1   **Novel engineered chimeric engulfment receptors trigger T-cell effector functions against**
2   **SIV infected CD4+ T cells**

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5   Daniel Corey[1§*], Francoise Haeseleer[2,3•§], and Lawrence Corey[1,2,3]

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7   [1]CERo Therapeutics, San Francisco, CA, U.S.A.

8   [2]Department of Laboratory Medicine, University of Washington, Seattle, WA, U.S.A.

9   [3]Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle,
10 WA, U.S.A.

11

12 •Current affiliation: Outpace Bio, Seattle, WA, 98109 USA

13

14 § Authors made equal contribution

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16 *Correspondence should be addressed to D.C.
17 Daniel Corey
18 201 Haskins Way, Suite 230
19 South San Francisco, CA
20 94080
21 dcorey@cero.bio
22
23

24 **Short title:** (50 characters; 45) CER T cells can kill SIV-infected CD4 T cells

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ABSTRACT

Adoptive therapy with genetically engineered T cells offers potential for infectious disease treatment in immunocompromised persons. HIV/simian immunodeficiency virus (SIV) infected cells express phosphatidylserine (PS) early post-infection. We tested whether chimeric engulfment receptor (CER) T cells designed to recognize PS-expressing cells could eliminate SIV infected cells. Lentiviral CER constructs comprised of the extracellular domain of T-cell immunoglobulin and mucin domain containing 4 (TIM-4), the PS receptor, and engulfment signaling domains were transduced into primary rhesus macaque (RM) T cells. We measured PS binding and T-cell engulfment of RM CD4+ T cells infected with SIV expressing GFP. As chimeric antigen receptor (CAR) T cells induce PS and subsequent TIM-4 binding, we evaluated in vitro killing of CAR and CER T-cell combinations. We found that recombinant TIM-4 bound to SIV infected cells. In vitro, CER CD4+ T cells effectively killed SIV infected cells, which was dependent on TIM-4 binding to PS. Enhanced killing of SIV infected CD4+ T cells by CER and CAR T-cell combinations was observed. This installation of innate immune functions into T cells presents an opportunity to enhance elimination of SIV infected cells and offers potential to augment functional cure of SIV/HIV infection.

2

49 **INTRODUCTION**

50 Cell therapies are being investigated to treat a large variety of diseases. Chimeric antigen

51 receptor (CAR) T cells are cells engineered to recognize and kill clinically relevant targets. CAR

52 T-cell therapies are an example of successful cell therapy, especially to treat different types of

53 hematologic malignancies[1, 2]. New cells therapies are being investigated to broaden the number

54 of diseases where they might be applicable and explore the potential of other effector cell types

55 such as macrophages and natural killer cells and other CAR[3-6]. The application of the

56 knowledge resulting from in vivo studies and clinical trials with CAR T cells could lead to more

57 successful cell therapies targeting HIV reservoirs that remain in HIV-infected patients and in

58 controlling viral rebound[7-9].

59 A common feature displayed by cells that have become apoptotic because of viral or parasitic

60 infections, aging, or altered metabolism is the redistribution of phosphatidylserine (PS) to the

61 outer leaflet of their plasma membrane[10]. For example, HIV infection was shown to trigger the

62 exposure of PS by activating scramblases[11]. The exposed PS was also shown to facilitate

63 fusion between the viral and cell membrane[11, 12]. In addition, because HIV viruses are produced

64 by apoptotic CD4 T cells, HIV virions contain PS in their envelope that stimulates clearance

65 mechanisms and facilitate entry into other cell types such as macrophages[12, 13].

66 The exposure of PS on the surface of apoptotic cells is a key "eat me" signal triggering

67 engulfment by phagocytes[10]. Several PS receptors have been identified, including the T-cell

68 immunoglobulin and mucin domain containing 4 (TIM-4) receptor, and anti-TIM-4 antibodies can

69 block this engulfment process by macrophages[14, 15].

70 In this study, we developed novel chimeric engulfment receptors (CER) that take advantage of

71 TIM-4 binding PS [16]. These CERs are composed of the extracellular TIM-4 domain and one or

72 more intracellular signaling domains from receptors involved in innate immune responses to

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73   pathogens; e.g., Toll-like receptors (TLR) [17]. Addition of innate immune function such as

74   phagocytosis, antigen presentation, and greater lytic and non-cytolytic killing offers the potential

75   of enhancing immune responses to chronic HIV infection. These CERs expressed in T cells

76   provided the capability of eliminating simian immunodeficiency virus (SIV) infected cells in vitro.

77   This investigation provides the initial rationale for use of CER T cells in in vivo models of

78   nonhuman primate lentiviral infection to determine if the addition of functional killing and other

79   innate functions such as enhanced antigen presentation and reversal of endogenous T helper

80   responses can be improved through adoptive transfer experiments of T cells with enhanced

81   engulfment function.

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83

84   **RESULTS**

85   **TIM-4 binds to SIV infected CD4+ T cells**

86   Exposure of PS occurs when HIV infects CD4+ T cells [11]. To visualize TIM-4 binding to PS

87   exposed on the surface of SIV infected cells, we created a fluorescent SIVmac239 virus [18]. The

88   coding sequence for the enhanced green fluorescent protein (EGFP) was introduced between

89   the matrix and capsid domains of the SIVmac239 Gag protein and flanked by SIV protease

90   cleavage sites (SIVGAGGFP) (**Figure S1**).

91

92   To test if TIM-4 bound to SIV infected cells, we used a TIM-4 Fc chimera composed of the TIM-

93   4 extracellular domain fused to the N-terminus of the human IgG Fc region. When CD4+ T cells

94   were infected with SIVGAGGFP, strong binding of TIM-4 Fc to SIVGAGGFP+ cells was

95   detected 1 hour post infection while no binding was observed on SIVGAGGFP- gated cells

96   (**Figure 1A**). Binding of the labeled anti-IgG antibodies to SIVGAGGFP+ cells was not observed

97   in the absence of TIM-4 incubation, thus confirming interaction of TIM-4 to SIV infected cells

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98   (**Figure 1B**). As a positive control, TIM-4 bound to cells undergoing apoptosis triggered by

99   treatment with camptothecin (**Figure 1C**).

100   The CCR5 coreceptor is necessary for SIV infection of CD4+ T cells. CCR5 antagonist TAK-779

101   blocks the interaction between CCR5 and SIV and inhibits PS exposure induced by R5-tropic

102   virions [11, 19, 20]. Incubation of SIVGAGGFP with CD4+ T cells in the presence of TAK-779

103   decreased virion binding to target cells and also decreased TIM-4 binding to infected T cells

104   (**Figure 1D**). These results indicate that TIM-4 detects PS exposed on the surface of SIV

105   infected cells.

106   **CER T cells can kill SIV infected cells upon infection**

107   We initially constructed a series of prototype CER in order to evaluate if differences in signaling

108   domains altered functional activity in vitro. All DNA constructs used the TIM-4 extracellular

109   domain. As TLRs are known to enhance endosomal transfer and trafficking, some of the

110   constructs tested contained a TLR signaling domain (**Figure 2A**). The DNA constructs included

111   a truncated version of the epidermal growth factor receptor (EGFR), which can be detected on

112   the cell surface using an anti-EGFR monoclonal antibody, to assess the efficiency of lentiviral

113   transduction into T cells. We also introduced a membrane anchored fusion inhibitory peptide

114   derived from gp41 (C46) to protect the CD4$^+$ CER T cells against SIV infection [21, 22]. We first

115   investigated if CD4+ or CD8+ CER T cells would be efficient in eliminating SIV infected T cells.

