Functional Tuning of CARs Reveals Signaling Threshold above Which CD8+ CTL Antitumor Potency Is Attenuated due to Cell Fas–FasL-Dependent AICD

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Abstract

Chimeric antigen receptor (CAR) development is biased toward selecting constructs that elicit the highest magnitude of T-cell functional outputs. Here, we show that components of CAR extracellular spacer and cytoplasmic signaling domain modulate, in a cooperative manner, the magnitude of CD8+ CTL activation for tumor-cell cytolysis and cytokine secretion. Unexpectedly, CAR constructs that generate the highest in vitro activity, either by extracellular spacer length tuning or by the addition of cytoplasmic signaling modules, exhibit attenuated antitumor potency in vivo, whereas CARs tuned for moderate signaling outputs mediate tumor eradication. Recursive CAR triggering renders CTLs expressing hyper-active CARs highly susceptible to activation-induced cell death (AICD) as a result of augmented FasL expression. CAR tuning using combinations of extracellular spacers and cytoplasmic signaling modules, which limit AICD of CD8+ CTLs, may be a critical parameter for achieving clinical activity against solid tumors. Cancer Immunol Res. 3(4): 368–79. ©2015 AACR.

Introduction

Approaches to cancer immunotherapy, whereby T cells are genetically modified to express chimeric antigen receptors (CAR), are the subject of considerable early-phase clinical trials (1). Whereas dramatic antitumor potency is observed in patients treated with CD19-specific CAR T cells for B-cell malignancies, such as acute lymphoblastic leukemia and non–Hodgkin lymphomas, challenges to achieve similar responses in patients harboring solid tumors are considerable (2–4). At present, the development and clinical testing of CAR-redirected T-cell adoptive therapy in cancer patients is largely empiric and constrained by a variety of technical parameters that affect feasibility of executing clinical phase I trials. Two parameters related to cell products that can be defined with greater precision are the composition of T-lymphocyte subset(s) and the tuning of CAR signaling for functional outputs that maximize their antitumor activity. Our group has studied the therapeutic activity of CAR-expressing central memory T cells, a stable antigen-experienced component of the T-cell repertoire having stem cell–like features and capacity to repopulate long-lived functional memory niches following adoptive transfer (5–9).

Moving beyond the targeting of CD19-expressing B-cell malignancies, a significant challenge for the field is the identification and vetting of cell-surface target molecules that are amenable to CAR T-cell recognition with tolerable “on” target “off” tumor reactivity (10, 11). Once identified, however, approaches to tune new CARs for signaling outputs are presently rudimentary. Parameters that are generally perceived as central to CAR development are the affinity of the target molecule CAR antigen-binding domain and the signaling modules of the cytoplasmic domain. We and others have described the significant impact of the extracellular spacer in contributing to CAR T-cell performance and the growing appreciation that CAR spacers need to adjust the biophysical synapse distance between a T cell and a tumor cell to one that is compatible for T-cell activation (12–14). Given that each new scFv and target molecule define a unique distance from the tumor cell plasma membrane, the adjustment of CAR spacers is unique to each construct and derives via empiric testing of libraries of spacer length variants.

The present study evaluates the contribution of both extracellular spacer length and cytoplasmic signaling module selection on the performance of a CAR specific for a tumor-selective epitope on CD171 (L1-CAM) that is recognized by monoclonal antibody CE7 and was previously tested as a first-generation CAR in a clinical pilot study (15–17). Using in vitro functional assays for CAR-redirected effector potency, we observed a quantitative hierarchy of effector outputs based on spacer dimension in the context of second- and third-generation cytoplasmic signaling domains. We observed a striking discordance in CAR T-cell performance in vitro versus in vivo due to fratricidal activation-induced cell death (AICD) of the most functionally potent CAR formats. These data reveal new and potentially clinically relevant parameters for...
inspection in the development of CAR T-cell immunotherapy for solid tumors.

