™ Corporation, Michigan) to maintain their high activity. The information of all supplies and reagents used for our validation runs is listed in **Table 4** and **Table 5**.

Table 5. Test result of retroviral transduction efficiency of TCR viral particles

	Harvest 1	Harvest 7
Transduction Efficiency (%)	79.0	78.3
	78.7	79.2
	79.5	80.1
	78.6	78.5

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Table 6. List of supplies for validation runs at KJCCT

Item Description (supplies)	Manufacturer / Vendor	Catalog/Product Number	Lot number
24-well cell culture plate	Greiner Bio-One	662160	E19123B8
24-well suspension culture plate	Greiner Bio-One	662102	E20083JT
1.5 ml microcentrifuge tube	Axygen MCT	MCT-150-C-S	04120078
Instant Sealing Sterilization Pouches	Fisher/Fisher	01-812-54	2020-06-28
Needle Free Spike	OriGen Biomedical	CBS	SE21804
DynaMa-15 Magnet	Invitrogen/Fisher	12-301-D	3320
Sterile Sampling Bags	Ward's/VWR	470236-308	03262020
50 ml conical centrifuge tube	Thermo Scientific/Fisher	339653	JAAF9Z8119
15 ml conical centrifuge tube	Thermo Scientific/Fisher	339651	K7AF6Z7117
blunt fill needle, 18g, 1.5in	BD/VWR	305180	1095828
Alcohol Prep Pads (# 154818-H5)	Honeywell Safety/VWR	89186-138	476902
1 ml serological pipet	Fisher	13-678-11B	02420044
5 ml serological pipet	Fisher	13-678-11D	06220007
10 ml serological pipet	Fisher	13-678-11E	07020024
25 ml serological pipet	Fisher	13-678-11	06820008
30ml syringe	BD/Fisher	302832	9053930
serological transfer pipettes, sterile, 5ml, Cat. #105	Ashton Pumpmatic Inc/VWR	25470-008	12101
96-Well Polystyrene Plates, White Opaque, sterile	Thermo Scientific/Fisher	PI15042	E191234M
20-200 ul tip, filtered	Fisher	02-707-430	20410202
100-1000 ul tip, filtered	Fisher	02-707-404	20120720
cryovial	Thermo Scientific/Fisher	5000-0020	1277450
0.1-10 ul tip, filtered	Fisher/Fisher	02-707-442	20080029
CryoMACS Freezing Bag 50 (10-20ml)	MACS/MACS	200-074-400	7210100786
G-Rex®10M Open System, Sterile Fluid Path	WilsonWolf/Wilson Wolf	P/N 80110	211220-2
250 ml bottle top filter, 0.22um (CA membrane: cGMP, low protein binding) #430767	Corning/VWR	28199-774	36121006

Shandon™ Straight Sharp/Sharp Dissecting Scissors	Epredia/Fisher	28301	NA

Table 7. List of reagents for validation runs at KJCCT

Item Description (Reagent)	Reagent Grade	Manufacturer / Vendor	Catalog/Product Number	Lot number
TexMACS™ GMP Medium (Phenol Red)	GMP	MACS/MACS	170-076-309	7210100119
CryoStor® CS10	USP	Biolife Solutions/Stemcell	210102	21218
1.077 g/ml Ficoll-Paque PREMIUM	GMP	GE Health/Sigma	17-5442-02	10296886
cGMP Human Serum Albumin 25%	GMP	Baxalta US Inc/CHLA Pharmacy	2G0201	LB060772 and LB060319
HEPES 1M	GMP	Millipore/Sigma	SRE0065-100ML	SLCJ7909
1X CTS DPBS	GMP	Gibco/Thermo	A12856-01	2257207
CTS GlutaMAX-I Supplement 100X	GMP	Gibco/Thermo	A12860-01	2257211
DynaMa-15 Magnet		Invitrogen/Fisher	12-301-D	3320
MACS® GMP CD3 pure, 0.2 mg/ml	GMP	MACS/MACS	170-076-124	6220100293
MACS® GMP CD28 pure, 0.5 mg/ml	GMP	MACS/MACS	170-076-117	6211000059
PROLEUKIN® (aldesleukin) rhIL2	GMP	Clinigen Inc./CHLA Pharmacy	NDC 76310-022-01	W054060
CTS Dynabeads CD3/CD28	GMP	Gibco/Thermo	40203D	A2-012003G
RetroNectin GMP grade CH296	GMP	Takara/Takara	T202	J2105
human AB serum, heat inactivated	USP	Valley Biomedical /Fisher	HP1022HI	21K2005
Retroviral particles MSGV- A2ESOTCR	GMP	Indiana University	18-1-VP-16	081018P (harvest 2 bag 24/25/27/28)

Two leukopaks from healthy donors (ND8 and ND9) were obtained from Gulf Coast blood center in Houston and their sterility was tested by KJCCT. Peripheral blood mononuclear cells (PBMCs) isolated from those leukopaks served as the source of T cells for transduction by retroviral particles of A2-ESO-1 TCR during two independent runs, mimicking the clinical situation with autologous peripheral blood samples from triple-negative breast cancer patients. A complete run for A2-ESO-1 TCR-T production in the cGMP facility included the following SOP (standard operating procedure) and was recorded on the batch records in detail.