116   Transduction of RM CD4+ and CD8+ T cells with CER21 or EGFR lentivirus led to high levels of

117   EGFR expression (**Figure 2B**). We developed a real-time fluorescence assay to evaluate CER

118   T-cell potency against freshly SIVGFP infected target cells expressing surface-exposed PS [23]. A

119   significant decrease in the number of GFP+ infected T cells were detected over time in the

120   presence of CD4+ CER T cells but not CD8+ CER T cells or EGFR T cells, indicating the

121   potency of CD4 CER T cells in killing SIV-infected cells (**Figure 2C**). To direct CER T cells to

122   major sites of SIV/HIV persistence, the cDNA encoding RM CXCR5, a homing receptor shown

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123   to promote cell trafficking to B-cell follicles in lymph nodes, was added to the lentiviral vector [24-

124   28]. About 16% of the EGFR+ transduced T cells expressed both EGFR and CXCR5 (**Figure**

125   **2D**). CD4+ CER T cells transduced with the CER21-CXCR5 lentivirus were efficient in killing

126   SIV infected targets (**Figure 2E**).

CER composed of a TLR8 signaling domain and CD3ζ activation domain is the most

128   **potent in killing SIV infected cells**

129   We designed 8 additional CERs by linking the extracellular domain of TIM-4 to various

130   intracellular signaling domains with either the TIM-4 or CD28 transmembrane domain. The

131   intracellular domain was composed of one or multiple engulfment signaling domains of TLR2,

132   TLR8, tumor necrosis factor receptor associated factor 6 (TRAF6), DAP10, DAP12, and/or

133   CD28. Some CERs also included TLR signaling domains together with the CD3ζ activation

134   domain (**Figure 3A**). High expression of the EGFR marker was observed for all 9 CER-

135   transduced RM CD4+ T cells (**Figure 3B**). When comparing the effector functions of these CER

136   T cells in the real-time fluorescence assay, we found diverse killing potency, with CER131

137   (TLR8-CD3ζ) and CER29 (TRAF6) exhibiting the greatest effector functions towards SIV

138   infected cells (**Figure 3C**).

Mutations in the PS binding site of TIM-4 impair CER T-cell effector function

140   In the immunoglobulin domain of TIM-4, a hydrophobic phenylalanine-glycine (FG) loop is

141   located in a cavity important for metal ion interaction and PS binding [29]. Mutations of 4 amino

142   acids, tryptophan, phenylalanine, asparagine and aspartic acid (WFND), present in this cavity

143   result in loss of phagocytosis of apoptotic cells [14]. In order to determine whether CER effector

144   functions are triggered upon specific recognition of PS by TIM-4, we generated CER constructs

145   with alanine mutations or deletion of all 4 WFND residues (**Figure 4A**). After assessment of the

146   transduction rate of CER T cells (**Figure 4B**), CER mutants were tested in the real-time killing

147   assay. Both mutants were impaired in their ability to eliminate SIV infected CD4+ T cells

148   compared with wild-type CER131 (**Figure 4C**).

149   **Additive killing activity of CER T cells and anti-SIV CAR T cells against SIV infected cells**

150   As cytotoxic T-cell killing has been shown to elicit surface exposed PS on target cells, we

151   evaluated potential additive effects between CER T cells and CAR T cells directed at SIV

152   infected CD4+ T cells. For these experiments, we utilized two previously constructed lentivirus

153   directed CAR T cells [30]. The first, ITS06, contains an scFv directed at the V1 region of SIV

154   envelope and the second, VRC26, contains a V2 loop-directed scFv, which cross-reacts with

155   HIV-1 in vitro and is representative of lower avidity but greater breadth. We evaluated

156   combinations of CD4+ CER T cells with CD8+ and/or CD3+ anti-SIV CAR T cells in killing

157   potency against SIV infected CD4+ RM cells. CD4+ CER T cells co-incubated with CD8+ ITS06

158   CAR T cells induced additive killing of SIV infected target cells (**Figure 5A**). A dose response

159   cytotoxic effect was observed using a E:T ratio of 5:1 for CER T cells and various E:T ratios of

160   ITS06 CAR T cells; most readily seen at a low E:T ratio of 1:20 CAR T cells. A similar

161   concentration dependent effect was seen in experiments using a combination of VRC26 CAR T

162   cells with CER T cells (**Figure 5B**). These latter experiments used a 40-fold higher

163   concentration of the VRC26 CAR T cells (E:T of 2:1) due to its reduced potency compared to

164   ITS06 CAR T cells. These data suggest additive in vitro killing between CER and CAR T cells.

165   **DISCUSSION**

166   Although CAR T-cell therapies have shown some efficacy in controlling viral replication, anti-

167   HIV/SIV CAR T cells have not reached the potential shown by CAR T cells targeting cancer

168   cells. Some success in delaying and reducing SHIV or HIV viremia has been primarily achieved

169   with CAR T cells based on the extracellular domain of the human CD4 receptor, while T cells

170   expressing CARs engineered with the scFv of broadly neutralizing antibodies were not

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171   successful likely because of anti-SIV antibodies blocking the interaction between the CAR scFv

172   and its epitope [22, 31-33]. As such, development of novel chimeric receptors targeting different

173   surface proteins and signaling through different immune pathways might lead to improved

174   elimination of HIV infected cells.

175   Here, we investigated the potential of receptors triggering engulfment signals for the clearance

176   of SIV infected cells. Phagocytes sense PS, the eat me signal, exposed at the surface of

177   apoptotic T cells such as cancer or infected cells, which leads to phagocytic engulfment [10].

178   Although multiple PS receptors have been identified, TIM-4 has been shown to be necessary for

179   apoptotic cell engulfment by macrophages [14, 15]. In this study, we demonstrated that TIM-4 can

180   bind PS exposed on the surface of SIV infected cells and is thus a good candidate for

181   engineering new receptors against SIV.