Materials and Methods

CAR construction and lentiviral production

CD171-specific CARs were constructed using (G4S)3 peptide-linked VL and VH segments of the CE7-IgG2 monoclonal antibody (18). The scFv was codon optimized and subsequently linked to variable spacer length domains based on 12AA [short spacer (SS)/"hinge-only"], 119AA [medium spacer (MS)/"hinge-CH3"] or 229AA [long spacer (LS)/"hinge-CH2-CH3"] derived from human IgG4-Fc. All spacers were linked to the transmembrane domain of human CD28 and to signaling modules comprising either the cytoplasmic domain (i) of 4-1BB alone (2G CAR) or (ii) of CD28 (mutant) and 4-1BB (3G CAR), with each signaling module fused to the human CD3-ζ endodomain (19). The cDNA clones encoding CAR variants were linked to a downstream T2A ribosomal skip element and truncated EGF receptor (EGFRt), cloned into the ephIV7 lentiviral vector, and CD171-CAR lentiviruses were produced in 293T cells (20).

Real-time PCR

Total RNA was extracted from T cells using the RNeasy Mini Kit (Qiagen). cDNA was synthesized by reverse transcription using the First Strand Kit (Life Technologies). RNA quantification was performed using Fast primers (IDT) and the CFX96 real-time detection system (Bio-Rad). Housekeeping gene actin was used as a control. Data were analyzed using CFX Manager Software version 3.0.

Protein expression

Western blot analysis. T cells were harvested, washed in PBS, and lysed in protease inhibitor (Millipore). Proteins were analyzed using SDS–PAGE followed by Western blotting using anti-CD247 (CD3-ζ, BD Biosciences). Signals were detected using an Odyssey Infrared Imager, and band intensities were quantified using Odyssey v2.0 software (LI-COR).

Flow cytometry. Immunophenotyping was conducted using fluorophore-conjugated mAbs: CD4, CD8, CD27, CD28, CD45RA, CD45RO, CD62L, CCR7 (Biolegend). Cell-surface expression of L1-CAM was analyzed using a fluorophore-conjugated mAb (Clone 014; Sino Biological). EGFRt expression was analyzed using biotinylated cetuximab (Bristol-Myers Squibb) and a fluorophore-conjugated streptavidin secondary reagent. To assess activation and AICD fluorophore-conjugated mAbs for CD25, CD69, CD137, CD178 (Fas Ligand) and CD95 (Fas, all Biolegend) were used. Caspase-3 activity was measured using CaspGlow (eBioscience) following the manufacturer’s protocol. Flow analyses were performed on an LSRFortessa (BD Biosciences), and data were analyzed using FlowJo software (TreeStar).

Generation of T-cell lines expressing CD171-CARs

Samples of heparinized blood were obtained from healthy donors after written informed consent following a research protocol approved by the Institutional Review Board of Seattle Children’s Research Institute (SCRI IRB #13795). Peripheral blood mononuclear cells (PBMC) were isolated using ficoll (GE Healthcare), and CD8+CD45RO+CD62L- central memory T cells (TCM) were isolated using immunomagnetic microbeads (Miltenyi). First, CD8+CD45RO+ cells were obtained by negative selection using a CD8 T-cell isolation kit and CD45RA beads, then enriched for CD62L, activated with anti-CD3/CD28 beads at a 5:1 T cell ratio (Life Technologies) and transduced on day 3 by centrifugation at 800 × g for 30 minutes at 32°C with lentiviral supernatant (multiplicity of infection = 5) supplemented with 1 mg/mL prolateamine sulfate (APP Pharmaceuticals). T cells were expanded in RPMI (Cellgro) containing 10% heat-inactivated FCS (Atlas), 2 mmol/L L-glutamine (Cellgro), supplemented with a final concentration of 50 U/mL recombinant human IL2 (Chiron Corporation), and 1 ng/mL IL15 (Miltenyi). The EGFRt subset of each T-cell line was enriched by immunomagnetic selection with biotin-conjugated Erbitux (Bristol-Myers Squibb) and streptavidin microbeads (Miltenyi; 21). CD171-CAR and mock control T cells were expanded using a rapid expansion protocol (9). T cells used for in vitro assays were derived by stimulation with CD3/CD28 beads (S1) followed by 2 rapid expansions (S2) and cryopreserved 14 days (D14) after the second rapid expansion stimulation.