Day 1: Isolation of human T cells from blood samples.

PBMCs were extracted from the leukopaks (ND8 or ND9) transported to the KJCCT facility. Briefly, 20–25 ml of anti-coagulated blood samples from patients or healthy donors and an equal volume of 1 × CTS DPBS were added to a 50 ml conical centrifuge tube and then slowly mixed with 20 ml of Ficoll-Hypaque PREMIUM to a new 50 ml conical centrifuge tube. After centrifugation at 800 × g for 30 min at room temperature, the mononuclear cell layer was transferred to a new 50 ml conical centrifuge tube. The cell pellets were resuspended in complete T cell culture medium containing RPMI 1640 medium with 10% (v/v) human AB serum (Valley Biomedical) supplemented with 1% (v/v) HEPES, 1% (v/v) GlutaMAX and 0.1% (v/v) 2-mercaptoethanol supplemented with recombinant human interleukin-2 (IL-2, 300 IU/ml) after washing with 1 × CTS DPBS twice. The resuspended cells were applied to bead-purification for CD3+ T cells. Briefly, the cells were diluted to approximately 1×10^7 cells/ml in wash buffer (1 x CTS DPBS containing 1% (v/v) HSA). Those PBMCs were sent to flow cytometry analysis to determine the percentage of CD3+ T cells. Based on this percentage, the volume of PBMCs applied to T cell isolation and activation were determined (containing 1×10^7 CD3+ T cells). Pre-washed and re-suspended CTS Dynabeads CD3/CD28 beads (4×10^8 beads/ml) were added to obtain a bead-to-cell ratio of 3:1 in a tube (75 µl of beads for 1×10^7 CD3+ T cells). The cells and the CTS Dynabeads were gently mixed at 1-3 rpm for 30 min at room temperature and then placed on a magnet for 1-2 min to capture the bead-bound CD3+ T cells. The supernatant containing non-isolated cells were discarded on the magnet and the tube containing the captured cells were removed from the magnet. Complete T cell medium was immediately added to the tube containing the captured cells to make a concentration of 1×10^6 cells per ml and the cell/bead complexes were gently re-suspended. The cell/bead complexes were transferred from the tube into a 24-well tissue culture plate at 1×10^6 cells per well.

Day 2–3: Activation of human T cells

Briefly, the cell/bead complexes were cultured in the complete T cell culture medium containing 300 IU/ml IL-2 in a 24-well tissue culture plate incubated at 37°C, 5% CO_2 for 48 h. The bead-stimulated CD3+ T cells were ready for transduction after being washed twice with the culture medium.

Day 4: Transduction of human T cells with retroviral particles for the first round

Briefly, 24-well non-tissue culture plates were coated with 0.5 ml of human recombinant RetroNectin per well (10 μg/ml in PBS) 1 day prior to the transduction and then incubated at 4°C overnight. After RetroNectin was removed, the plate was blocked with 2% (w/v) BSA in PBS at room temperature for 30 min. The plate was then washed twice with PBS containing 2.5% (v/v) HEPES. A2-ESO-1 TCR retroviral particles were thawed at room temperature and added to the RetroNectin-coated plates (2 ml per well) at a target multiplicity of infection (MOI) of 4 particles/cell. The plate was centrifuged at 2,000×g, 32°C for 2 h. After the viral supernatant was discarded, bead-stimulated T cells from the steps above were added to the plate (5×10^5/well) and cultured in a complete T cell culture medium containing 150 IU/ml IL-2 at 37°C, 5% CO_2 overnight.

Day 5: Transduction of human T cells with retroviral particles for the second round

A second round of transduction was performed with a fresh retroviral supernatant and RetroNectin-coated plates, as described above. The same T cells from previous day's transduction were added to the second RetroNection-coated plate, then tranduced for the second time.

Day 6–14: Expansion of A2-ESO-1 TCR-T

Briefly, 5-10 × 10^6 cells of A2-ESO-1 TCR-engineered T cells were added to the G-Rex10M containing 100 ml of the complete T cell medium with 35 ng/ml human anti-CD3 antibody and human anti-CD28 antibody respectively before being placed in a humidified 37°C, 5% CO_2 incubator. Then, 50 μl of IL-2 (300 IU/μl) was added to the G-Rex10M without disturbing the cells on days 7, 10, and 13. On day 14, the expanded T cells were gently aspirated using 25 ml pipettes and harvested and transferred into sterile 50 ml tubes using 25 ml pipettes.

