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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 will be to address the overarching challenge of eliminating mortality associated with metastatic breast cancer, specifically, the subtype, TNBC.

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 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 well as critical help and cytotopic function of D -ESO-1 TCR-transduced CD 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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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. 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. We have submitted the IND application for a clinical trial, and it has been 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	Completed
• To screen pathological samples for TNBC, contact and recruit study subjects	13-36	In progress

What was accomplished under these goals?

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

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

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

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 1**.

Table 1. 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 2** and **Table 3**.

Table 2. 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/WilsonWolf	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 3. 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 details.

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.

1) 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 4**.

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 4**.

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 4**.

2) 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 4**.

3) 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 4**.

Table 4. 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 $CD8^+$ T cells >50 %	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	

4) 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 4** and **Table 5**, which demonstrated specific response to ESO+/A2+ tumors (>1000pg/ml interferon-γ).

Table 5. 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 4 and Table 6**, which demonstrated the therapeutic effects against ESO+/A2+ tumors (>50% cytolysis).

Table 6. 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

5) 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 1** and **Table 4)**, indicating the absence of RCR in our final TCR-T cell products.

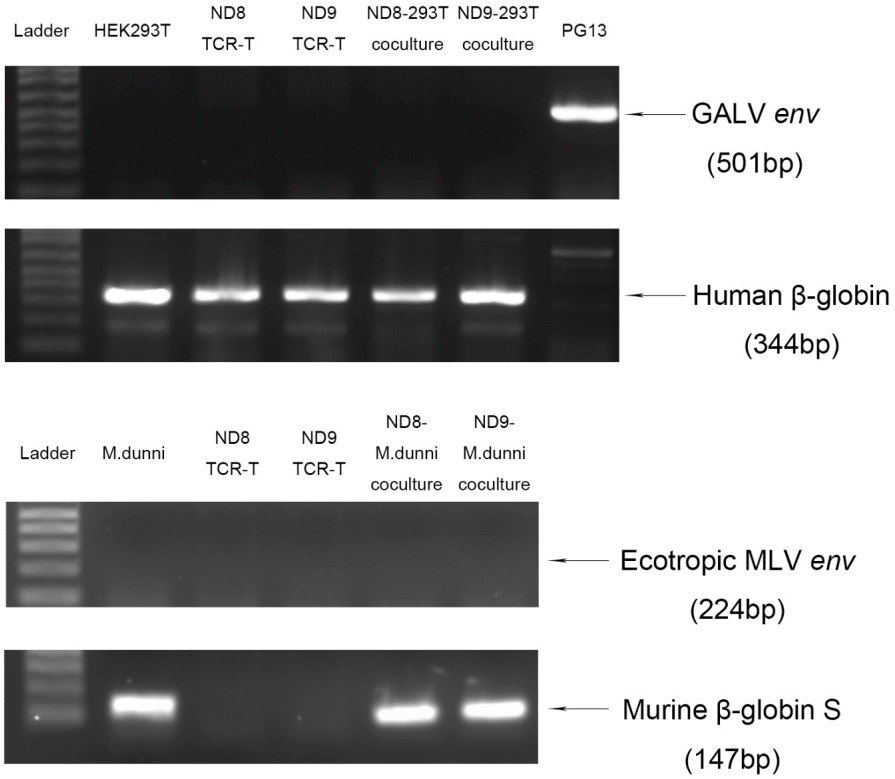

Figure 1. PCR test on env genes of GaLV in HEK293T and of ecotropic MLV in M. dunni after co-culture with A2-ESO-1 TCR-T product. Upper: No amplicons of GaLV env were visible from gDNA of HEK293T co-cultured with A2-ESO-1 TCR-T; Lower: No amplicons of ecotropic MLV env were visible from gDNA of M. dunni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

6) 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.

7) 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 4**.

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 2**).

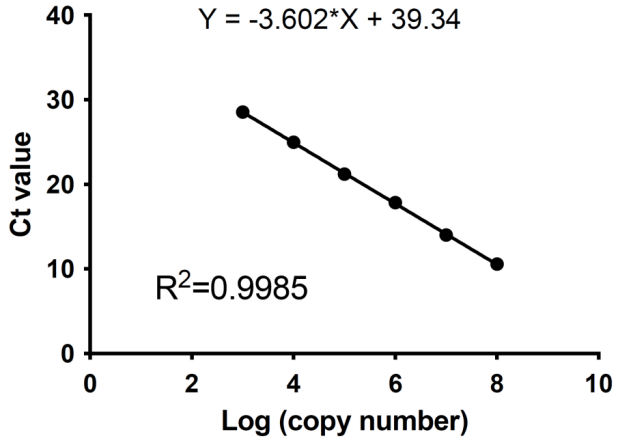

Figure 2. A representative standard curve to calculate the vector copy number (10^2-10^8 copies) from Ct value of qPCR. The R2 value confirms a perfect linear relationship between the vector copy number and the logarithm (log10) of Ct value.

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.