Cell lines

The neuroblastoma cell lines Be2 and SK-N-DZ were obtained from the ATCC. Be2-GFP-fluc EphIV7 and SK-N-DZ-GFP-fluc EphIV7 were derived by lentiviral transduction with the firefly luciferase (fluc) gene and purified by sorting on GFP. Both cell lines were further transduced with CD19t-2A-IL2_pHIV7 to generate IL2-secreting cell lines purified by sorting on CD19t. All neuroblastoma cell lines were cultured in DMEM (Cellgro) supplemented with 10% heat-inactivated FCS and 2 mmol/L of L-glutamine. EBV-transformed lymphoblastoid cell lines (TMCL) and TMCLs that expressed membrane-tethered CD3ζpilson-specific scFvFc derived from OKT3 mAb (TMCL-OK3; ref. 6) were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS and 2 mmol/L of L-glutamine.

CAR T-cell receptor signaling

After coculturing 1 × 10^6 effector and target cells for 4 to 8 minutes, cells were processed to measure Erk/MAP kinase 1/2 activity according to the 7-Plex T-cell Receptor Signaling Kit (Millipore). Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific).

In vitro T-cell assays

Cytotoxicity measured by chromium release assay (CRA). Target cells were labeled with ^51^Cr (Perkin Elmer), washed, and incubated in triplicate at 5 × 10^4 cells/well with T cells (S,R,D12,14) at various effector-to-target (ET) ratios. Supernatants were harvested after a 4-hour incubation for γ-counting using Top Count NXT (Perkin Elmer), and specific lysis was calculated (16).

Cytotoxicity measured by the biophotonic luciferase assay. Neuroblastoma cell lines containing GFP-fluc EphIV7 were cocultured with effector cells at a 5:1 ET ratio. The effector cells were on their first, second, or third round of tumor cell encounter as described above. To assess the amount of viable tumor cells left after T-cell encounter, α-luciferin was added, and after 5 minutes the biophotonic signal from the neuroblastoma cells was measured using an IVIS Spectrum Imaging System (Perkin Elmer).
Cytokine release. A total of 5 × 10^3 T cells (S1R2D12-14) were plated with stimulator cells at a 2:1 E:T ratio for 24 hours. IFNγ, TNFα, and IL2 in the supernatant were measured using Bioplex cytokine assay and Bioplex-200 system (Bio-Rad).

Stress test. To mimic recursive antigen encounters, we started a coculture of adherent target cells and freshly thawed nonadherent effector cells at a 1:1 E:T ratio. After 24 (round I) and 48 (round II) hours, T-cell viability was assessed using the Guava ViaCount Assay (Millipore), and nonadherent effector cells were moved to a new set of adherent target cells at a 1:1 E:T ratio. After rounds I, II, and III (72 hours), T cells were harvested and treated with a dead cell removal kit (Miltenyi) before further analysis.

Immunohistochemistry

Tumor samples were obtained from patients diagnosed with neuroblastoma and treated in the Department of Pediatric Hematology-Oncology of Seattle Children’s Hospital (SCRI IRB #13740).

Human neuroblastomas and mouse brains were harvested, fixed, processed, paraffin embedded, and cut into 5-μm sections. Antigen retrieval was performed using Diva decloaker RTU (Biocare Medical). Primary antibodies were incubated with sections overnight at 4°C and diluted in blocking buffer as follows: rat monoclonal anti-human CD3 (Clone CD3D-12; Bio-Rad) 1:100, mouse monoclonal anti-human Ki67 (Clone MIB-1; Dako) 1:200, rabbit polyclonal anti-human cleaved caspase-3 (Biocare Medical) 1:100, rabbit polyclonal anti-human Granzyme B (Covance) 1:200, and mouse monoclonal anti-human CD171 (clone 14.10; Covance) 1:200, and mouse monoclonal caspase-3 (Biocare Medical) 1:100, rabbit polyclonal anti-human Granzyme B (Covance) 1:200, and mouse monoclonal anti-human CD171 (clone 14.10; Covance) 1:200. Secondary antibodies (Life Technologies) were incubated with sections for anti-human CD171 (clone 14.10; Covance) 1:200, and mouse monoclonal caspase-3 (Biocare Medical) 1:100, rabbit polyclonal anti-human Granzyme B (Covance) 1:200, and mouse monoclonal anti-human CD171 (clone 14.10; Covance) 1:200. Secondary antibodies (Life Technologies) were incubated with sections for anti-human CD171 (clone 14.10; Covance) 1:200, and mouse monoclonal anti-human CD171 (clone 14.10; Covance) 1:200. Secondary antibodies (Life Technologies) were incubated with sections for anti-human CD171 (clone 14.10; Covance) 1:200, and mouse monoclonal anti-human CD171 (clone 14.10; Covance) 1:200. Secondary antibodies (Life Technologies) were incubated with sections for anti-human CD171 (clone 14.10; Covance) 1:200, and mouse monoclonal anti-human CD171 (clone 14.10; Covance) 1:200.