Day 14: Formulation and cryopreservation of A2-ESO-1 TCR-T

Briefly, harvested, expanded A2-ESO-1 TCR-T cells were formulated in the cryoprotectant (CryoStor® CS10) at 2 × 10^7/ml. Then, 2 ml of the bulk harvest consisting of cells and cryoprotectant was taken from the harvested T cells before the final formulation for sterility testing and other release testing. The formulated cells were aliquoted into labeled CryoMACS® Freezing Bag (10 ml per bag) and cryopreserved under controlled-rate freezing in the LN_2 storage tank (≤ −150°C).

After two batches of A2-ESO-1 TCR-T (ND8 and ND9) were produced at the KJCCT facility independently, multiple tests for quality control (QC) were carried out to confirm the quality of our products of validation runs that meet the requirements for clinical use by the FDA.

Sterility, mycoplasma and endotoxin

Sterility test is required to ensure viable contaminating microorganisms are not evident in our products. This test detects a wide range of microorganisms through the use of both aerobic and anaerobic culture bottles as well as the ability to incubate bottles at both 20-25°C and 30-35°C, as is the case in USP <71> sterility tests. The in-process and final samples of ND8 and ND9 were sent to quality assurance (QA) at KJCCT and the tests were conducted with the expectation that no microorganisms grow within 14 days. Negative test result was obtained by QA at KJCCT, as shown in **Table 7**.

Mycoplasma can cause contamination during cell therapy processing or manufacturing. This test uses a selective biochemical test that exploits the activity of mycoplasmal enzymes indicative of the presence or absence of mycoplasma producing results in 30 min. This test with in-process and final samples of ND8 and ND9 was conducted by QA at KJCCT with the expectation of complete negative results. Negative test result was obtained by QA at KJCCT, as shown in **Table 7**.

Endotoxin test is the most critical quality control test required by the FDA for all drugs in their final stages of formulation. Endotoxins are invariably associated with every gram-negative bacteria, so they cause severe reactions in humans and animals and retain high toxic activity even present at low concentration. This test using the Endosafe® nexgen-PTS™ to detect endotoxin in the cryopreserved samples of ND8 and ND9 was conducted by QA at KJCCT with the expectation of < 5 EU per kg of donor's weight. Negative test result was obtained by QA at KJCCT, as shown in **Table 7**.

1) Purity and transduction efficiency

The purity of ND8 or ND9 A2-ESO-1 TCR-T products was confirmed by testing the percentage of expressing CD8+ and CD4+ T cells in the final products. The procedure for testing CD3+ (CD8+, CD4+) percentage was describedin the batch records. Briefly, the A2-ESO-1 TCR-T cells were stained with PE anti-human CD3 Antibody (eBioscience, 12-0037-42), FITC anti-human CD8a Antibody (eBioscience, 11-0086-42), and APC anti-human CD4 antibody (eBioscience, 17-0049-42) for 30 min at room temperature and then analyzed using flow cytometry. The purity of A2-ESO-1 TCR-T was calculated by the percentages of CD8+ and CD4+ T cells with the expectation of >70% CD8+ and CD4+ T cells in total. The transduction efficiency of ND8 and ND9 was determined by staining with PE anti-human CD3 Antibody (eBioscience, 12-0037-42), FITC anti-human CD8a Antibody (eBioscience, 11-0086-42) and APC anti-human TCR Vβ13.1 Antibody (Biolegend, 362407) and flow cytometry analysis with expectation that >50% of CD8+ or CD4+ T cells express A2-ESO-1 TCR. Our test results showed there were 85.79% CD3+ T cells in viable cells in ND8 final products, among which 83.2% were CD8+ T cells and there were 84.61% CD3+ T cells in viable

cells in ND9 final products, among which 81.6% are CD8+ T cells. Besides, 81.9% of ND8 viable cells were CD3+ A2-ESO-1 TCR+ T cells and 70.7% of ND9 viable cells were CD3+ A2-ESO-1 TCR+ T cells. Both the purity and transduction efficiency of ND8 and ND9 final products were much higher than expected. Test result was shown in **Table 8**.

2) Viability

The viability of A2-ESO-1 TCR-T was confirmed using flow cytometry analysis after propidium iodide (PI) staining. Briefly, the A2-ESO-1 TCR-T cells were stained by PI (Invitrogen, P3566) for 10 min at room temperature and then analyzed. The viability of A2-ESO-1 is determined by (100% minus the percentage of PI-positive cells). Our test results showed 72.1% viable cells in ND8 and 82.5% viable cells in ND9, which meet the requirements of >70% viable cells. Test result was shown in **Table 8**.