182   The TIM-4 PS binding protein in combination with other apoptotic cell clearance pathways led to

183   a range of increased killing by the CER T cells. TLRs, a family of pattern recognition receptor

184   proteins that recognize pathogen-associated molecular patterns and lead to activation of

185   immune signaling pathways, have also been used to boost the immune response against

186   cancer cells and tested as part of CAR T cells [6, 34, 35]. The TLR8 signaling pathway, including

187   MyD88 and TRAF6, results in NF-κB expression and protection against RNA viruses [36]. DAP10

188   and DAP12 are involved in kinase signaling cascades for a variety of immune response

189   receptors [37, 38]. The CD28 costimulatory domain together with the CD3ζ domain of the T-cell

190   receptor triggers T-cell activation upon antigen recognition, and has been extensively used as

191   signaling component of CAR T cells to promote cytokine production and cytolysis [39, 40]. We

192   found that out of all the innate signaling constructs we tested, the TLR8-CD3ζ combination and

193   TRAF6 CER T cells killed target cells most efficiently. PS binding by TIM-4 on the surface of

194   CER T cells was essential for killing and mutation in the PS binding pocket abolished activity.

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195   The process triggered by the CER T cells to eliminate SIV infected cells has not been

196   investigated. Prior studies suggest that killing by T cells engineered to express chimeric antigen

197   receptors for phagocytosis (CAR-Ps) appears to be related to the cell's ability to nibble plasma

198   membrane fragments of other target cells (i.e., trogocytosis) [3, 41-43]. CERs with diverse signaling

199   domains provide new functionality to CD4+ T cells, and it is possible that different processes are

200   triggered by each receptor [16]. For example, CER131 that includes a CD3ζ activation domain

201   might also trigger the activation of natural T-cell effector functions. Although no receptors similar

202   to the CERs described in this study have been investigated and tested in T cells, CAR-Ps are

203   another type of engulfment receptor composed of a specific scFv fused to intracellular signaling

204   domains that contain immunoreceptor tyrosine-based activation motifs. These CAR-Ps when

205   expressed in macrophages induce engulfment of specific targets, including cancer cells [3, 44].

206   However, for cell therapy, gene transfer into primary macrophages as well as manufacturing

207   primary macrophages for infusion would likely be more complex and expensive than for T cells.

208   While our data showing such an approach to eliminate SIV infected cells are provocative,

209   several limitations exist. Whether SIV infected T cells are inhibited and put into endosomal

210   pathways and "directed" are not yet known, although evidence for this has been seen with tumor

211   cell lines [16]. Similarly, whether elimination is achieved by the CD4+, CD8+ or both populations of

212   T cells remains to be determined. In ongoing experiments, most engulfment activity appears

213   restricted to the CD4+ T-cell populations[45]. While a high safety profile has been shown in small

214   animal models, whether off target effects will occur in NHP remains to be seen[46].

215   In conclusion, we engineered novel types of chimeric receptors that provide CD4+ T cells the

216   ability to eliminate SIV infected cells in vitro. These genetically engineered CER T cells

217   enhanced in vitro cellular damage caused by high and low affinity CAR T cells, suggesting the

218   approach could be evaluated in vivo. Lastly, in vivo studies will be required to define if the

219   potency and ability of engulfment to enhance antigen presentation and endogenous immune

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220   responses will occur in vivo with limited toxicity. To date, extensive animal model experiments in

221   mice in tumor models have shown no evidence of hematologic or systemic toxicity[45, 46].

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223   **MATERIALS AND METHODS**

224   **Enrichment of CD4+ and CD8+ RM T lymphocytes**

225   Frozen peripheral blood mononuclear cells (PBMCs) from Indian genetic background RM

226   (*Macaca mulatta*) were obtained from the Oregon National Primate Research Center in

227   accordance with standards of the Center's Institutional Animal Care and Use Committee and

228   the *National Institutes of Health Guide for the Care and Use of Laboratory Animals.*

229   Immunomagnetic negative selection (Easy Sep NHP, STEMCELL Technologies, Cambridge,

230   MA) was used to enrich in CD4+ or CD8+ T cells from PBMCs that were cultured in X-vivo 15

231   media (Lonza) supplemented with 10% FBS, 100 U/ml Pen/Strep, 1 x glutamax and 50 IU/ml

232   human recombinant IL-2 (Peprotech, Cranbury, NJ). Enriched CD4+ and CD8+ T cells were

233   activated with ImmunoCult NHP CD2/CD3/CD28 T-cell activator (STEMCELL Technologies)

234   and incubated 3 days at 37°C in humidified 5% CO_2.

235

236   **Production of SIVGAGGFP and SIVGFP and CD4 T-cell infection**

237   To generate the fluorescent SIVmac239 virus (SIVGAGGFP), we introduced cDNA encoding

238   enhanced green fluorescent protein (EGFP) together with protease cleavage sites and

239   restriction sites between the matrix and capsid domains of Gag of the full-length

240   SIV_{MAC239} proviral DNA using PCR and the NEBuilder HiFi DNA Assembly (New England

241   Biolabs, Ipswich, MA) and used the same strategy as Hubner et al to generate the HIV Gag-

242   iGFP [18]. The junction between MA and EGFP is as follows: 5'-

243   <u>CCATCTAGCGGCAGA</u>*GGAGGAAATTACCCAGTACAACAA*<u>ACGCGT</u>ATGGCTAGCAAGGGC

244   -3' where the MA coding sequence is underlined, the protease cleavage site is in italics, the *MluI*

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245   restriction site is in underlined italics and followed by the EGFP sequence. The junction between

246   EGFP and CA is as follows: 5'-

247   GACGAGCTGTACAAG*TCTAGA*GGAGGAAATTACCCAGTACAACAAATAGGTGGTAACTAT-

248   3' where the EGFP coding sequence is followed by the *XbaI* restriction site in underlined italics,

249   the protease cleavage site in italics and the underlined CA coding sequence. Virus production

250   for SIVGAGGFP and SIVGFP, a recombinant SIVmac239 virus with an IRES-EGFP cassette

251   downstream of the *nef* gene illustrated in our previous work, was performed as previously

252   described [23]. Briefly, HEK293T cells were transfected with 20 μg of the SIVGAGGFP or SIVGFP

253   plasmid using the calcium phosphate method. The fluorescent viral supernatant was collected

254   48 hours later, cleared by centrifugation, filtered, and concentrated. Stocks of SIVGAGGFP or

255   SIVGFP viruses were titrated using TZM-bl cells as described by Wei et al [47]. Infection of CD4

256   targets was performed by adding ~ 20μl of concentrated fluorescent SIV viruses to 10^5 CD4

257   cells (MOI:0.5) or control Jurkat 76 cells [48] plated on retronectin-coated 96-well plates followed

258   by spinoculation for 2 hours at 1,200 x g. The SIV-infected cells were assessed for infection

259   using flow cytometry after gating on lymphocytes and single cells.

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261   **TIM-4 binding assays**

262   Binding of TIM-4 to infected cells was tested using a TIM-4 Fc chimera composed of the

263   extracellular domain of TIM-4 fused to the N-terminus of the Fc region of human IgG (Abcam,

264   Waltham, MA). One μg of TIM-4 in PBS containing 1% BSA was incubated on ice for 30

265   minutes with 10^5 washed CD4 T cells infected as indicated above with SIVGAGGFP. After

266   washing, the cells were incubated for 15 minutes on ice with an Alexa Fluor 647-anti-IgG

267   antibody (clone M1301G05, Biolegend, San Diego, CA). Control experiments were performed

268   by skipping the incubation with TIM-4. Binding of TIM-4 to SIVGAGGFP infected cells was

269   analyzed by flow cytometry after gating on lymphocytes, single cells and GFP+ cells. When

270   indicated, the CD4 T cells were incubated with 10 μm TAK-779 (Medchemexpress, Monmouth

271   Junction, NJ) for 15 minutes before adding the SIVGAGGFP virus and infection of cells. Control

272   apoptotic cells were prepared by incubating CD4 T cells with 2 μM camptothecin (Sigma-

273   Aldrich, St. Louis, MO) overnight at 37°C.

274   **Generation of lentiviral transfer plasmids and transduction into RM T cells**