Slides were imaged on an Eclipse Ci upright epifluorescence microscope (Nikon) equipped with a Nuance multispectral imaging system and analyzed with InForm analysis software (Perkin Elmer).

Experiments in NOD/SCID/γc−/− mice

NSG mouse tumor models were conducted under SCRI IACUC-approved protocols.

Intracranial NSG mouse neuroblastoma xenograft model. Adult male NOD.Cg-Pkrdc<tm12Kgrmi/v>/SzJ [NODsci gamma (NSG)] mice were obtained from the Jackson Laboratory or bred in house. Mice were injected intracranially (i.c.) on day 0 with 2 × 10^6 IL-2-secreting, fluc-expressing Be2 or SK-N-DZ tumor cells 2 mm lateral, 0.5 mm anterior to the bregma, and 2.5 mm deep from the dura. Mice received a subsequent intratumoral injection of 2 × 10^6 CAR-modified CD8<sup>+</sup> T<sub>(ECM)</sub> either 7 (therapy response model) or 14 (stress test model) days later. In the stress test model, mice were euthanized 3 days after T-cell injection, and brains were harvested for immunohistochemistry (IHC) analysis.

For bioluminescent imaging of tumor growth, mice received intraperitoneal (i.p.) injections of β-luciferin (4.29 mg/mouse). Mice were anesthetized with isoflurane and imaged using an IVIS Spectrum Imaging System 15 minutes after β-luciferin injection. Luciferase activity was analyzed using Living Image Software Version 4.3 and the photon flux analyzed within regions of interest (all Perkin Elmer).

Statistical analyses

Statistical analyses were conducted using Prism software (GraphPad). Data are presented as means ± SD or SEM, as stated in the figure legends. The Student t test was conducted as a two-sided unpaired test with a confidence interval of 95%. Statistical analyses of survival were conducted by the log-rank test. Results with a P value of less than 0.05 were considered statistically significant.

Results

Magnitude of CAR-triggered cytolytic and cytokine functional outputs can be incrementally modulated based on CAR extracellular spacer size

The biophysical synapse between a CAR-expressing T cell and a tumor cell is influenced by the epitope location on the tumor cell-surface target molecule relative to the distance from the tumor cell’s plasma membrane. We hypothesized that CAR extracellular spacer size tuning to accommodate a functional signaling synapse is a key attribute to engineering bioactive CARs. To assess the impact of CD171-specific CAR extracellular spacer size, we assembled a set of spacers using modular domains of human IgG4 as follows: "long spacer" (LS) IgG4 hinge-CH2-CH3, "medium spacer" (MS) IgG4 hinge-CH3 fusion, and "short spacer" (SS) IgG4 hinge. Each spacer variant was fused to a CD28-transmembrane domain followed by a second-generation (2G) 4-1BB:zeta-endomodomain that was linked to the cell-surface EGFRt tag (Fig. 1A; ref. 21). We generated sets of spacer variant 2G-CAR<sup>-</sup>/EGFR<sup>+</sup> human CD8<sup>+</sup> central memory–derived effector T-cell lines (T<sub>ECM</sub>) from purified CD8<sup>+</sup>CD45RO<sup>+</sup>CD62L<sup>+</sup> central memory precursors by immunomagnetic selection (Supplementary Fig. S1A and S1B). Following expansion, lentivirally transduced T<sub>ECM</sub> cells were further enriched for homogeneous levels of EGFRt expression by cetzumab immunomagnetic positive selection (21). We confirmed the similar surface expression levels of each of the CAR spacer variants by anti-murine F(ab’)-specific and EGFR-specific flow-cytometric staining and protein expression quantified by Western blot for CD3ζ of each T-cell line (Fig. 1B and C).

We first sought to determine whether the magnitude of 2G-CAR-triggered in vitro activation of CD8<sup>+</sup> T<sub>(ECM)</sub> is influenced by spacer domain size. The human neuroblastoma cell lines Be2 and SK-N-DZ were selected based on L1-CAM expression levels that are similar to those of clinical tumor specimens (Supplementary Fig. S2). Following activation by CD171<sup>+</sup> human neuroblastoma cells, CD171-specific 2G-CAR<sup>-</sup>/LS-CD8<sup>+</sup> T<sub>(ECM)</sub> exhibited 3.1-fold higher levels of phospho-ERK (P = 0.003), and 5.7-fold higher percentage of cells expressing the activation marker CD137 (P = 0.003), as compared with their CD171-CAR<sup>-</sup>/SS counterparts (Fig. 1D and E). 2G-CAR<sup>-</sup>/MS exhibited intermediate levels of phospho-ERK and CD137 induction as compared with LS and SS 2G-CARS. Next, we sought to determine whether spacer size also modulated the magnitude of antitumor cytolytic activity in an L1-MS-S<sup>-</sup> pattern. Using a 4-hour CRA, we observed that lysis of CD171<sup>+</sup> neuroblastoma target cells followed the same potency gradient of LS>MS>SS against both CD171<sup>+</sup> Be2 and CD171<sup>+</sup> Be2 and CD171<sup>+</sup> SK-N-DZ cell lines (Fig. 1F and Supplementary Fig. S3A). Furthermore, activation for cytokine secretion followed the same incremental output hierarchy such that 2G-CAR<sup>-</sup>/LS produced 8.4-fold higher amount of IFNγ (P = 0.003), 6.3-fold more IL2 (P < 0.0001) and 6.1-fold higher levels of TNFα (P = 0.0001).
Figure 1. CAR extracellular spacer tunes antitumor effector outputs of CD8\(^+\) CTLs. A, schematic of CD171-specific 2G-CAR extracellular domain spacer variants. B, human CD8\(^+\) T\(_{E(CM)}\) cell-surface expression of 2G SS, MS, or LS spacer variants and EGFR\(_t\) detected with antimurine F(ab) and cetuximab. C, expression levels of 2G-CAR detected by CD3-\(\zeta\)-specific Western blot. D, 2G-CAR-induced levels of phospho-ERK upon coculture with CD171\(^+\) Be2 neuroblastoma cells at a 1:1 E:T ratio (\(n \geq 3\) per condition). E, 2G-CAR activation-induced CD137 surface expression upon tumor coculture as in D. F, 2G-CAR-induced levels of IFN\(\gamma\), IL2, and TNF\(\alpha\) upon coculture with CD171\(^+\) Be2 neuroblastoma cells at a 1:1 E:T ratio (\(n \geq 3\) per condition). Fold cytokine production comparison is relative to SS 2G-CAR as in F. * \(P < 0.05\).
Inverse correlation of spacer-modulated CAR-redirected CTL functional activity in vitro with in vivo antitumor potency

To delineate the relationship of the observed potency of CAR signaling based on in vitro assays to therapeutic activity in vivo, we performed adoptive transfer experiments in NSG mice with established human neuroblastoma xenografts stereotactically implanted in the cerebral hemisphere (Fig. 2A). Surprisingly, Be2 tumor-engrafted mice treated with intratumoral injection of 2G-CAR(LS) exhibited no therapeutic activity, necessitating animal euthanasia approximately 3 weeks after tumor inoculation (Fig. 2B and C). In comparison, biophotonic tumor animal euthanasia approximately 3 weeks after tumor inoculation (Fig. 2B and C). In contrast with the LS>MS>SS hierarchy of tumor activity, in vivo antitumor potency may not always be achieved by spacer tuning to achieve optimal antitumor functional outputs but can modulate downstream signaling events that result in not only differential magnitudes of antitumor functional outputs but coordinated increases in susceptibility to AICD. The balance between these two processes for optimal in vivo antitumor activity may not always be achieved by spacer tuning to achieve the highest levels of CAR-signaling outputs, as exemplified by our comparison of 2G-CAR(LS) and 2G-CAR(SS) structural variants.

Augmentation of CAR cytoplasmic endodomain composition reverts short spacer CD171-CAR to AICD-prone variant upon recursive tumor encounter

Third-generation CARs contain two costimulatory endodomain modules in series with the CD3-ζ activation module and have been reported to augment the magnitude of cytokine and cytokine production levels over their second-generation counterparts (23). We assembled a CD171-specific 3G-CAR through the addition of a CD28 endodomain to the 2G 4-1BB:7zeta-endodomain (Fig. 4A). CD8+ T_E(CM) expressing comparable levels of 2G-CAR(SS) and 3G-CAR(SS) were derived from purified T_CM precursors by immunomagnetic selection (Fig. 4B and C). 3G-CAR(SS)-CD8+ T_E(CM) demonstrated an 8.4-fold higher induction of CD137 expression upon tumor contact than their second-generation counterparts (P <
A 1.3-fold increase in cytolytic activity against Be2 targets (E:T of 1:10; $P = 0.0001$; Fig. 4E) and 5.1-fold more IL2 and 2.5-fold more TNFα secretion ($P < 0.0001$ and $P = 0.003$; Fig. 4F).

Next, we assessed whether the 3G endodomain, in the context of an extracellular short spacer, could selectively enhance antitumor activity in vivo without exacerbation of AICD. Surprisingly, the in vivo antitumor activity of 3G-CAR(SS)-CD8+ TECM against Be2 targets (E:T of 1:10; $P = 0.0001$; Fig. 4D), a 1.3-fold increase in cytolytic activity against Be2 targets (E:T of 1:10; $P = 0.0001$; Fig. 4E) and 5.1-fold more IL2 and 2.5-fold more TNFα secretion ($P < 0.0001$ and $P = 0.003$; Fig. 4F).
both Be2 (Fig. 5A) and SK-N-DZ (Fig. 5B) was inferior, though not to a statistically significant degree to their 2G-CAR(SS) counterparts. These findings were not attributable to differences in short-term persistence of CAR T cells within tumors based on similar densities of human CD3$^+$ T cells detected 3 days after adoptive transfer (Fig. 5C). Despite the finding of higher frequencies of Granzyme B$^+$ 3G-CAR(SS) T cells compared with 2G-CAR(SS) intratumoral T cells, we again observed augmented numbers of third-generation T cells with activated caspase-3, suggesting that the augmented costimulation through a combined effect of CD28 and 4-1BB was capable of hyperstimulation resulting in heightened AICD, despite the context of a short spacer extracellular domain (Fig. 5D). This was confirmed by comparing their performance using the in vitro stress test assay. Following each round of tumor stimulation, we observed higher frequencies of CD25$^+$ CD69$^+$ T cells in the 3G-CAR(SS)-T-cell population (Fig. 6A) accompanied by increased frequencies of dead T cells through successive rounds of activation (Fig. 6B). Augmented AICD was again associated with heightened levels of FasL expression by surface staining and mRNA content, which in turn coincided with increased levels of activated caspase-3 (Fig. 6C–E). These data demonstrate that overtuning of CAR-signaling outputs based on intracellular signaling domain composition negatively impacted on a tuned short spacer dimension in a combinatorial manner by enhancing FasL-mediated T-cell AICD.

Discussion

CARs are capable of mediating multiplexed signaling outputs that trigger redirected antitumor T-cell effector function (24–26). It stands to reason that the tuning of CARs for effective T-cell antitumor activity will be more stringent in solid tumor
applications, and that empiric designs of CARs based on limited understanding of the impact of their composition on in vivo antitumor function will only hamper progress in human clinical applications. Here, we systematically interrogate CAR structure function in human central memory – derived CD8⁺ effector CTLs focusing on the combinatorial effects of extracellular spacer dimension in the context of cytoplasmic signaling module composition. By surveying CAR-signaling strength using in vitro assays, we have identified a potency hierarchy of CAR structural variants. These analyses have revealed a range of CAR-signaling outputs permissive for in vivo antitumor activity above which in vivo potency is attenuated by heightened AICD.
The evolution of CAR design has proceeded to date via a largely empiric process, and has focused predominantly on the augmentation of signaling outputs through combinatorial modules of costimulatory receptor cytoplasmic domains fused in series to immunoreceptor tyrosine-based activation motif (ITAM)-containing activation domains (27–29). Comparisons of the function of CTLs expressing first-, second-, or third-generation CARs have typically been made in the context of a “stock” extracellular spacer domain preferred by a particular laboratory, ranging from full-length IgGs to relatively short CD8α hinges or membrane-proximal portions of CD28 (30–32). Our group and others have studied the impact of spacer dimension on CAR signaling and functional activity (14, 19). Unlike a T-cell receptor contact with peptide-loaded HLA class I or II, which defines a scripted biophysical gap between T-cell plasma membrane and target-cell plasma membrane that is permissive for assembly of a supramolecular activation complex, CARs do not conform to this dimensional relationship as a consequence of the target molecule’s structural dimensions, the scFv’s epitope location on the target molecule, and the CAR’s spacer size (33). While the first two dimensions are unique to each selected antigen and antibody-binding domain, the CAR spacer is size tunable and can compensate to some extent in normalizing the orthogonal synapse distance between CAR T cell and target cell. This topography of the immunological synapse between a T cell and a target cell also defines a distance that cannot be functionally bridged by a CAR due to a membrane-distal epitope on a cell-surface target molecule that, even with a short spacer CAR, cannot bring the synapse distance in to an approximation for signaling (13). Likewise, membrane-proximal CAR target antigen epitopes have been described for which signaling outputs are only observed in the context of a long spacer CAR (34).

Using a CD171-specific scFv-binding domain derived from the CE7 mAb, we first assessed the impact of extracellular spacer size on signaling outputs from a 4-1BB:ζ second-generation CAR. We observed incremental gains of function in signaling outputs based on in vitro assays as spacer size increased from the short IgG4 hinge spacer, to an intermediate hinge:CH3, to the full-length IgG4-hinge:Fc spacer. Because prior studies have revealed reduced survival of LS CAR T cells due to interaction between FcγRIIa cells in the lung and the Fc portion of the CAR after intravenous injection (14, 35), we used for in vivo testing a direct intratumoral route of CD8+ CTL administration to study the direct effect of spacer length on CAR T cells within a solid tumor that provides IL2 locally, as a surrogate for infusional IL2 and/or codelivery of CD4+ Th1 T cells. Unexpectedly, the antitumor potency of intratumorally injected CAR-CD8+ CTLs due to interaction between FcγRIIa cells in the lung and the Fc portion of the CAR after intravenous injection (14, 35), we used for in vivo testing a direct intratumoral route of CD8+ CTL administration to study the direct effect of spacer length on CAR T cells within a solid tumor that provides IL2 locally, as a surrogate for infusional IL2 and/or codelivery of CD4+ Th1 T cells. Unexpectedly, the antitumor potency of intratumorally injected CAR-CD8+ CTLs was inversely correlated to spacer size (i.e., SS > MS > LS) and in vitro functional potency. Given these findings, we hypothesized that commonly employed in vitro assays that assess CAR-T-cell function upon a single limited-duration tumor cell encounter fail to detect the subsequent fate of CART cells upon recursive tumor exposure, as would be predicted to occur within solid tumors in vivo. To better assess this possibility, we devised an in vitro assay in which CART cells are recursively exposed to equal numbers of biophotonic reporter gene-expressing tumor cells. Tumor-cell killing can thereby be quantified biophotonically and retrieved CART cells can be interrogated for activation status, viability, and caspase activity.
after each round of tumor coculture. We observed, upon three recursive tumor encounters, disproportionate increases in the frequency of T cells undergoing apoptosis among 2G-CAR(LS) T cells as compared with 2G-CAR(SS)-T-cells. The exaggerated AICD correlated with heightened LS CAR-induced expression of FasL and activated caspase-3 relative to SS CAR. AICD in LS CAR T cells was reduced by siRNA knockdown of FAS or FasL before exposure to tumor cells. These in vitro findings reveal a T cell-intrinsic Fas–FasL-dependent mechanism of AICD that correlates with limited intratumoral persistence of LS CAR T cells. In aggregate, these data demonstrate that the nonsignaling extracellular spacer is a major tunable CAR design element that affects not only signaling activity but persistence of CAR T cells in solid tumors in a recursive tumor encounters, disproportionate increases in the frequency of T cells undergoing apoptosis among 2G-CAR(LS) T cells as compared with 2G-CAR(SS)-T-cells. The exaggerated AICD correlated with heightened LS CAR-induced expression of FasL and activated caspase-3 relative to SS CAR. AICD in LS CAR T cells was reduced by siRNA knockdown of FAS or FasL before exposure to tumor cells. These in vitro findings reveal a T cell-intrinsic Fas–FasL-dependent mechanism of AICD that correlates with limited intratumoral persistence of LS CAR T cells. In aggregate, these data demonstrate that the nonsignaling extracellular spacer is a major tunable CAR design element that affects not only signaling activity but persistence of CAR T cells in solid tumors in a recursive manner, independent of interactions with Fcε cells of the reticulo-endothelial system (14).

Given the relation between spacer dimension and in vitro survival in the context of a 4-1BB:zeta CAR, we sought to understand whether the short spacer dimension would be generically optimal in the context of the augmented signaling outputs of a third-generation CD28-4-1BB:zeta CAR endodomain format. Consistent with observations made by multiple other groups, the CD171-specific 3G-CAR(SS) stimulated heightened levels of cytolytic activity and cytokine synthesis compared with the 2G-CAR(SS) upon in vitro tumor stimulation. However, the augmented signaling outputs of the 3G-CAR in the context of its short spacer also increased FasL expression, exacerbated apoptosis as indicated by increased levels of activated caspase-3 and resulted in higher frequencies of cell death. Correspondingly, we observed impaired in vivo antitumor efficacy of the 3G-CAR(SS) T cells, as compared with the 2G-CAR(SS) due to attenuated in vivo intratumoral survival. While CD28 costimulates T cells upon initial antigen activation and enhances T-cell viability by deflecting AICD through Nuclear Factor of Activated T Cells (NFAT)-regulated increases in cFLIPShort, published studies have also revealed that recursive CD28 costimulation of previously activated T cells can reduce their subsequent survival via augmented FasL expression and, consequently, increased AICD (36). It is interesting, therefore, to speculate whether recursive CD28 signaling mediated by anti-CD19 4-1BB:zeta CAR-T-cells is responsible for the relatively short persistence duration in treated ALL patients, as compared with the often prolonged persistence of anti-CD19 4-1BB:zeta-treated patients in reported clinical trials (2, 37). In aggregate, these data demonstrate that in vivo potency of CAR-redirected T cells is dependent, in part, on identifying permissive combinations of size-optimized extracellular spacer domains in the context of a particular cytoplasmic signaling domain composition. Furthermore, we describe an in vitro assay for assessing the proclivity of a CAR construct to induce AICD in primary human CD8+ TILs upon recursive activation events. These studies, using a solid tumor model system, reveal that ‘overtuning’ of CARs based on in vitro functional assays can lead to the selection of constructs that exhibit suboptimal in vivo potency due to excessive AICD.

There is as yet no predictive structural model that can reliably direct a priori how CARs should be built based on target molecule...
epitope location relative to the plasma membrane of the tumor cell. Moreover, commonly used surrogate in vitro bioassays may instruct away from a definitive choice of CAR composition that results in the greatest differential between high-level functional antitumor CAR-T-cell outputs and low-level AICD. Our work here demonstrates that a CAR structural library screen technique using the in vitro stress assay may be a valuable additional parameter to integrate into CAR engineering. It is conceivable that genetic strategies might limit the susceptibility of hyperactive CAR constructs to undergo AICD, such as fast overexpression of cFLIP or Toxo, or, vector-directed synthesis of siRNAs that knock down FasL or Fas [38, 39]. Additional secondary consequences of CAR overexpression also require interrogation, such as the predilection of hyperactive CARs to trigger expression of inhibitory receptors, such as PD-1, capable of enforcing an exhausted T-cell functional phenotype [40, 41]. Our data demonstrate that in a solid tumor model that (i) CAR structure function in vitro testing using commonly employed functional assays can misdirect the selection of candidate constructs as common practice is to focus on those constructs that display the highest functional activity, and (ii) potency tuning of CAR-directed effector CTLs has an upper limit above which gains in the magnitude of effector outputs are negated by augmentation in AICD upon recursive triggering through the CAR. These results have guided our selection of a CD171-specific short spacer CAR for a phase 1 study in children with relapsed/refractory neuroblastoma.

Disclosure of Potential Conflicts of Interest

M.C. Jensen reports receiving commercial research support from, has ownership interest (including patents) in, and is a consultant/advisory board member for Juno Therapeutics, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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CAR-Mediated Activation Induced Cell Death


Cancer Immunology Research

Functional Tuning of CARs Reveals Signaling Threshold above Which CD8 + CTL Antitumor Potency Is Attenuated due to Cell Fas–FasL-Dependent AICD

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