Table 8. Batch analyses of A2-ESO-1 TCR-T cells qualification and proposed clinical batches

Test	**Acceptance Criteria**	**Batch number**		
		ND8	**ND9**	**Clinical 1, etc**
Cell numbers ($\times 10^6$)	Report result	93	125	
Cell viability	>70%	72.1%	82.5%	
Cell purity	Percentage of CD3+ viable T cells >90%	91.0%	91.2%	
A2-ESO-1 TCR transduction efficiency	Percentage of TCR+ viable $CD4^+$ and $CD8^+$	81.9%	70.7%	
T cell Potency IFN-γ release Cytotoxicity	IFN-γ release > 1000 pg/ml Cytolysis > 50%	IFN-γ release > 1000 pg/ml Cytolysis > 50%	IFN-γ release > 1000 pg/ml Cytolysis > 50%	
Sterility	No growth	No growth	No growth	
RCR- PCR	No amplicons for env genes of GALV and Ecotropic MLV	No amplicons	No amplicons	
Vector copy number per cell	1-3 copies/cell	2.06±0.47	1.89±0.55	

3) Potency (Functional tests)

The potency of A2-ESO-1 TCR-T products was confirmed by the cytokine secretion assay and the cytotoxicity assay after incubating the TCR-T cells with TNBC cell lines in vitro. The procedure for measuring cytokine secretion and cytotoxicity was recorded in the batch records. Briefly, TNBC cell lines (MDA-MB-231 with NY-ESO-1 overexpression), other tumor cell lines (586mel and 624mel) and HEK293T cells pulsed with ESO peptides were seeded at 5×10^4 /well in a 96-well plate and co-cultured with 1×10^5 A2-ESO-1 TCR-T cells at 37℃, 5% CO_2 overnight. The following day, 50 µl of

the supernatant was collected from each well to measure cytokine release (interferon-γ) with ELISA. The testing results of the cytokine secretion of ND8 and ND9 derived T cells were shown in **Table 8** and **Table 9**, which demonstrated specific response to ESO+/A2+ tumors (>1000pg/ml interferon-γ).

Table 9. Results of cytokine secretion assay of ND8 and ND9

Interferon-γ secretion (pg/ml)	T cell alone	HEK293T	HEK293T/ ESO PEP157-165	MDA-MB-231	MDA-MB-231/ ESO	586mel (A2-ESO+)	624mel (A2+ ESO+)
ND8	48.20104	34.56902	2896.045	56.08791	1844.284	94.81683	1123.211
	44.43144	32.27754	3207.116	49.32991	1728.91	92.15386	1173.933
ND9	33.0421	33.0421	2779.57	31.51222	2402.412	69.19138	1500.287
	35.33141	28.44303	3694.885	39.1334	2670.293	66.94558	1274.42

Briefly, for the cytotoxicity test, the A2-ESO-1 TCR-T cells (effectors) and targeted cell lines including MDA-MB-231 with NY-ESO-1 overexpression, 586mel and 624mel were mixed at an E/T ratio of 20:1 and then co-cultured at 100 µl/well in a 96-well plate at 37°C, 5% CO_2 for at least 4 h. After the incubation, the LDH concentration in the supernatant of each well was measured with the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega, G1780) following the manufacturer's instructions, and the percentage of targeted cell lysis was calculated accordingly. The testing results of the cytotoxicity assay of ND8 and ND9 T cells were shown in **Table 8 and Table 10**, which demonstrated the therapeutic effects against ESO+/A2+ tumors (>50% cytolysis).

Table 10. Results of cytotoxicity assay of ND8 and ND9

Cytolysis (%)	MDA-MB-231			MDA-MB-231/ESO		
ND8	20.01916	8.237548	17.43295	66.01563	67.57813	84.76563
ND9	14.27203	11.68582	13.69732	77.34375	83.59375	82.8125
Cytolysis (%)	**586mel (A2- ESO+)**			**624mel (A2+ ESO+)**		
ND8	18.9916	19.41176	22.77311	58.47545	53.82429	67.77778
ND9	11.20448	10.5042	10.5042	53.74677	73.90181	64.59948

4) Replication competent retrovirus (RCR

The potential pathogenicity of replication competent retrovirus (RCR) requires vigilant testing to exclude the presence of RCR in vector-based human gene therapy products, even though an RCR may present a minimal direct safety risk to humans and there have been no reports of RCR contaminations in previous retroviral and lentiviral transduced-T cell therapy. Our manufacturing process indicated that ecotropic murine leukemia virus (MLV) and gibbon ape leukemia virus (GALV) might be potentially introduced due to the usage of Phoenix-Eco (ATCC, CRL-3214) and PG13 (ATCC, CRL-10686) cell lines during the production of retroviral particles of A2-ESO-1 TCR, even though they were tested negative as the QC of retroviral particles. The RCR tests were conducted after the co-culture of final TCR-T products with HEK293T cells or M. dunni cells, which were susceptible to GALV or MLV infection respectively. Briefly, 1 ×

10^5 of T cells were seeded with 1×10^5 of HEK29T cells or M. dunni cells, respectively, in 10-cm dish and co-cultured for 2 weeks at 37℃, 5% CO_2. Genomic DNAs were extracted from those expanded cells and other control cell lines as templates for PCR. Primers for detecting the envelop gene of GALV or MLV integration in the genome of HKE293T or M. dunni were designed from their genomic sequences in NCBI database (GALV, NC_001885; MLV, KJ668270). After the PCR all amplicons were examined on the 2% agarose gels. Our results demonstrated that no amplicons (501bp for GALV *env* and 224bp for ecotropic MLV *env*) were observed in both ND8 and ND9 samples (**Figure 21** and **Table 8)**, indicating the absence of RCR in our final TCR-T cell products.