275   All CER constructs were generated by standard PCR cloning techniques and the PCR products

276   were assembled using the NEBuilder HiFi DNA Assembly (New England Biolabs). CER21 was

277   generated by linking the extracellular and transmembrane domain of human TIM-4 (GenBank[TM]

278   accession number AAH08988.1, residues 1 to 335) with the signaling domain of the human toll-

279   like receptor 8 (TLR8) (GenBank[TM] accession number AAQ88663.1, residues 849 to 1041). The

280   CER are fused to a truncated EGFR as a marker to identify transduced cells via a Thosea

281   asigna virus 2A (T2A) self-cleavage peptide. The C46 inhibitory peptide preceded by a T2A self-

282   cleavage peptide and a signal peptide and linked through an IgG2 hinge to the membrane

283   spanning domain of CD34 [49] was also added as a PCR product to the CER construct. When

284   indicated, a cDNA encoding the rhesus CXCR5 (Sino Biological, GenBank[TM] accession number

285   XP_001100017.2) preceded by a porcine teschovirus-1 2A (P2A) cleavage site was also added

286   downstream of the C46 to build the CER-CXCR5. Control EGFR and EGFR-CXCR5 were

287   generated by removing the CER from the above construct using the NEBuilder HiFi DNA

288   Assembly (New England Biolabs). CER 104 and CER 131 were generated by adding the human

289   DAP 12 signaling domain (GenBank[TM] accession number NP_001166985, residues 51 to 102)

290   or the human CD3z activation domain (GenBank[TM] accession number NP_932170.1, residues

291   52 to 164), respectively, downstream of the TLR8 domain in CER21. CER132 was constructed

292   by adding the human DAP 10 signaling domain (GenBank[TM] accession number NP_055081,

293   residues 70-93) downstream of the TLR8 and DAP12 signaling domains in CER104. The CER

294   133 is composed of the human TLR2 signaling domain (GenBank[TM] accession number

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295   AAY85648, residues 610 to 784) and CD3z activation domain. CER 129 is composed of the

296   signaling domain of the human TNF receptor-associated factor 6 (TRAF6) (GenBank[TM]

297   accession number AAH31052, residues 1 to 274). CER140 and CER137 were generated by

298   linking the extracellular domain of TIM-4 (GenBank[TM] accession number AAH08988.1 residues

299   1 to 314) to the transmembrane domain and signaling domain of human CD28 with or without

300   the CD28 hinge, respectively (GenBank[TM] accession number NP_006130.1, residues 114 to

301   220 (with hinge) or residues 153 to 220 (without hinge). These CER or control EGFR constructs

302   were cloned together with a woodchuck hepatitis virus posttranscriptional regulatory element

303   (WPRE) into a SIV-based lentiviral vector (a generous gift from Dr. Nienhuis, St June Children's

304   Research hospital, Memphis, TN and Dr. Miyazaki, Osaka University, Japan) [50, 51]. The

305   production of recombinant lentiviruses was performed as previously described [23]. Briefly, Lenti-

306   XTM 293T cells (Takara Bio) in DMEM media containing 10% FBS and 100 U/ml Pen/Strep

307   were transfected using the standard calcium phosphate method with 15 μg of the CER transfer

308   vector, 6 μg of the pCAG-SIVgprre (*gag/pol* and *rev* responsive element [RRE]), 4 μg of the

309   *rev/tat* expression plasmid pCAG4-RTR-SIV and 3 μg of the pMD2.CocalG (glycoprotein G of

310   the cocal virus) [52]. After overnight incubation, cells were washed and added fresh media. One

311   and 2 days later, lentivirus-containing media was collected, cleared by centrifugation at 1,000 x

312   g for 5 minutes followed by filtration on a 0.45 μm Millipore filter, and concentrated (50 X) by

313   ultracentrifugation at 74,000xg for 2 hours at 4°C. The lentivirus stocks were titrated by

314   transduction of Jurkat cells cultured in RPMI media supplemented with 10% FBS and 100 U/ml

315   Pen/Strep using spinoculation for 2 hours at 1,200xg. The percentage of transduced Jurkat cells

316   was quantified by flow analysis for EGFR using the anti-EGFR cetuximab mAb (Erbitux, PE-

317   conjugated at Juno Therapeutics, Seattle, WA).

318   For the transduction of CD4+ or CD8+ T cells, cells were mixed on retronectin (Takara Bio)-

319   coated plates with CER lentivirus at a MOI of ~ 20 followed by spinoculation for 2 hours at

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320   1,200xg. Cells were washed about 24 hours after transduction and expanded in fresh complete

321   X-vivo 15 media. Four days after transduction, T cells were analyzed for EGFR expression by

322   flow cytometry using PE-anti-EGFR, BV421 anti-CD4 (OKT4, Biolegend) and APC-Cyanine7

323   anti-CD8 (SK1, Biolegend). EGFR expression was analyzed using FlowJo and sequential gating

324   on lymphocytes, single cells and then CD4+ or CD8+ T cells.

325   **Real-time monitoring of cell infection to assess CER T-cell potency in eliminating SIV-**

326   **infected CD4**

327   The targets were prepared at the time of the assay. A master mix was prepared by adding

328   SIVGFP at a MOI of ~ 0.5 to 4. 10^5 CD4 T cells/ml. Fifty µl/well of the mix (20,000 CD4 T cells +

329   SIVGFP) were distributed into BioCoat poly-D-lysine coated flat bottom 96-well plate and

330   spinoculated. CER T cells or control cells were then added at the effector:target (E:T ratio) of

331   5:1 in triplicate wells. Plates were incubated at 37°C in the IncuCyte S3 LiveCell Analysis

332   System (Sartorius) and five images of each well were recorded every 3 hours and analyzed with

333   the IncuCyte image analysis software to determine the number of infected cells becoming

334   fluorescent overtime.

335

336   **ITS06 CAR T-cell preparation**

337   The design and assembly of the ITS06 CAR and VRC26 CAR was previously described [30]. The

338   ITS06 CAR was composed to the ITS06 scFv in a VH-VL orientation and a medium spacer of

339   119 amino acids linked to a CD28 transmembrane domain, a 4-1BB intracellular costimulatory

340   domain, and a CD3z activation domain and was also fused via a T2A peptide to a truncated

341   EGFR as a marker to identify transduced cells. The VRC26 CAR vector consisted of the scFv of

342   the VRC26.25 bnAb and a short spacer of 12 amino acids linked to a CD28 transmembrane

343   domain, a 4-1BB intracellular costimulatory domain, and a CD3z activation domain and fused to

344   a truncated EGFR via a T2A peptide. Both the ITS06 CAR and the VRC26 CAR were also

345   fused to a T2A-C46-peptide-P2A-CXCR5 cassette as described above for the CER vector. The

346   production of recombinant lentiviruses for the CAR and the transduction of T lymphocytes with

347   the CAR lentiviruses was performed as described above for the preparation of the CER T cells.

348   Transduction efficiency was determined by flow cytometry analysis using PE-anti-EGFR.

349

350   **Real-time assay to assess the additive effect of CER T cells and CAR T cells against SIV-**

351   **infected cells**

352   The real time assay to monitor infection of RM CD4 T cells was performed as described above

353   except that instead of CER T cells, a mix of CER T cells at a fixed ratio (E:T of 5:1) and various

354   amounts of CAR T cells as indicated in the figures were added together to the SIV-infected

355   targets.

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357   **Statistics**

358   Data of the real time assays are presented as the mean ± standard error of the mean. Statistical

359   significance was analyzed by Student's t test at time indicated in the figure legend and

360   compared to controls.

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368   licensed to Cero Therapeutics; he is employed by Cero Therapeutics. LC is on the Scientific

369   Advisory Board of Cero Therapeutics. FH declares no conflicts of interest.

370

371   **AUTHOR CONTRIBUTIONS**

372   Co-first authorship is listed alphabetically. DC conceived the idea of using TIM-4 and designed

373   the CER T cells including in vitro screens for engulfment activity for the lead constructs. FH

374   designed and performed all the in vitro experiments and wrote the first draft of the manuscript.

375   LC planned the studies and collaboration. FH and LC had unrestricted access to all data. All

376   authors agreed to submit the manuscript, read and approved the final draft and take full

377   responsibility of its content.

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613 **FIGURE LEGENDS**

614

615 **Figure 1. TIM-4 binds to SIVGAGGFP infected CD4+ T cells.** A) Flow analysis of recombinant

616 human TIM-4 binding to cells infected with SIVGAGAGFP for 15 minutes (red), 1 hour (blue), or

617 2 hours (orange). Lower plots show binding when gated on uninfected (SIVGAGGFP-) cells

618 (left) or infected (SIVGAGGFP+) cells (right). B) Same experiment as in A without addition of

619 TIM-4. Uninfected (SIVGAGGFP-) cells (left) or infected (SIVGAGGFP+) cells (right). C) TIM-4

620 binding to CD4+ T cells incubated in the presence (blue) or absence (red) of 2 μM camptothecin

621 for 24 hours. D) Overlaid flow cytometry histograms of SIVGAGGFP infected CD4+ T cells in

622 the presence (blue) or absence (red) of TAK779; uninfected CD4+ T cells are shown in orange

623 (upper panel). Lower plots show binding when gated on uninfected (SIVGAGGFP-) cells (left) or

624 infected (SIVGAGGFP+) cells (right).

625 **Figure 2. Assessment of CER T-cell potency in killing SIV infected cells.** A) Schematic

626 diagram of the CER construct with the extracellular domain of TIM-4 linked to the TLR8

627 signaling domain (CER21), CER21 with CXCR5 (CER21-CXCR5), the EGFR control vector

628 (EGFR), and EGFR with CXCR5 (EGFR-CXCR5). B) Flow cytometry analysis of CD4+ and

629 CD8+ T cells transduced with either CER21 or EGFR gated on expression of EGFR. Numbers

630 in plots indicate the percentage of EGFR+ cells after gating on lymphocytes and singlets.

631 Control untransduced CD4+ T cells are also shown. C) SIVGFP infected CD4+ T cells were

632 mixed with CER T cells (green) or control EGFR T cells (green) at t=0. The E:T ratio was 5:1 for

633 both CD4 (left) and CD8 (right) assays. Five images were taken in triplicate wells every 3 hours.

634 The error bars indicate the standard error to the mean. D) Flow cytometry analysis of CD4+ T

635 cells transduced with CER21-CXCR5 or EGFR-CXCR5 for expression of EGFR and CXCR5. E)

636 Assessment of CER21-CXCR5 T-cell potency in killing SIV infected cells as described in C.

637   **Figure 3. Comparison of effector functions of diverse CERs.** A) Schematic diagram of 8

638   CER constructs with the extracellular domain of TIM-4 linked to one or multiple intracellular

639   signaling domains (ICD) originating from TLR8, TLR2, DAP10, DAP12, TRAF6, CD28 or CD3z;

640   and negative controls without ICD (TIM-4–no ICD) or EGFR-CXCR5. B) CD4+ T cells

641   transduced with CER lentiviruses. C) Assessment of CER T-cell potency in killing SIV infected

642   cells as described. Five images were taken in triplicate wells every 3 hours. The error bars

643   indicate the standard error to the mean. *P* values <0.05 for CER29, CER131, CER140, CER132

644   and CER21 T cells compared with EGFR-CXCR5 T cells (Student's t-test). *P* values <0.05 for

645   CER29, CER131, CER140 and CER132 T cells compared with TIM-4-no ICD T cells.

646   **Figure 4. CER131 containing TIM-4 PS binding site (PSBS) mutations lose ability to kill**

647   **SIV infected CD4+ T cells.** A) Amino acid sequence of the wild-type TIM-4 FG loop including

648   four residues important for PS binding (green). Residues replaced by alanine (brown) in CER-

649   4A or deleted (blue) in CER–ΔBS. B) Flow cytometry analysis of CD4+ T cells transduced with

650   CER131 WT and CER131 mutants for EGFR expression. C) Comparison of the potency of

651   CD4+ T cells expressing CER131 WT (green), CER-4A (black) and CER–ΔBS (blue) in killing

652   SIV infected cells as described in Figure 2C. The E:T ratio was 6:1.

653   **Figure 5. Additive killing activity of CER T cells and SIV directed CAR T cells against SIV**

654   **infected CD4+ T cells.** A) Killing of SIV infected RM T cells by different ratios of CD4 CER and

655   CD8 CAR T cells: EGFR CER with EGFR CAR T cells (red), CER21 with EGFR CAR T cells

656   (green), CER21 with ITS06 CAR T cells (blue), and EGFR CER with ITS06 CAR T cells (gray)

657   at E:T ratios indicated. Five images were taken in triplicate wells every 3 hours for all time

658   points. B) Killing of SIV infected RM CD4+ T cells by the combination of CD4+ CER21 T cells

659   and VRC26 CD3+ CAR T cells.

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662 **Supplemental Material**

663 **Supplemental Figure 1. Generation of a fluorescent SIVmac239 virus.**

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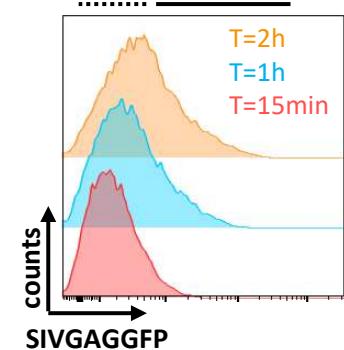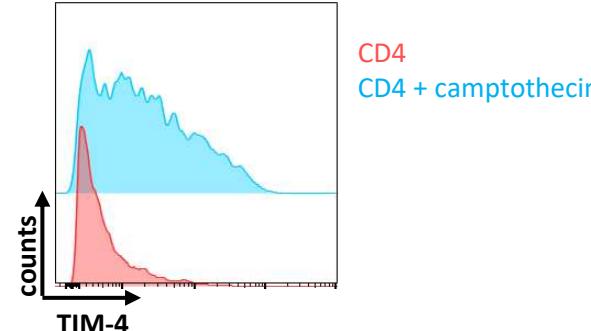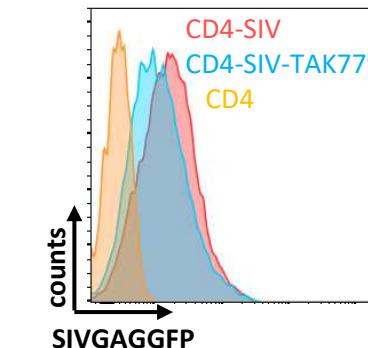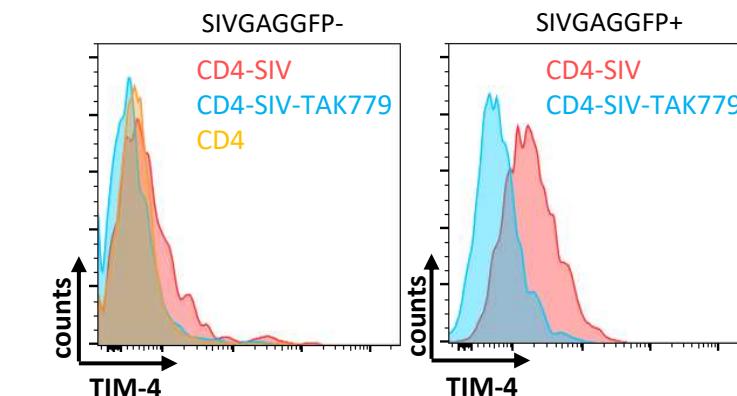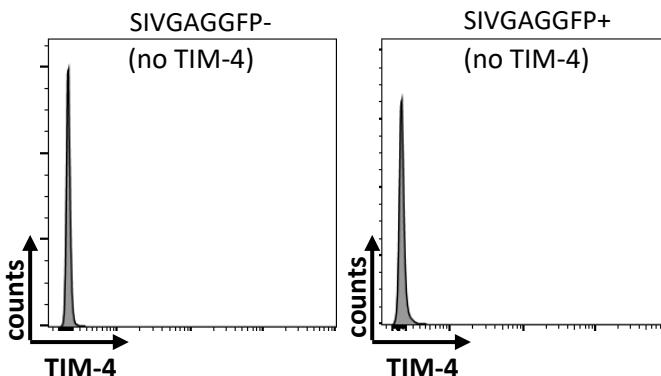
Figure 1. TIM-4 binds to SIVGAGGFP infected CD4+ T cells. A) Flow analysis of recombinant human TIM-4 binding to cells infected with SIVGAGAGFP for 15 minutes (red), 1 hour (blue), or 2 hours (orange). Lower plots show binding when gated on uninfected (SIVGAGGFP-) cells (left) or infected (SIVGAGGFP+) cells (right). B) Same experiment as in A without addition of TIM-4. Uninfected (SIVGAGGFP-) cells (left) or infected (SIVGAGGFP+) cells (right). C) TIM-4 binding to CD4+ T cells incubated in the presence (blue) or absence (red) of 2 mM camptothecin for 24 hours. D) Overlaid flow cytometry histograms of SIVGAGGFP infected CD4+ T cells in the presence (blue) or absence (red) of TAK779; uninfected CD4+ T cells are shown in orange (upper panel). Lower plots show binding when gated on uninfected (SIVGAGGFP-) cells (left) or infected (SIVGAGGFP+) cells (right).
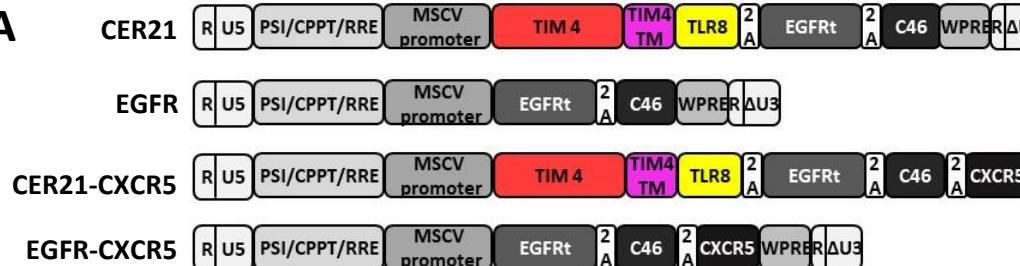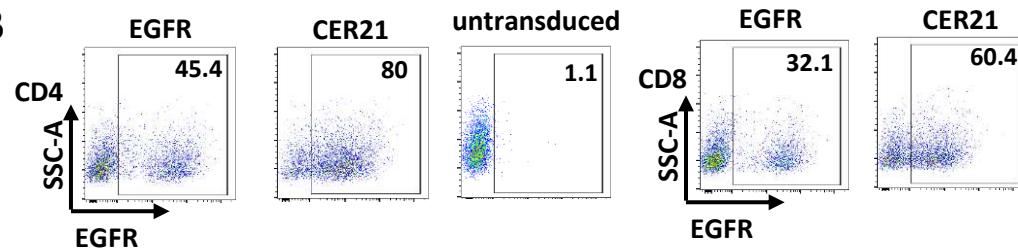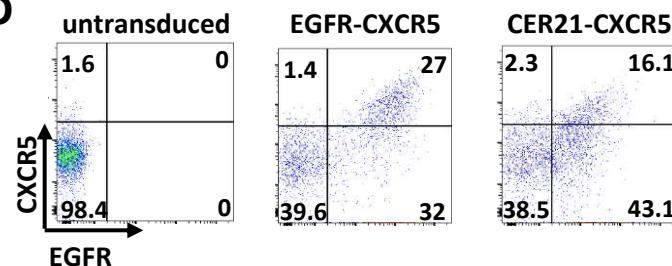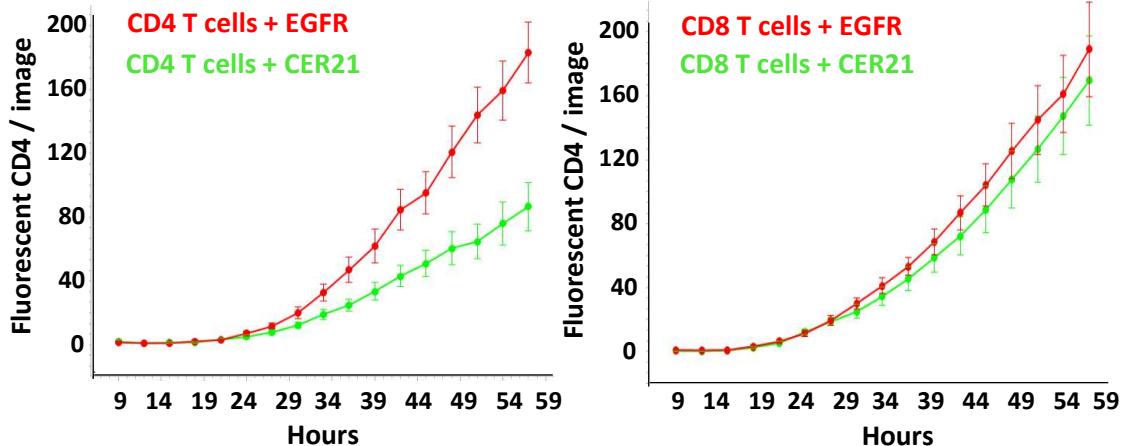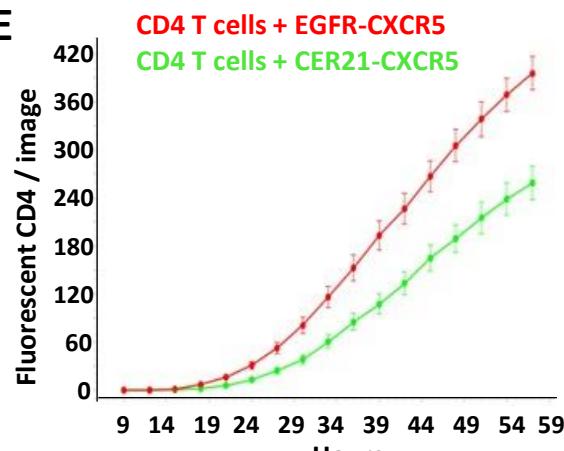
Figure 2. Assessment of CER T-cell potency in killing SIV infected cells. A) Schematic diagram of the CER construct with the extracellular domain of TIM-4 linked to the TLR8 signaling domain (CER21), CER21 with CXCR5 (CER21-CXCR5), the EGFR control vector (EGFR), and EGFR with CXCR5 (EGFR-CXCR5). B) Flow cytometry analysis of CD4+ and CD8+ T cells transduced with either CER21 or EGFR gated on expression of EGFR. Numbers in plots indicate the percentage of EGFR+ cells after gating on lymphocytes and singlets. Control untransduced CD4+ T cells are also shown. C) SIVGFP infected CD4+ T cells were mixed with CER T cells (green) or control EGFR T cells (green) at t=0. The E:T ratio was 5:1 for both CD4 (left) and CD8 (right) assays. Five images were taken in triplicate wells every 3 hours. The error bars indicate the standard error to the mean. D) Flow cytometry analysis of CD4+ T cells transduced with CER21-CXCR5 or EGFR-CXCR5 for expression of EGFR and CXCR5. E) Assessment of CER21-CXCR5 T-cell potency in killing SIV infected cells as described in C.
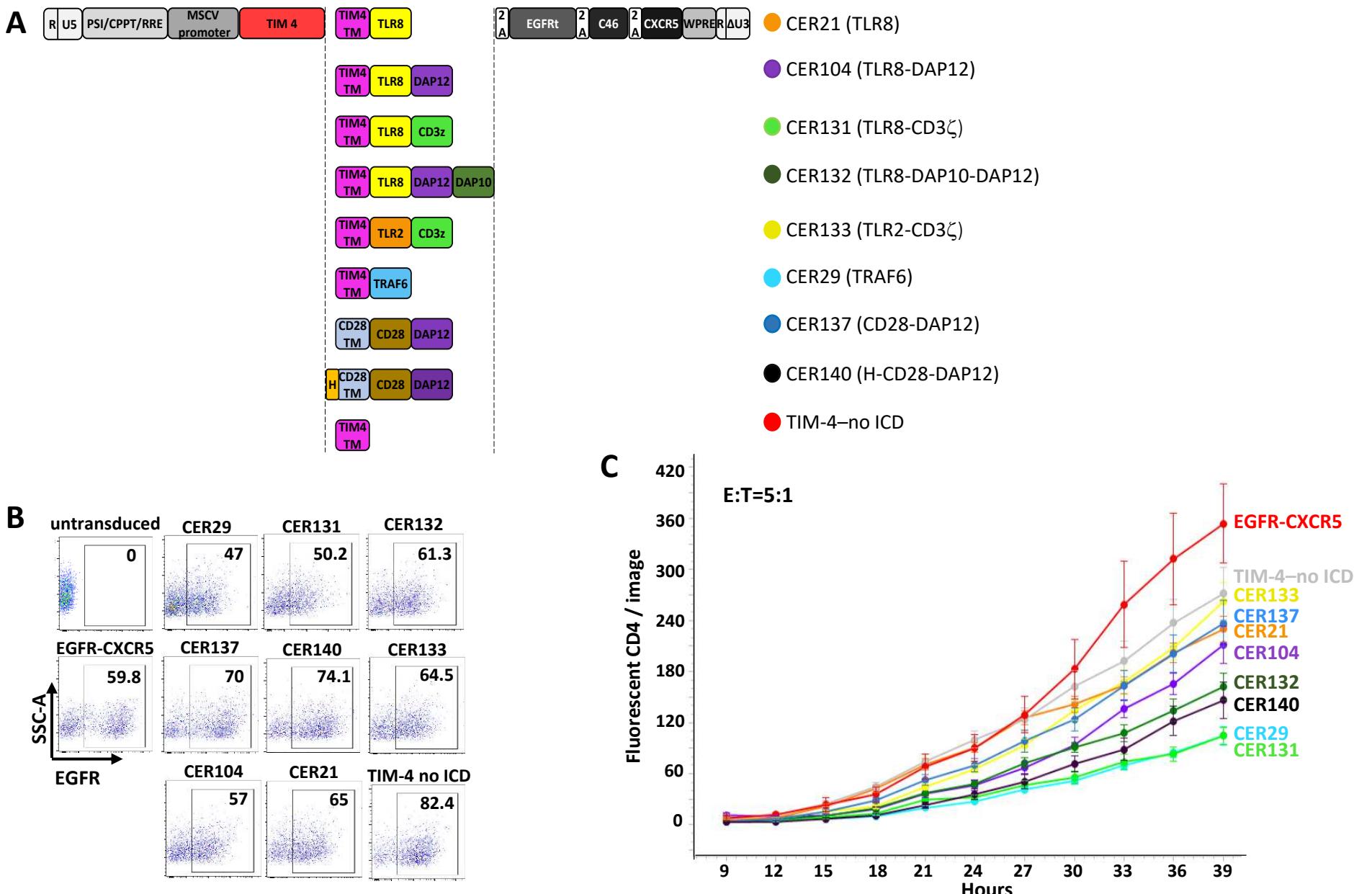
Figure 3. Comparison of effector functions of diverse CERs. A) Schematic diagram of 8 CER constructs with the extracellular domain of TIM-4 linked to one or multiple intracellular signaling domains (ICD) originating from TLR8, TLR2, DAP10, DAP12, TRAF6, CD28 or CD3z; and negative controls without ICD (TIM-4–no ICD) or EGFR-CXCR5. B) CD4+ T cells transduced with CER lentiviruses. C) Assessment of CER T-cell potency in killing SIV infected cells as described. Five images were taken in triplicate wells every 3 hours. The error bars indicate the standard error to the mean. *P* values <0.05 for CER29, CER131, CER140, CER132 and CER21 T cells compared with EGFR-CXCR5 T cells (Student's t-test). *P* values <0.05 for CER29, CER131, CER140 and CER132 T cells compared with TIM-4-no ICD T cells.

