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# TIM-4-Chimeric Engulfment Receptor (CER) T Cells Elicit Phosphatidylserine-Dependent Cytotoxic and Innate-Like Function and Synergize with Approved PARP Inhibitors in an Ovarian Cancer Model

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## INTRODUCTION

Engineered T cells have emerged as a powerful anti-cancer therapy, mainly for the treatment of hematologic tumors. Here, we describe a solid tumor model of multi-modal Chimeric engulfment receptor (CER) T cell function. CER T cells recognize the stress-induced, tumor-agnostic ligand phosphatidylserine (PS) and overcome limitations of current adoptive T-cell therapies by facilitating antigen capture, processing, and presentation, and imparting target-dependent cytotoxic function. CER T cells were assessed in combination with approved small molecule inhibitors of poly (ADP-ribose) polymerase (PARP) enzymes in a PARP inhibitor-sensitive ovarian cancer tumor cell line.

CER T cells integrate features of both the innate (engulfment and antigen presentation) and adaptive (T-cell activation) immune systems. Introduction of a CER into a T cell allows tumor recognition via an inducible stress ligand and triggers capture of tumor cell fragments by the CER T cell. T-cell signaling domains of the CER then mediate activation of T-cell cytotoxicity directly against the tumor. An additional component of the CER design also enhances the T cell's ability to process and present tumor antigen, which has the potential to stimulate a broad immune response.

Herein we show that CER T cells recognize a specific phospholipid and produce cytokines in response to target engagement in both a cell-free system and a drug-induced model, and process and present antigen, and, in combination with small molecule drugs, kill target cells.

## OBJECTIVES

- To design and manufacture novel CER T cells that target a drug-inducible phospholipid on cancer cells.
- To determine characteristics and function of CER T-cell in vitro using cell-based and cell-free systems.

## METHODS

The induction of surface PS expression on A2780 ovarian cells treated with the PARP inhibitors olaparib and niraparib was measured by flow cytometry (FC) using annexin V.

CER T cells containing the T-cell immunoglobulin and mucin domain containing-4 (TIM-4) receptor fused to various transmembrane and intracellular signaling domains were generated using healthy donor T cells.

**CER T-cell activation was assessed by measuring induction of IFN-γ by plate- and cell-based assays**

- Plate-based assay:
  - Single induction: varying concentrations of PS or phosphatidylethanolamine (PE), a control membrane phospholipid not recognized by wild type TIM-4 (wtTIM-4), were coated onto high-binding tissue culture plates. CER T cells were added and IFN-γ levels were measured 24 hours after ligand exposure by ELISA. EC50 concentrations were determined by nonlinear regression (agonist vs. response — variable slope (4 parameters)).
  - Induction by recursive stimulation: CER T cells were added to PS coated plates (2.5-20 µg/mL PS coating). Five days later, CER T cells were transferred to uncoated plates with fresh T cell optimizer media for 24 hours. Then, CER T cells were transferred again to a fresh PS coated plate. Supernatant was collected to assess IFN-γ induction by ELISA at various timepoints during restimulation (24, 72 and 120 hours).
- Cell-based assay: cytokine induction was assessed by multiparametric ELISA using conditioned supernatants harvested after 120 hours from the cytotoxicity assay described below.

CER T-cell cytotoxic function was evaluated using the Incucyte® live-cell analysis system in co-culture assays with A2780 cells.

- A2780 cells engineered to constitutively express mCherry were pretreated with 1.5 µM olaparib, 0.5 µM niraparib or no drug for 96 hours.
- CER T cells (expanded for 6 days after transduction) in T-cell optimizer media containing IL-2, IL-7, and IL-15 were co-cultured with A2780 cells at an E:T ratio of 1:2 in the presence of the same concentrations of olaparib or niraparib.
- Expression of TIM-4, CD45RA, and CCR7 as well as CD4/CD8 ratios were evaluated by FC using commercially available fluorochrome-conjugated antibodies.
- Proliferation was measured by FC using precision count beads to determine the absolute counts of viable T cells at the end of the co-culture. T-cell proliferation is reported as the fold expansion of T cell counts at 120 hours relative to 0 hours.

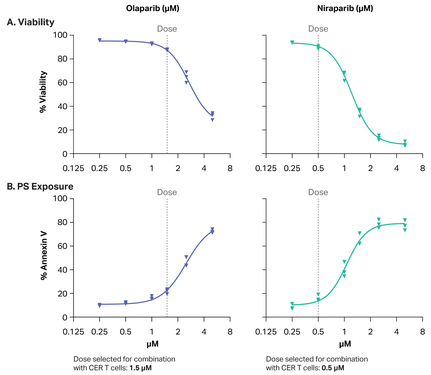
Antigen-presenting cell (APC)-like function of CER T cells was assessed after a 48-hour co-culture with PS-positive E7 oncoprotein-positive cells. T cells were separated and co-cultured for 4 days with cell trace violet-labeled E7 TCR T cells. The activation marker HLA-DR on E7 TCR T cells was analyzed using flow cytometry.

- PS-positive SCC152 squamous cell carcinoma cells were engineered by knockout of the TMEM30A gene (a flippase chaperone required for PS internalization).
- The role of HLA-I in APC-like function was determined by performing the same experiment in the presence of HLA-I blocking or the corresponding isotype antibodies.

## RESULTS

**Subtherapeutic doses of niraparib and olaparib induce PS exposure on viable A2780 cells (Figure 1)**

**FIGURE 1** PARP inhibitors Induce PS on A2780 Ovarian Cancer Cells.



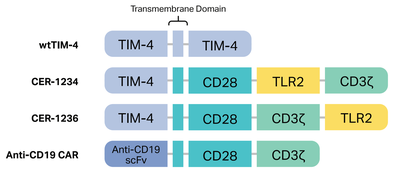

Viability (A) and PS exposure (B) of A2780 cells treated with niraparib or olaparib (both at 0.25 to 6 µM) at 96 hours. Based upon these curves, a dose of 1.5 µM was selected for olaparib and 0.5 µM was selected for niraparib as noted by the dotted line.

**CER Constructs Were Designed to Target PS via a Naturally Occurring Receptor**

CERs were designed as single-chain chimeric proteins (**Figure 2**) composed of different combinations of the following domains:

- The TIM-4 extracellular domain that binds PS.
- The transmembrane domain of TIM-4 or CD28.
- The intracellular CD28 and CD3ζ signaling domain that serve as T-cell activators.
- The intracellular TLR-2 signaling domain to enhance APC-like function.

**FIGURE 2** CER design.




*CER, chimeric engulfment receptor; TIM-4, T-cell immunoglobulin and mucin domain containing-4; TLR-2, Toll-Like Receptor-2.*

Phenotypes of CER T cells were generally consistent across 2 donors (**Table 1**).

- High proportions of CER T cells express the engineered TIM-4 receptor.
- CD4/CD8 ratios were similar after transduction and expansion of CER T cells vs untransduced T cells.
- CER T cells exhibit a predominantly naive and central memory phenotype as measured by CCR7 and CD45RA expression.

### RESULTS CONTINUED

**TABLE 1** CER T-cell Characteristics.

| Parameter | Untransduced | | wtTIM-4 | | CER-1234 | | CER-1236 | |
|---|---|---|---|---|---|---|---|---|
| | Donor 38 | Donor 41 | Donor 38 | Donor 41 | Donor 38 | Donor 41 | Donor 38 | Donor 41 |
| TIM-4+ (%) | — | — | 71.1 | 72.9 | 67.1 | 85.5 | 69.9 | 73.4 |
| T-cell CD4/CD8 ratio | 1 | 3.3 | 0.9 | 3.3 | 1.1 | 2.9 | 1.3 | 4.5 |
| T-cell Subsets (%) | | | | | | | | |
| Naïve (CD45RA+/CCR7+) | 39 | 14.7 | 37.4 | 15.4 | 32.8 | 20.8 | 45.2 | 25.1 |
| Central memory (CD45RA–/CCR7+) | 30.5 | 33.5 | 29.7 | 34 | 30.7 | 34.6 | 33.7 | 47.1 |
| Effector memory (CD45RA–/CCR7–) | 13.1 | 31.9 | 13.4 | 29.4 | 17.1 | 26.8 | 8.9 | 18.2 |
| Effector (CD45RA+/CCR7–) | 17.4 | 19.9 | 19.5 | 21.2 | 19.4 | 17.8 | 12.3 | 9.6 |

*CER, chimeric engulfment receptor; wtTIM-4, wild type T-cell immunoglobulin and mucin domain containing-4.*

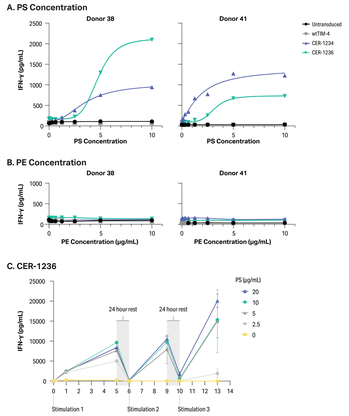
**CER T Cells produce concentration-dependent IFN-γ Responses Upon Stimulation with PS (Figure 3, A and B)**

- CER T cells show target-specific cytokine induction to PS, but not to PE.
- Saturation of IFN-γ induction by CER T cells occured at 5 to 10 µg/mL of PS.
- wtTIM-4, which lacks intracellular T-cell signaling moieties, did not induce IFN-γ upon PS stimulation.

**CER-1236 T cells are Capable of Recursive Stimulation In Vitro (Figure 3C)**

- CER-1236 T cells elicited repeated IFN-γ response upon 3 serial rounds of exposure to PS.

**FIGURE 3** CER T cells are activated and produce IFN-γ in response to PS engagement in a cell-free in vitro system.




*CER, chimeric engulfment receptor; SD, standard deviation.*

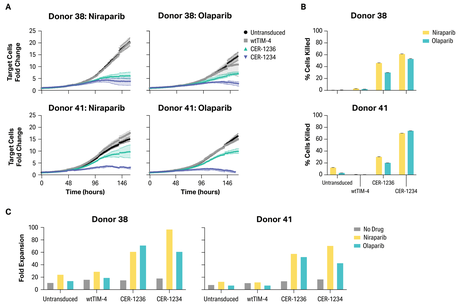
IFN-γ production was induced in CER-1236 and CER-1234 T cells stimulated with PS (A), but not with PE (B). PS EC50 values for CER-1234 and CER-1236 from Donors 38 and 41 were as follows: CER-1234, 3.52 µg/mL (SE ± 0.13) and 1.88 µg/mL (SE ± 0.48), respectively; CER-1236, 4.74 µg/mL (SE ± 0.02) and 3.16 µg/mL (SE ± 0.09), respectively. (C) Recursive IFN-γ induction with 3 serial stimulations at the indicated time points following a 24-hour rest after the first stimulation.

### RESULTS CONTINUED

**CER T Cells Demonstrate Synergistic Cytotoxic Activity With Niraparib and Olaparib Against A2780 Cells**

- Tumor cell killing was enhanced by the combination of CER-1236 or CER-1234 T cells with niraparib or olaparib (**Figure 4, A and B**).
  - wtTIM-4 T cells (no intracellular signaling domain) show no additive effect with niraparib or olaparib.
  - CER-1236 and CER-1234 T cells alone exhibited limited cytotoxic activity.
- CER-1234 and CER-1236 proliferated in co-cultures treated with PARPi compared to co-culture with wtTIM-4 expressing T cells or no drug.

**FIGURE 4** CER T Cells in Combination with PARP Inhibitors Are Cytotoxic Against A2780 Cells.



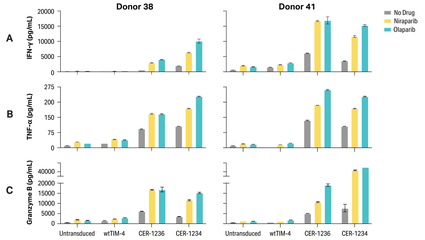

*Normalized to 0 days, 0 hours, and 0 minutes of co-culture. A2780 cells express mCherry. E:T ratio is 1:2 for A and B, E:T ratio is 1:1 for figure C. Niraparib = 0.5 µM, Olaparib = 1.5 µM. CER, chimeric engulfment receptor.*

(A) Reduced proliferation of A2780 cells is observed 120 hours after co-culture with CERs and PARPi measured by incucyte for 2 donors. (B) At 120 hours, the ratio of cells remaining in treated samples compared to untreated A2780 samples was quantified using the incucyte data from **Figure 4A**. (C) Proliferation of CER T cells in co-culture assays.

**CER T Cells produce IFN-γ Responses Upon Stimulation with PARP ihibitor-treated A2780 Cells**

- TNF-α (**Figure 5A**), IFN-γ (**Figure 5B**), granzyme B (**Figure 5C**) were induced by CER T cells but not by untransduced or wtTIM-4 T cells.
- TNF-α, IFN-γ, and granzyme B were induced in the presence of PARP inhibitor but not by vehicle.

**FIGURE 5** CER T cells are activated and produce IFN-γ in response to Co-culture with PARP inhibitor-treated A2780 Ovarian Cancer Cells.



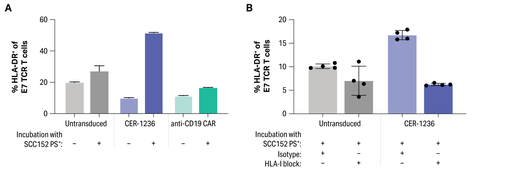

*CER, chimeric engulfment receptor; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha. E:T = 1:1, Niraparib = 0.5 µM, Olaparib = 1.5 µM.*

### RESULTS CONTINUED

**CER T Cells Demonstrate Enhanced Antigen-Presenting Cell-Like Function in an in vitro System**

- In an E7 HLA2-restricted TCR system, CER-T cells supported greater activation of autologous E7 TCR T cells, indicative of enhanced E7 antigen presentation by CER-1236 T cells, when compared to untransduced T cells or anti-CD19 CAR T cells (**Figure 6A**).
- CER T-cell-induced E7 TCR activation is dependent on antigen presentation via the HLA class I system (**Figure 6B**).

**FIGURE 6** CER-1236 T cells Activate E7 TCR T cells via Presentation of Tumor Cell-Derived E7 Antigen in an HLA-I-dependent Manner.




*PS+ = SCC152 cells engineered to constitutively expose phosphatidylserine on the cell surface. CER, chimeric engulfment receptor; CTV, cell trace violet; TCR, T cell receptor.*

(A) Following co-culture with E7 oncoprotein positive/PS-positive SCC152 target cells, CER-1236, but not untransduced T cells or anti-CD19 CAR T cells, induced HLA-DR on E7 TCR cells. (B) In a separate experiment, induction of HLA-DR on E7 TCR cells following co-culture of CER-1236 with E7 oncoprotein positive/PS-positive SCC152 cells was blocked by HLA-I blocking antibody, but not a control isotype antibody. Bars depict percentage of E7 TCR T cells that express the activation marker, HLA-DR. Error bars show SEM.

## CONCLUSIONS

CER T cells offer the potential for combination with multiple targeted therapies and application to both hematologic and solid tumors.

- Novel CER T cells containing a TIM-4 extracellular domain and CD28, CD3ζ, and TLR2 intracellular signaling domains are activated to produce cytokines and kill solid tumor cells in response to engagement with their target, PS, in vitro.
- PS induced by PARPi on viable A2780 cells.
- CER T cells synergize with subtherapeutic doses of PARPi to kill ovarian cancer cells in vitro.
- The ability of CER T cells to become reactivated upon additional antigen exposure in vitro suggests a durable effect in vivo.
- CER T cells can acquire antigen, a function that was considered to be a barrier in their ability to process and present antigen to naive T cells.[1] This engineered APC function of CER T cells may lead to initiation of secondary immune responses in vivo.

## REFERENCES

1. Lanzavecchia A, et al. Nature. 1988;334:530.

## DISCLOSURES

MB, AB, ST, RE, HN, J, FF, HK, and BC are employees of CERo Therapeutics.
JMR is an employee of CERo Therapeutics, reports stock or other ownership in CERo Therapeutics and Gilead Sciences and holds patents with Gilead Sciences. DC is an employee of CERo Therapeutics and reports stock or other ownership in CERo Therapeutics.