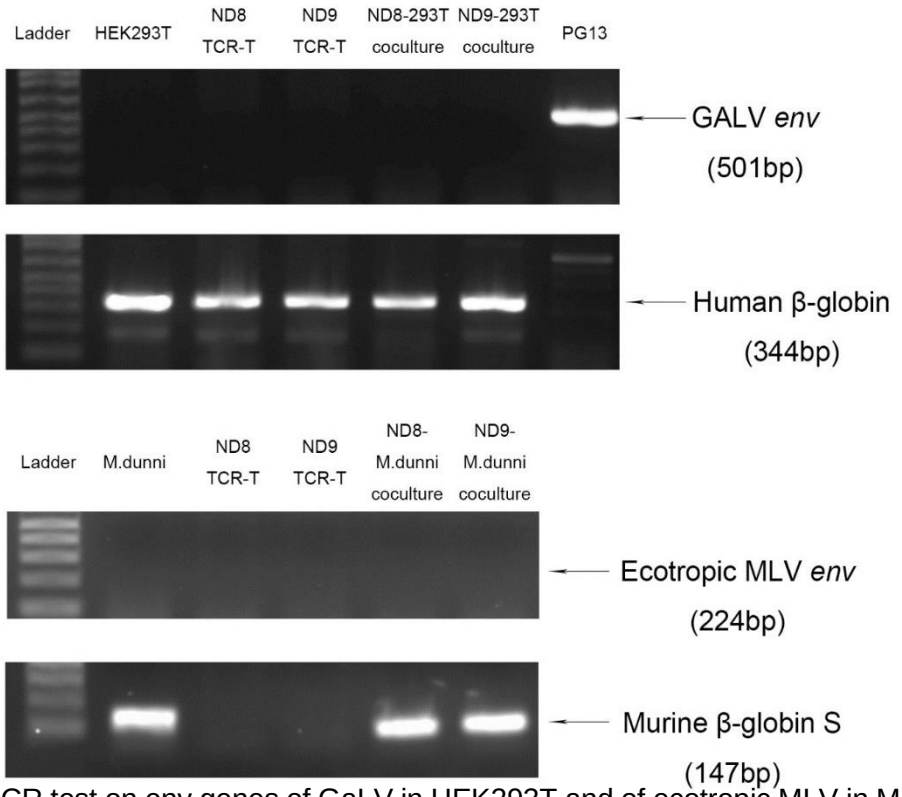

Figure 21. PCR test on env genes of GaLV in HEK293T and of ecotropic MLV in M. dunni after co-culture with A2-ESO-1 TCR-T product. Upper: No amplicons of GaLV env were visible from gDNA of HEK293T co-cultured with A2-ESO-1 TCR-T; Lower: No amplicons of ecotropic MLV env were visible from gDNA of M. dunni co-cultured with A2-ESO-1 TCR-T.

5) Residual beads

The usage of CD3/CD28 beads during the T cell activation may introduce residual beads in our final products, even though there was one action of removing the beads after the activation step. For the safety of the patients, the numbers of residual beads in the infused cell products cannot exceed 100 beads per 3×10^6 cells. The measurements of residual beads in ND8 and ND9 T cell products were conducted. Briefly, 1×10^6 cells were lysed with 10% Triton X-100 and incubated for 20 min. The cell lysate was spun at high speed for 5 min. After removing the supernatant, all cell debris was resuspended in 15 μl of distilled water. 10 μl of the suspension was mixed well with 10 μl of Trypan blue solution and 10 μl of the mixture was added on the cell

cytometer and observed under microscope. Only three bead-like particles in ND8 and two bead-like particles in ND9 were observed on the slides, indicating that there were at most 9 beads per million cells in ND8 and 6 beads per million cells in ND9, which meets the safety requirement for residual beads.

6) Vector copy number

The vector copy number in transduced T cells are determined by Quantitative PCR (qPCR). Briefly, genomic DNAs are extracted from 1 × 10^6 viable TCR-T cell products using Quick-gDNA™ Miniprep Kit (Zymo Research, D3024) following the manufacturer's protocol. The genomic DNAs are applied as templates for PCR amplification in Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, A25742) with MSGV vector transgene-specific primers (Forward: 5'-CCCTCTCTCCAAGCTCACTT; Reverse: 5'-CGGTCCAGTTGTTCTTGGTA). The real-time qPCR is run in the Applied Biosystems™ QuantStudio 6 Flex. The vector copy number per cell is calculated based on the cycle threshold (Ct) value generated from the qPCR, the cell number and the transduction efficiency obtained from flow cytometry analysis as described above and shown in **Table 8**.