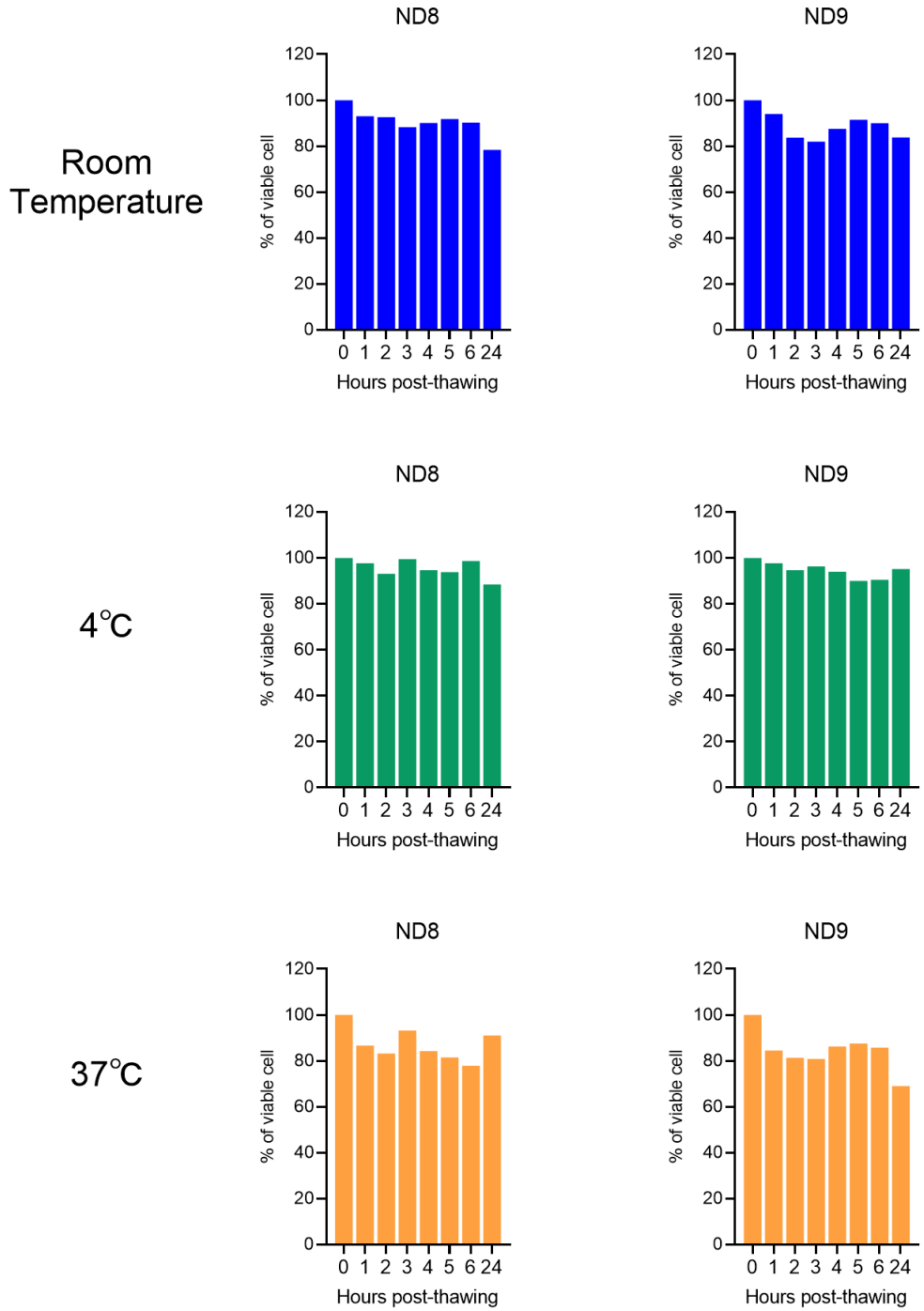

Figure 3. Post-thaw stability test on the lots of qualification runs. The lots of ND8 and ND9 TCR-T cell products were thawed after one-month cryopreservation at -150℃ and tested for cell survival under multiple storage conditions over 24 h. Data are presented as percentage of post thaw viable cells.

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 3** 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 finalize 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.

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.

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?

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

1. Obtain IRB approval by Houston Methodist and DoD HRPO.
2. Recruit cancer patients for clinical trial.
3. Produce A2-ESO TCR-T cell products in the cGMP facility at KJCCT at Houston Methodist Academic Institute using our SOPs.
4. Initiate a clinical trial for TNBC immunotherapy using A2-ESO-TCR T cells.

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

Nothing to Report

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

Our overall progress of project has been severely delayed due to COVID-19 pandemic. Despite these difficulties, we have now received IND approval by the FDA to initiate a phase I clinical trial using A2-ESO-1 TCR-T cells in clinical trials for triple negative

breast cancer.

In the next step, we will submit both IND and IRB protocol to the USAMRDC ORP Human Research Protection Office (HRPO) for review and approval before we start our clinical study. As the PI, I will continue to serve as adjunct professor at HMRI and closely work with the partnering PI Dr. Chang to initiate and conduct our clinical trial in TNBC using engineered A2-ESO-1 TCR-T cells.

Changes that had a significant impact on expenditures

Nothing to Report

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

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. 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

2. 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

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

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

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: