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378
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Chimeric Engulfment Receptor (CER) T Cells with a TLR2 Domain Synergize with an EGFR Inhibitor to Target NSCLC Cells In Vitro and Demonstrate APC-like Function

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CERo THERAPEUTICS

INTRODUCTION

Engineered T cells have emerged as a powerful anti-cancer therapy for the treatment of hematologic tumors. A barrier to successful adoptive cell therapy for solid tumors is target antigen heterogeneity and antigen escape. Activated T cells, including CAR T cells, have limited innate antigen capture/presentation capabilities[1]. Chimeric engulfment receptor (CER) T cells are engineered to express the T-cell immunoglobulin and mucin domain (TIM) 4 receptor along with intracellularly domains involved in both the innate and adaptive immune systems. The TIM-4 receptor allows tumor recognition via the stress ligand phosphatidylserine (PS) and binding to PS triggers capture of tumor cell fragments by the CER T cell, as well as activation of cytotoxicity. In addition, inclusion of a toll-like receptor (TLR) 2/toll-interleukin-1 (TIR) signaling domain is intended to maximize T-cell activation[2] and initiate secondary immune responses, leading to improved solid tumor clearance and durability of response. CER-T cells offer potential pairing with multiple small molecule drugs to enhance the surface levels of the target ligand. We tested for engulfment, cytokine induction, cytotoxicity, transcriptional activation, and antigen-presenting cell (APC)-like function, against EGFR-mutant NSCLC cells in combination with the epidermal growth factor receptor inhibitor (EGFRi) osimertinib.

OBJECTIVE

To characterize antigen-dependent phagocytic, cytotoxic, and APC-like functional responses of CER-1236, a novel CER T cell product with a TLR2 domain, in cell-free systems and NSCLC co-cultures.

METHODS

CER-1236 T cells that contain the TIM-4 receptor fused to transmembrane and innate and adaptive cell cytoplasmic signaling domains were engineered using healthy donor T cells (**Figure 1**).

Except as noted, all experiments used cryopreserved CER T cells.

Phagocytosis

- PS-coated beads were pre-stained with the pH-sensitive dye pHrodo red that shifts from minimally fluorescence at neutral pH to bright fluorescence at acidic pH (eg, lysosomes) - an increased fluorescence signal indicates bead internalization.
- Quantification of bead uptake was done by FACS and fluorescent microscopy.

CER T-cell activation by plate-bound PS and TIR inhibition studies

- CER T cells or untransduced T cells were incubated with plate-bound PS (10 µg/mL) for 48 h in the presence of various concentrations of the TLR2/TIR inhibitor C29 and cytokine levels in cell supernatants were measured by Ella.

CER T-cell activation in co-culture with EGFR-mutant H1975 Cells

- H1975 NSCLC cells were pretreated for 24 h with 100 nM Osimertinib, an EGFR inhibitor, or DMSO and then maintained in culture with 4.8 nM osimertinib or DMSO in the presence of untransduced T cells or CER-1236 T cells at an effector: target (E:T) of 1:4.
- CER T-cell cytotoxic function was evaluated using the Incucyte® live-cell analysis system.

RNASeq to evaluate transcriptional profiles

- Bulk RNA-sequencing was conducted on fresh CER-1236, CER-1251 TIM-4 binding-mutant, and untransduced T cells at rest and following activation by plate-bound PS.

Antigen-presenting cell (APC)-like functional assay using E7-TCR-T cells

- E7-expressing SCC152 squamous cell cancer cells were engineered by knockout of the TMEM30A gene (a flippase chaperone required for PS internalization).
- CER T cells were co-cultured with SCC152 TMEM30A KO cells for 48 h, then T cells were separated and co-cultured for 4 days with cell trace violet labeled-E7 TCR T cells.

RESULTS

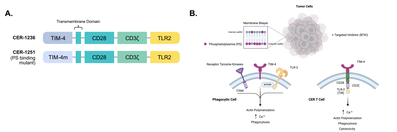
CER-1236 is Designed to Elicit Multiple Functions via its TIM-4 Phagocytic Receptor Together with Innate and Adaptive Intracellular Signaling Domains

CER-1236 combines attributes of both T cells and phagocytic cells into a single construct. The extracellular domain of the pro-phagocytic receptor TIM-4 was fused with CD28, CD3ζ and toll-like receptor (TLR)-2 toll/interleukin (TIR) signaling domains.

TIM-4 mediates uptake of tumor-associated antigens (**Figure 1B**, left) by cooperating with receptor tyrosine kinases and integrins. TIM-4 mediated phagocytosis depends on activation of RAC1 GTPase, which is similarly targeted by TLR signaling.[2,3,4]

CER-1236 T cells (**Figure 1B**, right) bind PS on tumor cells and initiate both phagocytosis and cytotoxicity, independent of cooperating receptors, by signaling through CD3ζ and CD28.

Figure 1 CER T Cell Design and Mechanism of Action



A) CER-1236 comprises the extracellular domain of the TIM-4 receptor linked to the CD28 transmembrane and indicated intracellular domains. **B)** Mechanism of Action.
Abbreviations: CER = chimeric engulfment receptor; TIM-4 = T-cell immunoglobulin and mucin domain 4; TIR = toll and interleukin-1 receptor; TLR-2 = toll-Like receptor 2; TMD = transmembrane domain.

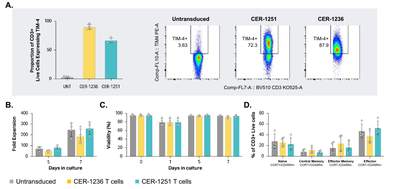
CER T cells Readily Express TIM-4, Proliferate, and Have an Early Memory Phenotype

A high proportion of CER T cells express CER-1236.

CER T-cell expansion and viability are similar to that of untransduced T cells during the manufacturing process.

CER T cells exhibit early memory phenotypes at Day 7 of manufacture.

Figure 2 CER-1236 Product Characteristics



A) Expression of TIM-4 at day 5 of manufacture measured by FACS (individual flow plots for each donor gated on live CD3+ cells are shown on the right). **B)** Expansion; **C)** Viability; **D)** Memory phenotypes at day 7 of manufacture by FC. Bars represents the mean ± standard deviation (std dev) of 4 donors. Statistics: one-way matched ANOVA with Geisser-Greenhouse correction and Tukey's multiple comparisons test with individual variances computed for each comparison.
Abbreviations: CER = chimeric engagement receptor; TIM = T-cell immunoglobulin mucin; UNT = untransduced.

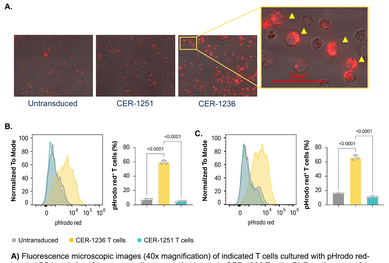
RESULTS (continued)

CER 1236 Demonstrates Target-Dependent Phagocytic Uptake of Agarose Beads

CER-1236 T cells expressing a functional TIM-4 receptor display robust engulfment activity. A large shift in fluorescence is observed by FACS with ~60% of CER-1236 T cells having acquired pHrodo red signal at 16 h and 40 h.

Untransduced T cells and CER-1251, which harbor a mutant TIM-4 receptor that fails to bind PS, have minimal bead engulfment.

Figure 3 CER-1236 Engulfment of PS-coated Agarose Beads



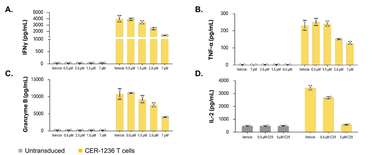
A) Fluorescence microscopic images (40x magnification) of indicated T cells cultured with pHrodo red-labeled PS beads for 16 h; arrows indicate engulfed beads in CER-1236 T cells. **B)** Engulfment at 16 h; **C)** Engulfment at 40 h. For B and C, CER T cells or untransduced T cells were incubated with beads at 10:1 cell:bead ratio for the indicated times and washed with pH 8 buffer; each bar represents the mean ± std dev for 3 CER T-cell preparations. Statistics: one-way ANOVA with Dunnett's post hoc test.
Abbreviations: CER = chimeric engagement receptor; PS = phospatidylserine; UNT = Untransduced.

The TLR2/TIR Domain is Critical for Maximal Activation of CER-1236 by Plate-bound PS

CER-1236 T cells elicit a target-dependent cytokine response to plate-bound PS but not PE, a closely related phospholipid.

The TLR2/TIR inhibitor C29 reduced cytokine production by plate-bound PS.

Figure 4 Effect of TLR2 Inhibition of CER-1236 Function in Response to Plate-bound PS



T cells were incubated in PS or PE-coated 96-well plates in the presence of various concentrations of the TLR2/TIR inhibitor C29 and cytokines were measured by Ella automated ELISA.
A) IFNγ; **B)** TNFα; **C)** Granzyme B; **D)** IL-2. Each bar represents the mean ± range for 1 donor.
Abbreviations: CER = chimeric engagement receptor; IFN = interferon; PE = phosphatidylethanolamine; PS = phospatidylserine.

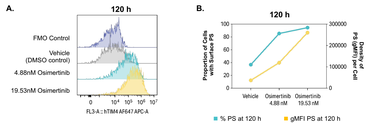
RESULTS (continued)

Surface Exposure of the Phagocytic Ligand PS on NSCLC is Induced by EGFR Inhibition and Can be Exploited Therapeutically by CER T Cells

Dose-dependent induction of surface PS observed on NSCLC cells after 120 h treatment with osimertinib

- Increased proportion of tumor cells with surface PS
- Increase in density of PS per cell, as indicated by the gMFI

Figure 5 Induction of Surface PS on H1975 NSCLC by the EGFRi Osimertinib



A) Histogram showing proportion of H1975 cells with surface PS after 120 hour treatment with 4.88 or 19.53 nM osimertinib. **B)** Increase in surface PS after treatment with Osimertininib.