The standard curve determining the correlation of the vector copy number and the cycle threshold (Ct) value of qPCR is generated with the multiple copy numbers of MSGV-A2-ESO-1 TCR plasmids and their Ct value in qPCR. The vector copy number shows a perfect linear relationship with the logarithm (log10) of Ct value (**Figure 22**).

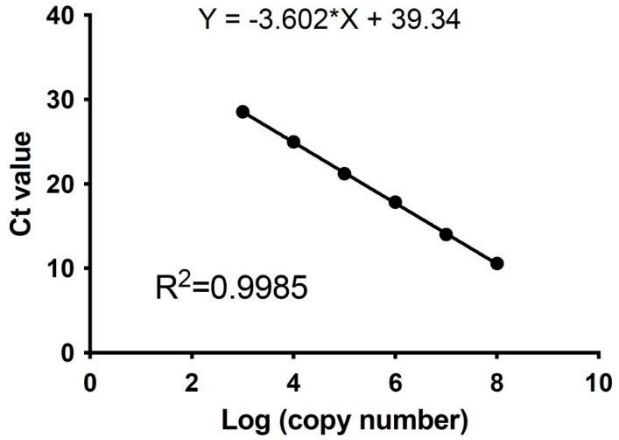

Figure 22. A representative standard curve to calculate the vector copy number (10^2-10^8 copies) from Ct value of qPCR. The R2 value confirms a perfect linear relationship between the vector copy number and the logarithm (log10) of Ct value.

7) Stability

The A2-ESO-1 TCR-T products were formulated in the cryoprotectant (CryoStor® CS10 at 2 × 10^7 cells/ml) and cryopreserved at -150°C after validation runs. Since it is anticipated that the clinical product will be stored for only a short period and then applied in patient infusion, the test on post-thawing stability of our ND8 and ND9 products was carried out.