A

PSBS: phosphatidylserine-binding site

	F	G

CER131 – wild type (WT)   DSGVYCCRIEVPGWFNDVKINVRLNQRA

CER131 – mutated PSBS (CER-4A)   DSGVYCCRIEVPGAAAAVKINVRLNQRA

CER131 - deleted PSBS (CER - ΔBS)   DSGVYCCRIEVPG----VKINVRLNQRA

B

C

E:T=6:1

CER131 – ΔBS
CER131 – 4A
CER131 - WT
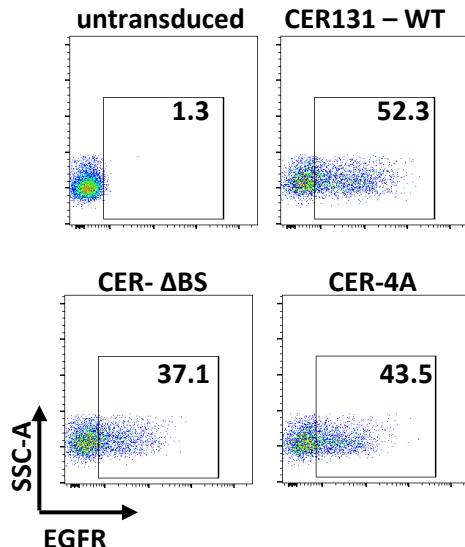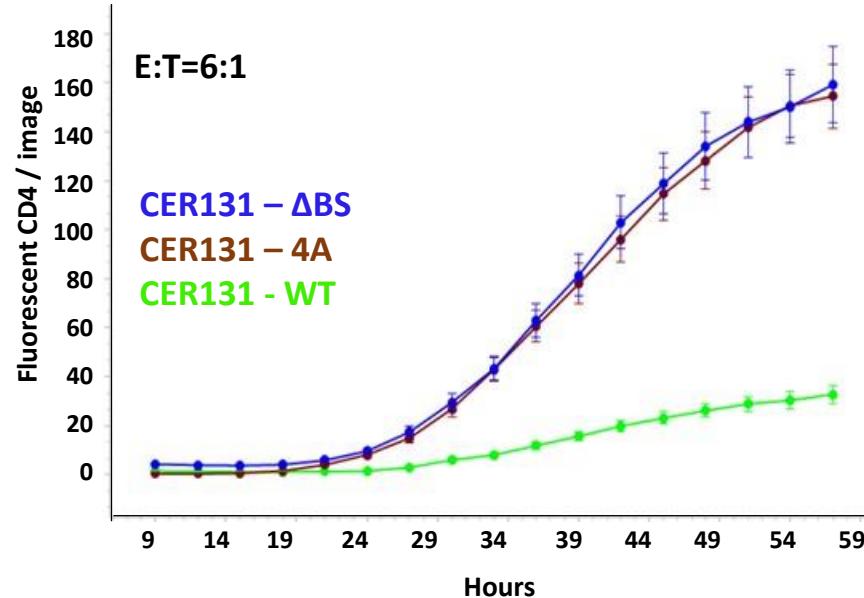
Figure 4. CER131 containing TIM-4 PS binding site (PSBS) mutations lose ability to kill SIV infected CD4+ T cells. A) Amino acid sequence of the wild-type TIM-4 FG loop including four residues important for PS binding (green). Residues replaced by alanine (brown) in CER-4A or deleted (blue) in CER–ΔBS. B) Flow cytometry analysis of CD4+ T cells transduced with CER131 WT and CER131 mutants for EGFR expression. C) Comparison of the potency of CD4+ T cells expressing CER131 WT (green), CER-4A (black) and CER–ΔBS (blue) in killing SIV infected cells as described in Figure 3C. The E:T ratio was 6:1.
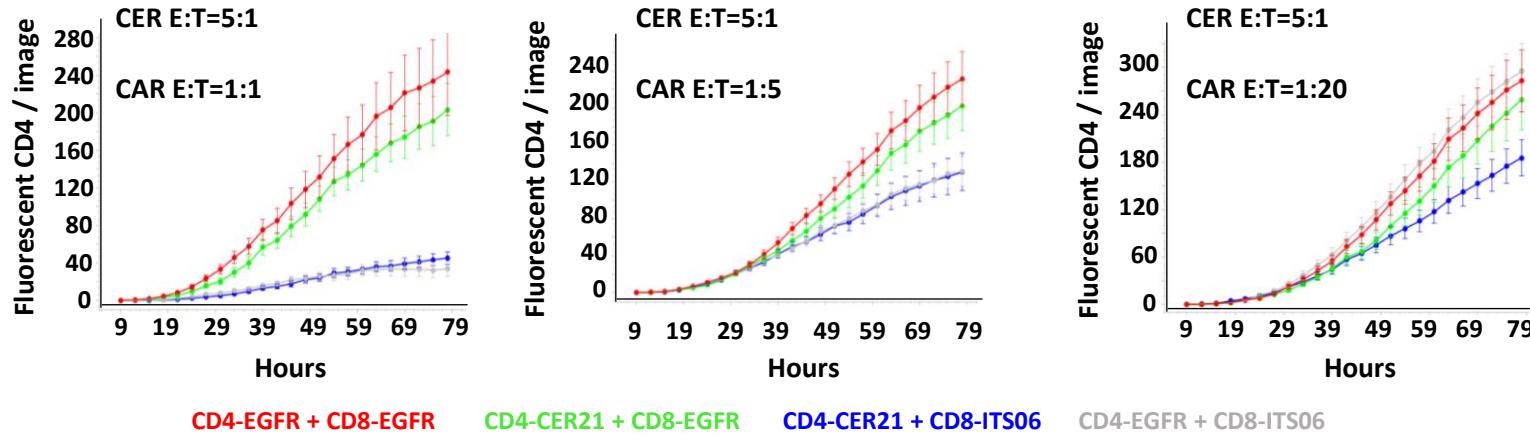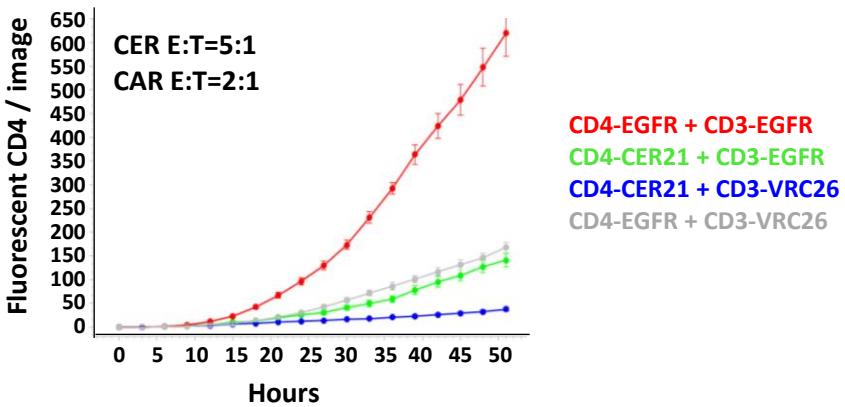
Figure 5. Additive killing activity of CER T cells and SIV directed CAR T cells against SIV infected CD4+ T cells. A) Killing of SIV infected RM T cells by different ratios of CD4 CER and CD8 CAR T cells: EGFR CER with EGFR CAR T cells (red), CER21 with EGFR CAR T cells (green), CER21 with ITS06 CAR T cells (blue), and EGFR CER with ITS06 CAR T cells (gray) at E:T ratios indicated. Five images were taken in triplicate wells every 3 hours for all time points. B) Killing of SIV infected RM CD4+ T cells by the combination of CD4+ CER21 T cells and VRC26 CD3+ CAR T cells.