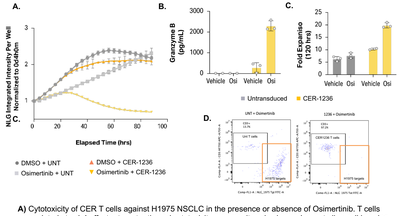
CER T Cells Demonstrate Osimertinib Induced Proliferation, Cytokine Production, and Cytotoxicity Against NSCLC Target Cells

CER-1236 in combination with subtherapeutic doses of osimertinib eliminated 97% of targets by 72 h at low effector:target ratios.

Neither CER T cells alone nor osimertinib alone eliminated target cells.

Osimertinib drives CER-1236 expansion and cytokine responses; minimal proliferation or cytokine responses were observed in the absence of drug.

Figure 6 CER-1236 T Cells Eliminate Osimertinib-treated H1975 NSCLC Cells in Vitro and Demonstrate Inducible Cytokine and Proliferation Responses



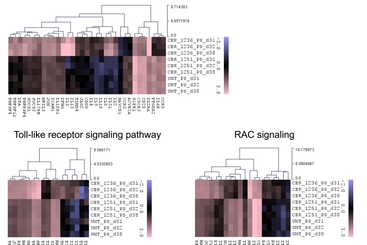
A) Cytotoxicity of CER T cells against H1975 NSCLC in the presence or absence of Osimertinib. T cells were plated at a 1:4 effector:target ratio and cytotoxicity was monitored using an Incucyte live-cell imaging system. Images were taken every 2 h. **B)** Granzyme B production at 96 h. Each bar represents mean of 3 technical replicates, ± SD. **C)** Proliferation: fold expansion of total CD3+ T cells at 120 h. **D)** Representative FACS plots showing proportion of CD3+ cells per well after 72 h of co-culture. Analysis was performed by gating on singlets followed by CD3+ cells. T-cell populations were checked using antibodies to CD4 and CD8.

RESULTS (continued)

RNA Sequencing Analysis Reveals a Distinct CER-1236 Transcriptional Program

Antigen stimulation of CER-1236 induces both an early polarizing Th1 signature and genes encoding key regulators of phagocytic clearance and co-stimulation.1706 genes were differentially expressed (FDR < 0.05, abs. fold change > 4) in CER-1236 stimulated groups compared to controls. IPA pathway analysis identified 242 pathways –log(p-value) > 1.3. Shown are heat maps from 3 independent donors from IPA gene sets for Th1 and Th2 T cell responses (top), Toll-like receptor signaling pathway (bottom, left), and RAC signaling (bottom, right).

Figure 7 IPA pathway analysis identifies pathways enriched for key regulators of phagocytic clearance and cytotoxicity



Heatmaps display both upregulated and downregulated differentially expressed genes from three independent donors. Enrichment is calculated by IPA analysis and expressed as log10 (counts).

CER T cells Exhibit TLR2-dependent, APC-like Activity in Response to Target Engagement

CER-1236, but not a CER containing a mutant TIM-4, exhibited antigen presentation and activation of HLA-matched E7 TCR T cells.

Maximal APC-like activity was dependent on activation of signaling through TLR2/TIR.

Figure 8 Activation of CER T Cell Function in Co-culture with Osimertinib-treated H1975 NSCLC cells

A) Schematic representation of in vitro assay design. **B)** Dose-dependent abrogation of CER-1236 APC-like antigen presentation by the TLR2-inhibitor C29.

CONCLUSIONS

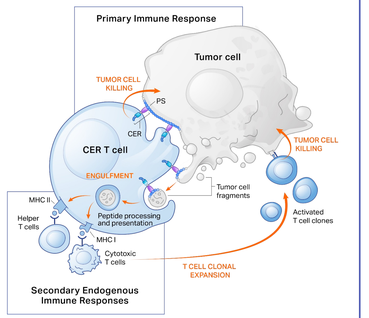
CER-1236 T cells, harboring a TLR2 signaling domain, are designed to elicit multiple functions to eliminate tumor cells via its TIM-4 phagocytic receptor.

CER-1236 demonstrates target-dependent phagocytic uptake and upregulation of key regulators of phagocytic clearance.

Drug treatment with osimertinib conditionally drives cytotoxic, cytokine, and proliferative responses to eliminate NSCLC cells in vitro.

CER T cells capture and present tumor cell antigen; optimal APC-like function requires signaling through the TLR2/TIR.

Antigen presentation, alongside inducible, target-specific cytotoxic function in single T cells, represent a potential advantage to initiate host immune responses against novel antigens for solid tumors.



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DISCLOSURES

This study was sponsored by CERo Therapeutics.

AA, AB, BC, DT, and DC are employees of CERo Therapeutics.

DC is an employee of CERo Therapeutics and reports stock or other ownership in CERo Therapeutics.

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