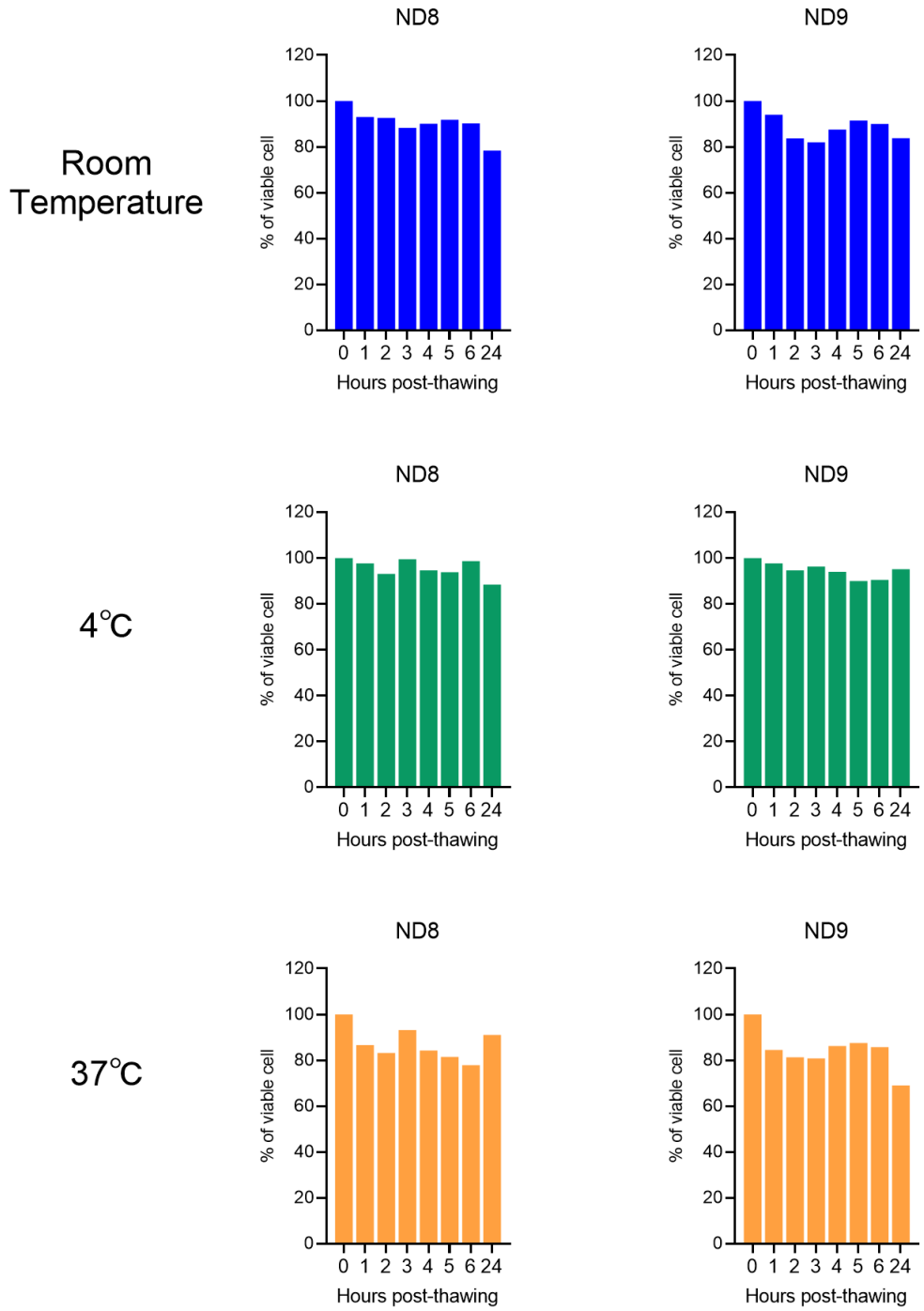

Figure 23. Post-thaw stability test on the lots of qualification runs. The lots of ND8 and ND9 TCR-T cell products were thawed after one-month cryopreservation at -150℃ and tested for cell survival under multiple storage conditions over 24 h. Data are presented as percentage of post thaw viable cells.

Briefly, ND8 or ND9 TCR-T cells were thawed from cryopreservation by 37°C water bath. The thawed cells were immediately mixed with 9 volumes of saline to mimic the clinical situation of cell infusion. Those diluted cells were aliquoted in parallel vials and incubated at room temperature and 4°C respectively. At the timepoint of 0, 1,2,3,4,5

and 6 hours post-thaw, one vial of cells was taken, and the cell viability was analyzed by PI staining and flow cytometry. Our results shown in **Figure 23** demonstrated that more than 90% of viable cells in both ND8 and ND9 were still alive within 6 hours post-thawing, no matter at room temperature or 4°C. Such viability was maintained up to 24 hours post-thawing.

Submission of IND to FDA

Once we completed the qualification runs at Ann Kimball W. Johnson Center for Cellular Therapy (KJCCT) facility and obtained results for manufacturing and testing for QA AND QC certification, we finalized the IND application.

The description of manufacturing process and results of qualification runs at KJCCT were included in the Module 3 of Common Technical Document (CTD) for IND submission, which provides the detailed quality information regarding the substances and products of our A2-ESO-1 TCR-T products. Besides Module 3, we also completed three standalone non-clinical study reports (Biopotency, Biodistribution and Toxicity) in Module 4. An Institutional Review Boards (IRB) protocol for a phase I clinical trial was also finalized and included in Module 5. The other materials such as Form FDA 1571, Form FDA 3674, Investigational drug labeling, General investigational plan for initial IND, Introduction to summary, Form FDA 1572, Investigator's CV and license, Informed Consent Form and copies of cited references were prepared and included in the IND submission package.

Our IND application was submitted on July 6th, 2022 and received by the FDA on July 8, 2022.

Communication and responses to the FDA for final approval on Aug. 5, 2022

Upon our submission, FDA team reviewed our IND application and raised multiple questions concerning the CMC information, the preclinical studies and our clinical protocols. We responded to FDA by submitting several additional supporting materials, results, and amendments. During the 30-day review period, we partnered with Houston Methodist clinical team led by Dr. Jenny Chang (Partnering PI) to address many questions related to clinical trials and statistical consideration of a clinical trials, recruitment criteria of TNBC patients and monitoring plan after T cell immunotherapy. With great help from FDA review team and their satisfactory with IND application, our IND application (IND 28656) has been approved by FDA to proceed with our clinical trial.

With the approval of our IND, the clinical team at Houston Methodist has submitted IRB for approval, which is currently pending for review and approval decision. The IRB will send to DoD for official HRPO review before recruitment of patients for our phase I clinical trial.

March 22, 2023 Update:
Houston Methodist Neal Cancer Center Protocol Review and Monitoring Committee

On January 11, 2023, the associated clinical trial protocol was reviewed by the Houston Methodist Neal Cancer Center's (HMNCC) Protocol Review and Monitoring Committee (PRMC)and was disapproved due to scientific merit and enrollment feasibility concern; hence this study was disapproved and will not move towards an IRB submission. After the receipt of this notice by PRMC, Chang informed Wang (PI) and Jamie Shortall, US Army Medical Research Acquisition Activity Grant Officer, via a conference call on February 13, 2023, that the proposed clinical trial will not proceed at Houston Methodist and the award will be relinquished. The corresponding letter by the PRMC Chair, Eric Bernicker, MD, is attached to this final progress report as an addendum.

After the approval of University of Southern California's (USC IND application (IND 28656) by FDA, Rongfu Wang, Partnering PI at USC, 1450 Biggy Street. Room 5503, Los Angeles, CA 90033 (rongfuwa@usc.edu) initiate the collaboration with Dr. Janice Lu, a Clinical Professor in Department of Medicine, Keck School of Medicine, USC. Dr. Lu is certified by the American Board of Internal Medicine and Medical Oncology, has extensive experience in clinical trials and breast cancer drug development.

In February 2023, USC submitted to an IRB protocol to Clinical Investigations Support Office (CISO) at USC. The CISO committee approved our IRB protocol to the Disease-Specific committee. After the review in the committee meeting in March, the reviewers raised 15 comments and we have made a major revision by incorporating T cell manufacturing, nonclinical study and human clinical study report (with additional over 50 pages). In the final version, the IRB contained the detailed information of the rationale, product development and testing, patient selection and treatment plan, and ethical considerations, which markedly improved the quality of this protocol. Detailed information about different sections in the IRB application is listed below.

2. Preparation of Investigator's Brochure

Meanwhile, Drs. Wang and Lu worked on the preparation of Investigator's Brochure for the upcoming clinical studies. The Investigator's Brochure is a comprehensive compilation of clinical and nonclinical data on the investigational product (A2-ESO-1 TCR-T) maintained by the drug developer. This document is important throughout the drug development process and is updated with new information as it becomes available. The purpose of preparing this document is to gather data relevant to the study of our product, including preclinical data and other clinical trials, to provide useful information and guidance to the management of study conduct throughout the upcoming clinical trial. Similarly, the data of their previous preclinical studies were organized and at least four previous clinical trials (NCT00670748, NCT00670748, NCT01352286, NCT01343043) were analyzed and summarized. Many key features of the protocol, such as dose justification, dose frequency, dosing interval, methods of administration and safety monitoring procedures were also included.

Key findings and results:

1) Completed the annual certification of the stability of GMP-grade retrovirus.
2) Performed the qualification runs at KJCCT for IND application.
3) Prepared and submitted our IND application.
4) Communicated with the FDA and addressed questions raised by review team.
5) Obtained IND approval by FDA (IND 28656), and worked on IRB submission.
6) HMNCC PRMC disapproved this study due to scientific merit and will not proceed with an IRB Submission at Houston Methodist. Houston Methodist will relinquish/terminate this award back to the DoD.
7) The proposed clinical trial will potentially proceed at University of Southern California, the institution that houses the Initiating Principal Investigator of this award, and will implement the proposed clinical trial of A2-ESO-TCR T cells therapy for TNBC patients, with Dr Janice Lu, also at University of Southern California, as the Clinical Trials Principal Investigator.

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

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

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

This will be a final report, as this award is to be relinquished by Houston Methodist Research Institute.

4. IMPACT:

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:

Changes in approach and reasons for change

This project was disapproved by the Houston Methodist Neal Cancer Center's Protocol Review and Monitoring Committee; hence the IRB submission will not proceed and the clinical trial will not commence in accordance with the original grant submission. This award will be relinquished by Houston Methodist Research Institute back to the DoD. Per Dr Rongfu Wang at USC, the proposed clinical trial will proceed at USC, after IRB approval.

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

This project was disapproved by the Houston Methodist Neal Cancer Center's Protocol Review and Monitoring Committee; hence the IRB submission will not proceed and the clinical trial will not commence in accordance with the original grant submission. This award will be relinquished by Houston Methodist Research Institute. Per Dr Rongfu Wang at USC, the proposed clinical trial will proceed at USC, after IRB approval.

Changes that had a significant impact on expenditures

No funds have been expended for the clinical trial portion of this project.

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

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:

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

Journal publications.

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

5. Duan T, Du Y, Xing C, Wang HY, Wang RF. Toll-Like Receptor Signaling and Its Role in Cell-Mediated Immunity. ***Front Immunol***. 2022 Mar 3;13:812774. doi: 10.3389/fimmu.2022.812774. eCollection 2022. PMID: 35309296

6. Liu X, Xu Y, Xiong W, Yin B, Huang Y, Chu J, Xing C, Qian C, Du Y, Duan T, Wang HY, Zhang N, Yu JS, An Z, Wang R. Development of a TCR-like antibody and chimeric antigen receptor against NY-ESO-1/HLA-A2 for cancer immunotherapy. ***J Immunother Cancer.*** 2022 Mar;10(3):e004035. doi: 10.1136/jitc-2021-004035. PMID: 35338087

7. Xing C, Du Y, Duan T, Nim K, Chu J, Wang HY, Wang RF. Interaction between microbiota and immunity and its implication in colorectal cancer. ***Front Immunol.*** 2022 Jul 29;13:963819. doi: 10.3389/fimmu.2022.963819. eCollection 2022. PMID: 35967333

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

Nothing to Report

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Rongfu Wang, PhD Project
Role: PI, Professor
Contribution to Project: Dr. Wang supervises the whole project, plans experiments, and analyzes and interprets 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, and collects and analyzes data.

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 works on the T cell infection and data collection in both in vitro and in vivo functional assays.

Name: Motao Zhu
Project Role: Assistant Professor of Research
Contribution to Project: Dr. Zhu assisted in the experiment planning and data collection from the in vitro functional assays.

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

Name: Xin Liu, PhD
Project Role: Postdoctoral Fellow
Contribution to Project: Dr. Liu works on the animal preparation, T cell infection, and generation of constructs.

Name: Chuntang Fu
Project Role: Postdoctoral Fellow
Contribution to Project: Dr. Fu performed and collected data from the animal experiments.

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

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: