Research Article

Cancer Immunology Research

The Nonsignaling Extracellular Spacer Domain of Chimeric Antigen Receptors Is Decisive for *In Vivo* Antitumor Activity

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Abstract

The use of synthetic chimeric antigen receptors (CAR) to redirect T cells to recognize tumor provides a powerful new approach to cancer immunotherapy; however, the attributes of CARs that ensure optimal *in vivo* tumor recognition remain to be defined. Here, we analyze the influence of length and composition of IgG-derived extracellular spacer domains on the function of CARs. Our studies demonstrate that CD19-CARs with a long spacer from IgG4 hinge-CH2-CH3 are functional *in vitro* but lack antitumor activity *in vivo* due to interaction between the Fc domain within the spacer and the Fc receptor–bearing myeloid cells, leading to activation-induced T-cell death. We demonstrate that *in vivo* persistence and antitumor effects of CAR-T cells with a long spacer can be restored by modifying distinct regions in the CH2 domain that are essential for Fc receptor binding. Our studies demonstrate that modifications that abrogate binding to Fc receptors are crucial for CARs in which a long spacer is obligatory for tumor recognition as shown here for a ROR1-specific CAR. These results demonstrate that the length and composition of the extracellular spacer domain that lacks intrinsic signaling function can be decisive in the design of CARs for optimal *in vivo* activity. *Cancer Immunol Res; 3(2); 125–35. ©2014 AACR.*

Introduction

The adoptive transfer of human T lymphocytes that are genetically engineered to express chimeric antigen receptors (CAR) specific for surface molecules on tumor cells has the potential to treat advanced malignancies (1–3). CARs consist of an extracellular antigen-binding domain that is most commonly a single-chain variable fragment (scFv) of a mAb linked to intracellular signaling components such as CD3ζ alone or combined with one or more costimulatory domains. Research in CAR design has focused on identifying scFvs that when expressed in T cells confer recognition of malignant cells without serious toxicity to normal tissues, and on defining optimal intracellular signaling modules to activate T-cell effector functions (4–10). *In vitro* and *in vivo* studies in mice and humans have demonstrated that linking CD28 or 4-1BB costimulatory domains to CD3ζ in the CAR enhances cytokine production and promotes T-cell survival and proliferation after antigen engagement compared with CARs that contain CD3ζ alone (5, 6, 11–13).

Target recognition by a CAR is MHC independent and differs from that of a T-cell receptor. In MHC-restricted T-cell recognition, the fixed dimensions of the T-cell receptor and MHC molecules determine the spatial interactions of T cell and target cell, whereas with CAR-modified T cells (CAR-T cells), the interaction is influenced by the structure and density of the target molecule on the tumor and the location of the epitope that is recognized. It was appreciated that for optimal CAR-T-cell recognition, the sequences between the scFv and the T-cell membrane should provide flexibility, and the length of this spacer region may need to vary depending on the target molecule (14, 15). However, the specific requirements of the nonantigen binding components of the CAR extracellular domain to mediate tumor elimination *in vitro* and *in vivo* are understudied compared with the evaluation of intracellular CAR signaling domains.

The most advanced clinical evaluation of CARs involves the genetic modification of T cells to recognize the CD19 B-cell lineage molecule retained on B-cell malignancies. Some, but not all, clinical trials of CD19-CAR-T-cell therapy have demonstrated profound antitumor activity and on-target depletion of normal $CD19^+$ B cells (1–3, 16–19). However, direct comparison of results from these trials is difficult because of the small number and heterogeneity of patients in each study, and the variations in disease stage, preconditioning regimens, gene-transfer vectors, culture methodology, and composition of T-cell products. The CD19-CAR vectors used in these trials encoded

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different costimulatory domains (CD28 or 4-1BB), and the spacer domains between the scFv and the T-cell membrane differed in composition and length. For example, the CD19-CAR that demonstrated potent antitumor activity in patients with advanced chronic lymphocytic leukemia contained a short spacer sequence derived from CD8α that linked the scFv to the intracellular signaling domains (1, 18). By contrast, in another trial in which antitumor efficacy and CD19-CAR-T-cell survival were less impressive, the spacer domain was longer and derived from the IgG1 hinge and Fc (17). Here, we designed CD19- and ROR1-specific CARs with a modified IgG4 hinge and various components of the Fc region in the extracellular domain to examine the effect of spacer length and composition on *in vitro* and *in vivo* function. We show that the length of the nonsignaling spacer exerts a major effect on T-cell effector functions after target engagement, and that the composition of spacers that contain Fc domains must be altered or mutated to prevent *in vivo* interactions with cells expressing Fc gamma receptors (FcγR) that result in off-target activation of CAR-modified T cells and impair antitumor efficacy.

Materials and Methods

Human subjects

Blood samples were obtained from donors, who provided written informed consent for research protocols approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center (FHCRC). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Ficoll-Hypaque (Sigma).

Cell lines

293T cells (ATCC_CRL-11268) were cultured in DMEM with 10% FCS and 100 U/mL penicillin–streptomycin. K562 (ATCC_CCL-243), K562/ROR1 (20), K562/CD19 (21), Raji (ATCC_CCL-86), JeKo-1 (ATCC_CRL-3006), and JeKo-1-ffluc (20) cells were cultured in RPMI-1640 with 10% FCS and 100 U/mL penicillin–streptomycin (all culture media and supplements were purchased from GIBCO). Raji cells were transduced with lentivirus encoding an *ffluc/eGFP* fusion gene and then sorted for expression of eGFP to derive Raji-ffluc. Human CD64 cDNA (OriGene) was cloned into the retroviral vector plasmid pMP71 (22). K562 cells were transduced and sorted for $CD64^+$ cells. All cell lines were tested for transgene expression and the absence of mycoplasma before experiments.

Immunophenotyping

PBMCs and T cells were stained with one or more of the following conjugated mAbs: CD3, CD4, CD8, CD25, CD45, CD45RO, CD62L, CD69, and matched isotype controls (BD Biosciences). Transduced T cells were stained with biotin-conjugated anti-EGFR mAb (ImClone Systems Incorporated) and streptavidin–phycoerythrin (BD Biosciences). Single-cell suspensions of lungs and spleens from NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ (NSG) mice were stained with anti-Ly6C mAb (eBioscience), biotin-conjugated IgG4 protein (Abcam), and streptavidin–phycoerythrin. K562/CD64 cells were stained with anti-CD64 mAb (BioLegend) or biotin-conjugated IgG4 protein and streptavidin–phycoerythrin. Staining with propidium iodide (PI) was performed to distinguish live/dead cells. Flow cytometric analyses were done on a FACSCanto, cell sorting on a FACSAriaII, and data were analyzed using FlowJo software (Treestar).

Vector construction, preparation of lentivirus, generation of CAR-T cells, and *in vitro* functional assays

Construction of lentiviral vectors is described in Supplementary Materials and Methods. CAR and control T cells were generated by transduction with CAR/EGFRt (EGFR truncated)-, EGFRt-, or ffluc/eGFP-encoding lentivirus supernatants and enriched for $EGFRt^+$ cells as described (20). CAR-T cells were expanded with an irradiated B-lymphoblastoid cell line for CD19-CARs or using a rapid expansion protocol for R11-CARs (23). Cytotoxicity, cytokine release, and proliferation assays were performed as described (20).

Transfer of T cells in NSG mice

The FHCRC Institutional Animal Care and Use Committee approved all mouse experiments. Six- to eight-week-old NSG mice were obtained from the Jackson Laboratory or bred in-house. CAR/EGFRt-, CAR/EGFRt/ffluc/eGFP-, or EGFRt-modified T cells were injected via tail vein into tumor-free mice or mice engrafted i.v. with 5×10^5 tumor cells 7 days earlier. Bioluminescence imaging was performed as described (20). Peripheral blood was obtained by retro-orbital bleeding. Single-cell bone marrow suspensions were prepared from hind leg tibias and femurs. Erythrocytes in blood and suspensions of bone marrow, spleen, and lung were removed by incubation with ammonium–chloride–potassium lysing buffer (GIBCO) and subsequent washing.

Statistical analyses

Statistical analyses were performed with Prism Software (GraphPad). A two-sided Student *t* test was used with a confidence interval of 95%, and results with a *P* value of $P < 0.05$ were considered statistically significant.

Results

CD19-CARs with long and short extracellular spacers specifically recognize tumor *in vitro*

We constructed two lentiviral vectors comprising a scFv corresponding to the sequence of the CD19-specific mAb FMC63 in VL-VH configuration (4), and one of two spacers consisting of either the IgG4 hinge-CH2-CH3 domain (229 aa, long spacer) or only the hinge domain (12 aa, short spacer). Each spacer was linked to a CD28 transmembrane domain and a signaling module composed of CD3ζ with a membrane-proximal 4-1BB costimulatory domain (Fig. 1A). The transgene cassette included EGFRt to provide a selection and an *in vivo* tracking marker for CAR-T cells (24). We transduced $CD8^+$ $CD45RO^+$ $CD62L^+$ central memory T cells (T_{CM}) from healthy donors to ensure uniform T-cell composition for comparing different vectors, and because T_{CM}-derived cells persist *in vivo* after adoptive transfer (25, 26). CAR-T cells were enriched to high purity (Fig. 1B) and expanded briefly before *in vitro* and *in vivo* assays.

$CD8^+$ T_{CM} modified with the CD19-CARs containing either the short or long extracellular spacer domains expressed similar amounts of CAR protein as determined by Western blot of cell lysates (data not shown), and efficiently lysed $CD19^+$ Raji cells and K562 cells transduced to constitutively express CD19, but not native $CD19^-$ K562 cells (Fig. 1C). In response to stimulation

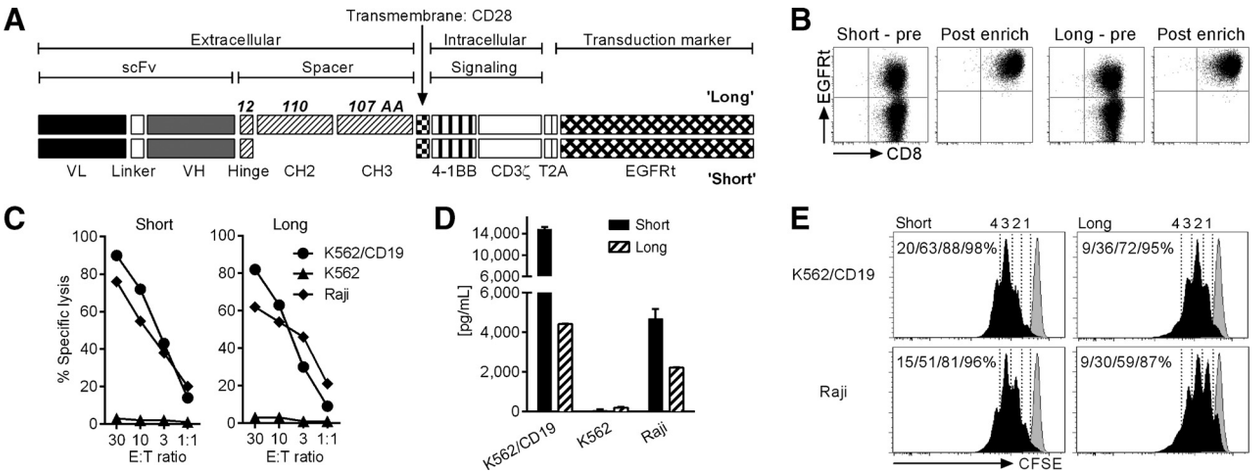

Figure 1.

CD19-CAR-T cells with short and long spacers show specific *in vitro* function. A, design of lentiviral transgene inserts encoding CD19-specific CARs with different extracellular spacer lengths. B, analysis of EGFRt expression on $CD8^+$ T_{CM}–derived T cells transduced with the lentiviral vectors encoding the CD19-CARs with short or long spacer domains before enrichment (pre) and after enrichment and expansion (post). Cytolitic activity (C), IFNγ production (D), and proliferation (E) of CD19-CAR-T cells after coculture with $CD19^+$ (K562/CD19, Raji) and control (K562) target cells. Proliferation after stimulation with $CD19^-$ K562 cells is shown as a comparison in each histogram (gray). Numbers above each histogram indicate the number of cell divisions the proliferating subset underwent, and the fraction of T cells in each gate that underwent ≥4/3/2/1 cell divisions is provided in the upper left of each plot. Data in B to E are representative of experiments with CAR-T cells derived from at least three different donors.

with K562/CD19 or Raji cells, T cells expressing the short spacer CAR produced higher levels of IFNγ compared with those expressing the long spacer CAR (Fig. 1D). CFSE-labeled T cells modified with each of the CD19-CARs proliferated following stimulation with either K562/CD19 or Raji cells; however, the average number of cell divisions was higher for CD19-CAR-T cells with a short spacer (Fig. 1E). These data demonstrate that CD19-CAR-T cells expressing two highly divergent spacer lengths are functional *in vitro*, but the data suggest that the short spacer CD19-CAR confers more efficient signaling after target recognition.

T cells expressing CD19-CARs with the long extracellular spacer fail to eradicate Raji tumors in NOD/SCID/$\gamma c^{-/-}$ (NSG) mice

To determine a T-cell dose for comparing the *in vivo* antitumor efficacy of the different CD19/4-1BB/CD3ζ-CAR constructs, we first treated mice engrafted with Raji-ffluc cells 7 days earlier with titrated doses of $CD8^+$ T_{CM}–derived T cells transduced with the short spacer CD19-CAR (Supplementary Fig. S1A). A dose of 2.5×10^6 CAR-T cells resulted in long-term tumor-free survival in 100% of mice, whereas 1×10^7 control T cells transduced with EGFRt only were ineffective (Supplementary Fig. S1B).

We then compared the antitumor activity of 2.5×10^6 CAR-T cells with short and long spacers. All mice treated with T cells expressing CD19-CARs with a short spacer had rapid and complete tumor regression and survived tumor-free for >56 days. Unexpectedly, the same dose of CD19-CAR-T cells expressing the long spacer that exhibited nearly equivalent *in vitro* function was as ineffective as control EGFRt T cells (Fig. 2A and B). To provide insight into the basis for poor efficacy, we analyzed the persistence of T cells in blood obtained from mice at scheduled intervals after T-cell infusion. On days 3 and 10 after adoptive transfer, all mice treated with $CD8^+$ T_{CM}–derived short spacer CD19/4-1BB/CD3ζ-CAR-T cells had higher levels of transferred T cells in the blood compared with mice treated with T cells expressing a long spacer CD19/4-1BB/CD3ζ-CAR (Fig. 2C).

Because of poor *in vivo* persistence of the CAR-T cells with a long spacer, we evaluated whether antitumor effects could be improved by increasing the T-cell dose or by including CD28 either alone or together with 4-1BB in the signaling domain of the CAR. T cells transduced with CARs containing the long spacer and the CD28 costimulatory domain lysed $CD19^+$ tumor cells and showed increased proliferation and cytokine production *in vitro* compared with T cells transduced with the long spacer CAR encoding only a 4-1BB costimulatory domain (Supplementary Fig. S2). However, even with a higher cell dose (1×10^7), T cells expressing the long/CD28 or the long/CD28_4-1BB CD19-CARs had no discernible antitumor effect *in vivo*, and only rare CAR-T cells were detected in the blood on days 3 and 10 after infusion (Fig. 2D and E). These data demonstrate that the *in vivo* antitumor reactivity of CD19-CARs constructed with Ig Fc spacer domains in NSG mice is dictated by the length and/or composition of the spacer, and not by the costimulatory domain.

T cells expressing CD19-CARs that possess long extracellular spacers undergo activation-induced cell death *in vivo*

We examined mechanism(s) underlying the markedly inferior *in vivo* antitumor activity of T cells that express CD19-CARs with long spacers. Because we observed lower numbers of transferred T cells expressing CD19-CARs with long spacers in the blood, we tested the possibilities that the T cells were not efficiently activated by the tumor *in vivo* or, conversely, that they underwent activation-induced cell death (AICD). T cells modified with each of the CD19/4-1BB/CD3ζ-CARs were labeled with CFSE, administered to NSG mice bearing Raji-ffluc tumors, and examined for activation, proliferation, and survival at tumor sites in the bone marrow and spleen. Immediately before infusion, T cells expressed low levels of the activation markers CD69 and CD25 (Fig. 3A). At 24 hours after T-cell infusion, there were statistically significantly lower numbers of T cells in mice treated with long spacer CD19-CAR-T cells compared with that in mice treated with the short spacer construct, even though the CD69 and CD25

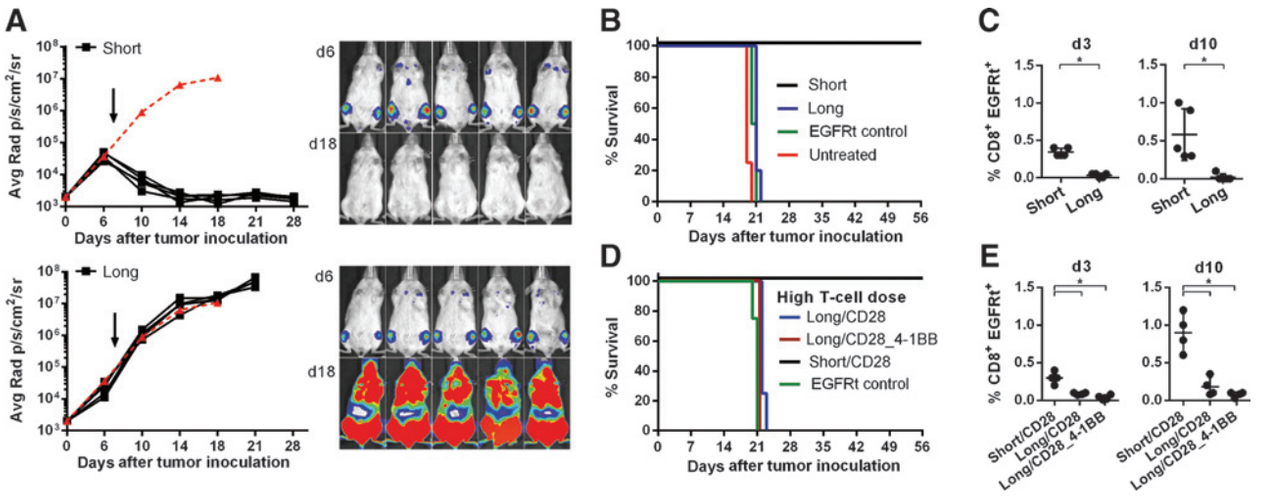

Figure 2.
CD19-CAR-T cells with a short spacer domain eradicate Raji tumors in NSG mice. A, NSG mice were inoculated with Raji-ffluc cells, and 7 days later treated with 2.5 × 10^6 T cells expressing short or long spacer CD19-CARs containing a 4-1BB/CD3ζ signaling module or with T cells expressing only EGFRt (4–5 mice per group). Arrows mark the day of T-cell transfer. Tumor growth was analyzed by bioluminescence imaging, and results from individual mice are plotted for short and long spacer CARs. Red triangles show the mean tumor burden in untreated mice at each time point. Images from one day before T-cell transfer (d6) and 11 days after transfer (d18) are shown for mice that had received short or long spacer CAR-T cells. B, survival of mice treated with short and long spacer 4-1BB/CD3ζ CAR-T cells was compared with mice that had received control T cells (EGFRt) or no T cells (untreated). C, frequency of $CD8^+$/$EGFRt^+$ T cells in the peripheral blood obtained at days 3 and 10 after T-cell transfer. *, statistically significant differences between groups. D, survival of Raji-ffluc–bearing NSG mice treated with a high dose (1×10^7) of CD19-CAR-T cells with long spacers (long/CD28, long/CD28_4-1BB), with a short spacer (short/CD28), or with control T cells (4 mice per group). E, frequency of $CD8^+$/$EGFRt^+$ T cells in the blood obtained at days 3 and 10 after T-cell transfer in mice treated in D.

activation markers were expressed at higher levels and in a greater proportion of cells modified with the long spacer CAR (Fig. 3B). There was no dilution of CFSE in any of the transferred T cells at 24 hours. By 72 hours, T cells expressing the short spacer CAR had undergone several cell divisions and increased by >10-fold in frequency in the bone marrow, whereas T cells expressing the long spacer CAR had undergone fewer cell divisions and the cell number did not increase (Fig. 3C). Moreover, we detected a much higher frequency of PI^+ T cells in the bone marrow and spleen, demonstrating that these T cells underwent AICD and as a result failed to control tumor growth (Fig. 3C–E).

AICD of CD19-CAR-T cells with a long IgG spacer is not dependent on tumor recognition

To determine if AICD of long spacer CD19-CAR-T cells resulted from recognition of $CD19^+$ tumor cells, we injected $CD8^+$ T_{CM}–derived T cells expressing each of the CAR constructs into tumor-free NSG mice and analyzed cell activation and persistence in the bone marrow, blood, and spleen after 24 hours. T cells expressing short spacer CARs were found at all sites and did not upregulate CD25 and CD69 as expected because tumor-free mice lack cells that express human CD19 (Fig. 4A and Supplementary Fig. S3A). Surprisingly, T cells with long spacer CARs showed an activated phenotype and were present at markedly lower levels. The same effect was observed when unselected T cells containing both $CD4^+$ and $CD8^+$ T-cell subsets were used (Supplementary Fig. S3B). To determine if the T-cell activation was dependent solely on spacer length and/or composition and did not result from altered specificity of the scFv in the long spacer format, we transferred T cells transduced with two different CARs (2A2 and R12) specific for the human tyrosine kinase receptor ROR1 (10), and constructed with short or long IgG4 spacers (20). In tumor-free NSG mice, we observed decreased persistence of ROR1-CAR-T cells with long spacers compared with short spacers for both of the ROR1-CARs (Supplementary Fig. S3C, data not shown).

T cells with long spacer CD19-CARs are sequestered in the lung and activated locally

We next examined the *in vivo* migration of T cells expressing CD19-CAR by transducing T cells with an *ffluc-eGFP* fusion gene in addition to the CAR, sorting $eGFP^+$/$EGFRt^+$ cells (Fig. 4B), and injecting them via the tail vein into tumor-free mice. One hour after injection, T cells with either the short or long spacer CAR were found predominantly in the lung (Fig. 4B). However, after 24 hours, short spacer CAR-T cells were present at high levels in blood and various tissue sites, whereas long spacer CAR-T cells still remained nearly entirely in the lung. Mice were sacrificed, and the lungs and spleens were removed and evaluated by bioluminescence imaging and flow cytometry to detect transferred T cells. T cells with short spacer CARs were found in the spleens and lungs, whereas T cells with long spacer CARs were present only in the lungs and had upregulated CD25 and CD69, indicating that they were activated locally in the absence of tumor cells (Fig. 4B).

A prior study had demonstrated that T cells modified with an IgG1 Fc spacer could activate human monocytes that expressed Fc receptors (FcR) *in vitro* as a result of binding to the Fc region of the CAR (27). Because the IgG4 long spacer construct included the entire Fc domain, we hypothesized that some cell population(s) in the lung might bind the Fc of the CAR and activate the CAR-T cells. To examine this possibility, we incubated a single-cell suspension of lung cells with biotinylated IgG4 protein and analyzed the cells by flow cytometry. A large fraction of cells in the lung that expressed the monocyte/macrophage marker Ly6C bound IgG4 protein (Fig. 4C). To test whether the $Ly6C^+$ cells were able to activate long spacer CAR-T cells *in vitro*, we sorted $Ly6C^+$ and $Ly6C^-$ cells from the spleen and cocultured them with

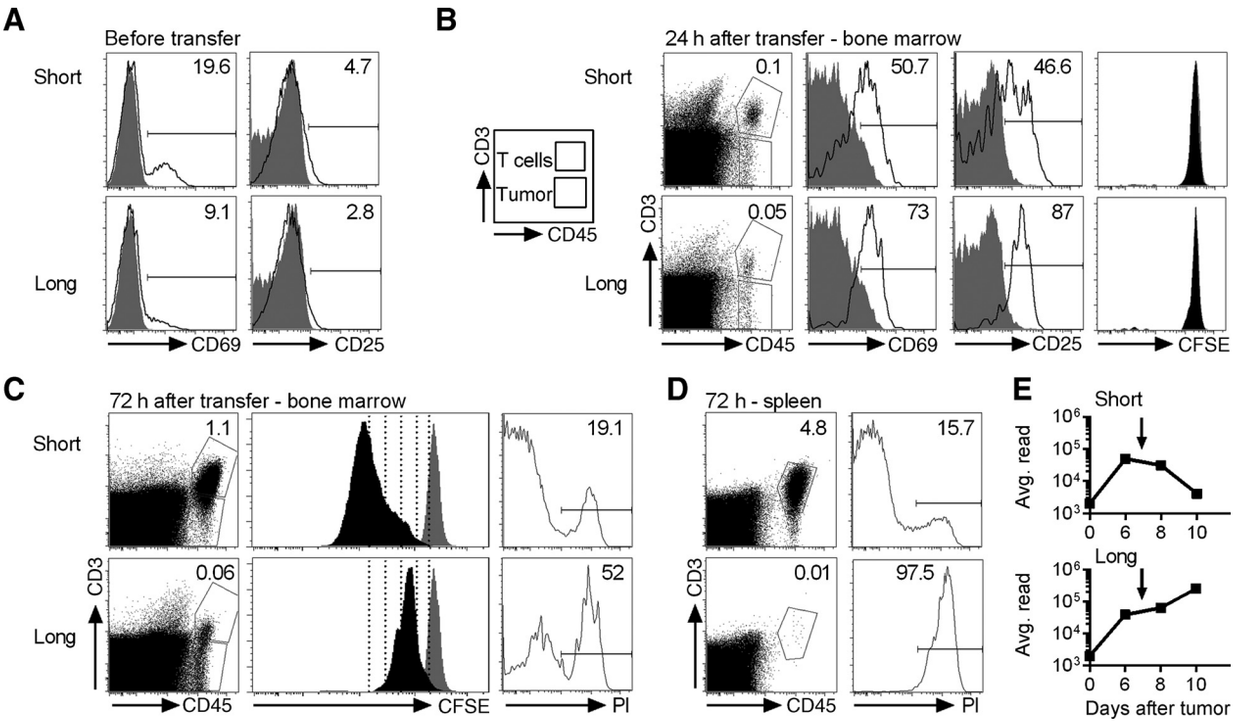

Figure 3.

CD19-CAR-T cells with a long spacer are activated *in vivo* but fail to increase in cell number. A, expression of activation markers CD69 and CD25 on CAR-T cells before transfer into NSG/Raji mice compared with isotype controls (gray). B and C, expression of activation markers and proliferation of CD19/4-1BB/CD3ζ-CAR-T cells after adoptive transfer. Flow cytometric analysis of bone marrow obtained 24 hours (B) and 72 hours (C) after T-cell transfer. Dot plots show anti-CD3 and anti-CD45 staining after gating on PI^- cells. The $CD3^-/CD45^+$ gate contains Raji tumor cells. Expression of CD25 and CD69 is shown for live (PI^-) $CD3^+/CD45^+$ T cells in comparison with isotype controls (gray). Proliferation of transferred T cells was analyzed by CFSE dye dilution and cell death by PI staining. D, frequency and PI staining of $CD3^+/CD45^+$ T cells in spleens obtained 72 hours after T-cell transfer. E, tumor growth in mice treated with T cells expressing CD19-CARs with a short or long spacer. Arrows mark the day of T-cell transfer. Data are representative of two independent experiments with 2 to 3 mice per group.

either long or short spacer CAR-T cells. T cells expressing the long spacer CAR upregulated CD25 after coculture with $Ly6C^+$ but not with $Ly6C^-$ cells, whereas CAR-T cells with short spacers were not activated by either subset of Ly6C cells (Fig. 4D, data not shown).

The FcR with the highest affinity for IgG4 is the FcγR1 (CD64; ref. 28). To determine if the interaction between the IgG4 domain of the CAR and CD64 was sufficient to activate effector functions in T cells, we transduced K562 cells with CD64 and confirmed its expression by staining with an anti-CD64 antibody and with IgG4 protein (Fig. 4E). $CD64^+$ and $CD64^-$ K562 cells were then cocultured with T cells expressing either short or long spacer CD19-CARs, and T cells that expressed the long but not the short spacer CAR produced IFNγ and were activated by and specifically lysed K562/CD64 cells (Fig. 4F and G).

CD19-CAR-T cells with CH2-deleted spacers are functional *in vitro* and *in vivo*

The CH2 domain of IgGs is crucial for binding to FcγRs; therefore, we evaluated a CAR in which the CH2 domain was deleted and the IgG4 hinge linked directly to the CH3 domain (intermediate spacer, 119 aa). We generated $CD8^+ T_{CM}$ expressing short, intermediate, and long spacer CD19/4-1BB/CD3ζ-CARs, and all three T-cell lines specifically lysed and produced IFNγ in response to $CD19^+$ K562 and Raji cells (Fig. 5A and B). Importantly, T cells expressing the intermediate spacer CAR did not recognize $CD64^+$ K562 cells, confirming the requirement for the CH2 domain for interaction with FcγRs. We injected 2.5×10^6 T cells expressing each of the CD19-CARs into Raji-ffluc–bearing NSG mice. T cells with intermediate spacer CD19-CARs eliminated Raji tumor *in vivo* as well as the short spacer CAR-T cells, and their persistence was similar to that of short spacer CARs (Fig. 5C and D). Long spacer CAR-T cells had no antitumor effect as observed previously. Thus, removing the CH2 domain of the Ig spacer was sufficient to abrogate the adverse consequences of FcγR binding *in vivo*.

CARs requiring long spacers can be modified to function *in vivo*

CARs specific for some molecules require a long spacer for efficient tumor recognition *in vitro*, presumably because the target epitope is located proximal to the tumor-cell membrane (15). To determine whether FcγR recognition of CARs with full-length IgG spacers could be eliminated, we modified amino acids within the CH2 domain previously shown to be important for binding of soluble Ig molecules to FcγR (29). We evaluated this first with CD19-CARs to facilitate *in vivo* analysis in the Raji tumor model. We designed two versions of the long spacer by replacing the first six amino acids of the CH2 domain with the corresponding five amino acids of IgG2 (designated long 4/2) and introducing an additional mutation at a glycosylation site at position 297 in the CH2 domain (30) to a conserved residue that is not subject to N-linked glycosylation (designated long 4/2NQ; Fig. 6A). CD19-CARs with short, long, long 4/2, or long 4/2NQ spacers were

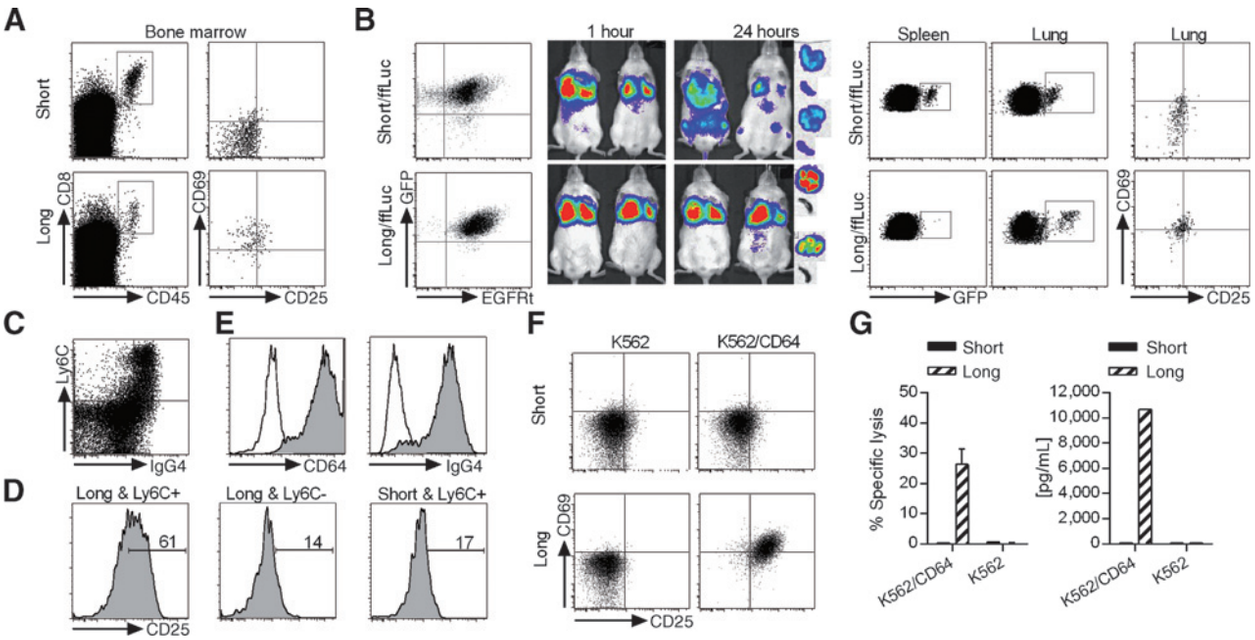

Figure 4.
CD19-CAR-T cells with a long spacer are activated in the lung. A, frequency of transferred T cells ($CD45^+/CD8^+$) and the expression of CD25 and CD69 in the bone marrow 24 hours after transfer of 1×10^7 CD19/4-1BB/CD3ζ-CAR-T cells into tumor-free NSG mice. B, T cells were transduced with ffluc/eGFP and short or long spacer CD19-CARs and enriched for $eGFP^+/EGFRt^+$ cells. T cells (1×10^7) were injected into tumor-free NSG mice, and localization of T cells was examined by bioluminescence imaging 1 and 24 hours later. Lungs and spleens were isolated, imaged separately, and analyzed for the presence of $eGFP^+$ T cells and the expression of CD25 and CD69 on $eGFP^+$ T cells. C, staining of a single-cell suspension of lung cells from NSG mice with IgG4 protein and anti-Ly6C mAb. D, analysis of CD25 expression on short and long spacer CD19-CAR-T cells after 24 hours of coculture with $Ly6C^+$ and $Ly6C^-$ cells isolated from spleens of NSG mice. E, staining of K562/CD64 cells with anti-CD64 mAb and IgG4 protein. Untransduced K562 cells were used as a control (white). F, CD25 and CD69 expression and (G) cytolytic activity and IFNγ release of CD19-CAR-T cells with short and long spacers after coculture with $CD64^+$ and $CD64^-$ K562 cells. Data are representative of at least two independent experiments.

expressed in $CD8^+$ T_{CM}, and transduced T cells were cocultured with K562/CD64 and stained for CD25. In contrast to CAR-T cells with the original unmodified long spacer, $CD64^+$ K562 cells did not activate CAR-T cells with either of the modified long spacers (Fig. 6B). The modifications did not affect CAR function because cytolytic activity, cytokine release, and proliferation of CAR-T cells with modified long spacers in response to K562/CD19 or Raji were comparable with CAR-T cells with unmodified spacers (Fig. 6C–E).

We then treated Raji-ffluc–bearing mice with 2.5×10^6 T cells expressing each of these CD19-CARs. As before, CAR-T cells with the short spacer were readily detected in the blood and eliminated Raji cells *in vivo* within 7 days, whereas CAR-T cells with the unmodified long spacer did not persist and had no antitumor activity (Fig. 6F–H). Surprisingly, CAR-T cells with long 4/2 spacers that were not activated by K562/CD64 cells *in vitro* did not persist *in vivo* or eliminate tumors. However, CD19-CAR-T cells with long 4/2NQ spacers persisted and exhibited antitumor activity (Fig. 6F–H). These data indicate that mutating known FcγR binding sites in the Fc region of the Ig spacer of CARs can abrogate binding to $CD64^+$ cells *in vitro*, but it is not always predictive of *in vivo* efficacy in NSG mice, as binding to other lower-affinity FcγRs may be retained.

A ROR1-specific CAR requires a modified long spacer to eliminate tumor cells *in vivo*

To confirm our results, we constructed ROR1-specific CARs with short, intermediate, and long spacers from the R11 scFv that recognizes a membrane-proximal epitope in the Kringle domain of ROR1 (31). As for CD19-CARs, the CD28 transmembrane and 4-1BB costimulatory domains were used and $CD8^+$ T_{CM} were transduced. Only R11-CAR-T cells with a wild-type or modified (4/2NQ) long spacer recognized $ROR1^+$ K562 cells *in vitro*, demonstrating that the location of the epitope recognized by R11 is not optimal for engagement by CAR-T cells with short or intermediate spacers (Fig. 7A–C). We tested the *in vivo* antitumor activity of R11-CAR-T cells in NSG mice that had been injected with the $ROR1^+$ mantle cell lymphoma line JeKo-1-ffluc 7 days earlier. As expected from the *in vitro* data, R11-CAR-T cells with short and intermediate spacers had no antitumor effect *in vivo*, although the T cells persisted after adoptive transfer (Fig. 7D and E). As for CD19-CARs, long spacer R11-CAR-T cells did not persist and failed to eradicate tumor cells *in vivo*. Only CAR-T cells with a long 4/2NQ spacer had an antitumor effect *in vivo*, and mice were tumor-free as early as 3 days after T-cell injection (Fig. 7D). These results showed that for CARs such as R11 that require a long spacer to function, modifications of the CH2 domain can abrogate FcγR binding and improve T-cell persistence and function *in vivo*.

Discussion

Artificial CARs usually are composed of an extracellular antigen-binding scFv, a spacer that provides separation of the scFv from the cell membrane and an intracellular signaling module that mediates T-cell activation. Within this conceptual framework, many variations have been described that are capable of eliciting antitumor functions in T cells *in vitro*, and several constructs have

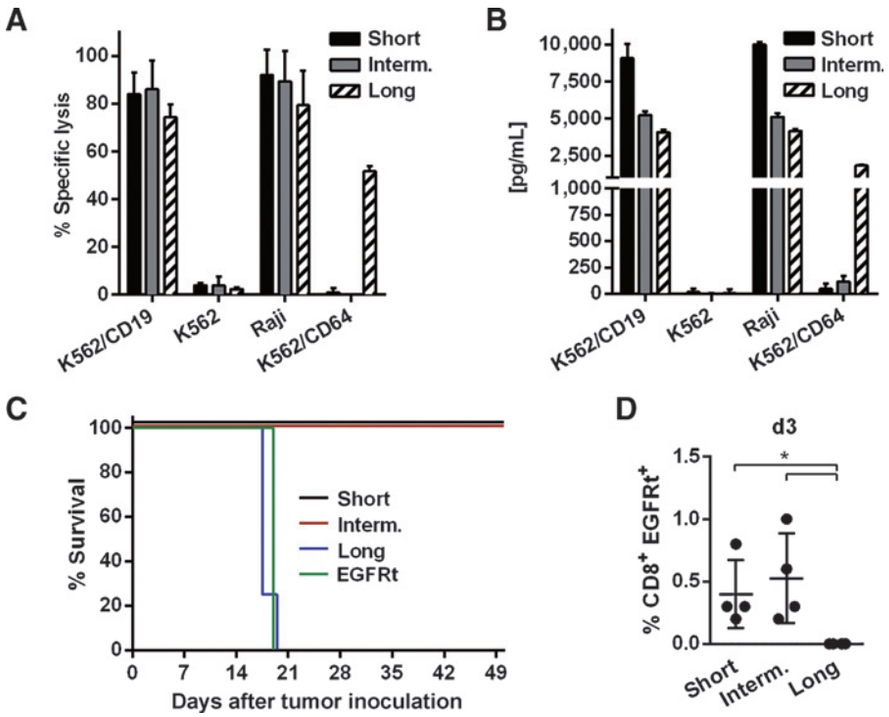

Figure 5.
CD19-CAR-T cells with a CH2-deleted spacer are functional *in vitro* and *in vivo*. A, cytolytic activity and IFNγ release (B) of CD19/4-1BB/CD3ζ-CAR-T cells were analyzed after coculture with Raji, K562, K562/CD19, and K562/CD64 cells. C, CAR-T cells (2.5×10^6) were injected into Raji-ffluc–bearing NSG mice (4 mice per group). Survival of mice was compared with mice receiving control EGFRt-T cells. D, persistence of T cells in blood was analyzed 3 days after T-cells transfer. *, statistically significant differences between groups. Data are representative of experiments with CAR-T cells derived from three different donors.

advanced to clinical trials (1–3, 32–35). Prior studies demonstrated that tailoring extracellular spacer length of CARs affects optimal tumor recognition for different target epitopes. A study of a CD22-specific CAR suggested that the distance of the target epitope from the cell membrane is an important parameter (36), and tumor recognition by 5T4- and NCAM-specific CARs that recognize membrane-proximal epitopes was improved with a long spacer (15). In the case of CD19-CARs, our data demonstrate that target recognition *in vitro* will accommodate a wide range of spacer lengths from 12 to 229 aa. This may reflect the structure and/or location of the CD19 epitope recognized by the FMC63 mAb or the high density of the CD19 molecule on tumor cells (37). Even though $CD19^+$ tumor cells are recognized *in vitro*, we observed more efficient cytokine production and proliferation of CAR-T cells that contain a short 12- aa spacer compared with those with intermediate (119 aa) or long (229 aa) spacers. We previously demonstrated a similar hierarchy of tumor recognition for ROR1-CARs derived from the 2A2 and R12 scFvs that recognize an epitope in the Ig/Frizzled domain of ROR1 and function optimally with a short extracellular spacer (20). However, the R11 ROR1-CAR that is specific for an epitope in the putatively more membrane-proximal Kringle domain of ROR1 requires a long spacer for recognition of $ROR1^+$ tumor cells. Thus, spacer length can affect tumor recognition depending on the molecule and epitope being targeted.

A spacer domain that is commonly used in CAR design consists of an IgG hinge region, typically IgG1 or IgG4, and the CH2-CH3 domain of IgG Fc (15, 17, 27, 33, 38). The use of the IgG Fc domain can provide flexibility to the CAR, lacks immunogenicity, facilitates detection of CAR expression using anti-Fc reagents, and allows removal of one or more CH2 or CH3 modules to accommodate different spacer lengths. Prior studies of the IgG1 Fc spacer demonstrated activation of CAR-T cells by FcγR-bearing cells and simultaneous cross activation of cytokine production by innate immune cells *in vitro*, although the specific FcγR that bound the IgG1 Fc component of the CAR was not identified in this study (27). This result was perhaps anticipated because the IgG1 isotype has been shown to have the highest affinity for FcγR binding. The authors demonstrated that replacement of IgG1 CH2 sequences with those of IgG2 that has lower binding affinity for FcγRs eliminated this problem *in vitro*, and speculated that the off-target activation of CAR-T cells might result in loss of antitumor activity due to AICD (27).

We used the IgG4 Fc as a spacer domain in CAR design because it has a lower binding affinity to the high-affinity FcγR1 (CD64) than IgG1 and IgG3 isotypes, and binds to other FcγRs with an affinity similar to or lower than that of IgG2 (28, 39). However, we found that CD19- and ROR1-CARs designed with the full-length IgG4 Fc spacer containing hinge, CH2, and CH3 domains, while functional *in vitro*, fail to mediate discernible antitumor activity in NSG mice. The lack of efficacy was independent of the costimulatory domain in the CAR and occurred with $CD8^+$ T_{CM} and unselected T cells containing both $CD4^+$ and $CD8^+$ subsets. Our studies demonstrate tumor-independent trapping of CAR-T cells in the lung. $Ly6C^+$ mononuclear cells that express FcγR and bind IgG4 are present in the lung, and they are capable of activating CAR-T cells through interactions with the Fc portion of the CAR. The few CAR-T cells that escape to the periphery display a highly activated phenotype, and a large proportion undergoes AICD, explaining the lack of antitumor efficacy. Prior studies have demonstrated that CAR-T cells constructed with full-length IgG4 and IgG1 spacers and specific for targets other than CD19 and ROR1 can mediate antitumor activity in NSG mice; however, these models used repeated injection of T cells shortly after tumor inoculation, and demonstrated only short-term persistence of CAR-T cells (40, 41). Our data reveal a significant limitation for interpreting the antitumor efficacy of CAR-T cells that use unmodified full-length Ig Fc spacer domains in NSG mouse tumor

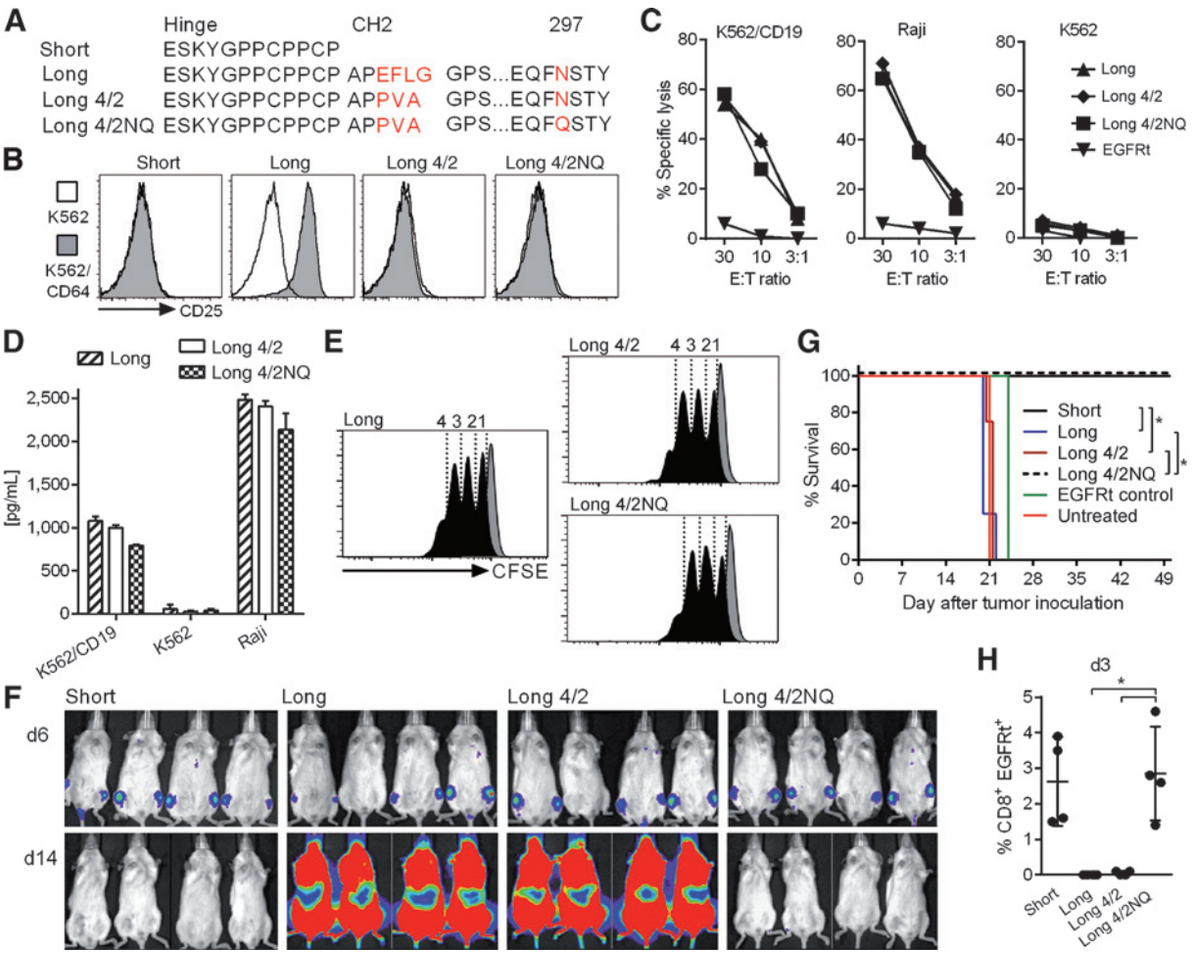

Figure 6.
CD19-CAR-T cells with long spacers comprised of a modified Fc domain persist *in vivo* and eliminate tumor cells. A, the amino acid sequence of the hinge and parts of the CH2 region for short, long, and long variants (4/2 and 4/2NQ). B, CD25 expression on $CD8^+$ T_{CM}–derived CD19/4-1BB/CD3ζ-CAR-T cells after 24-hour coculture with $CD64^+$ and $CD64^-$ K562 cells. Cytolytic activity (C), IFNγ production (D), and proliferation (E) of CD19-CAR-T cells with different spacers after stimulation with K562, K562/CD19, and Raji cells. Proliferation after stimulation with K562 cells is shown as a comparison in each histogram (gray). F, antitumor activity of 2.5×10^6 CAR-T cells with different spacers in Raji-ffluc–bearing NSG mice (4 mice per group). Results obtained 1 day before (day 6) and 1 week after (day 14) T-cell injection are shown. G, survival of mice treated with CD19-CAR-T cells, control T cells (EGFRt), and untreated mice. Statistical analyses were performed by the log-rank test; *, statistically significant differences ($P < 0.01$) between groups. H, persistence of T cells in blood 3 days after T-cell transfer. *, statistically significant differences between groups. Data in B to E are representative of experiments with CAR-T cells derived from two different donors.

xenograft models because of the adverse effects of this spacer design on CAR-T-cell survival and function. Candidate target molecules that require a long spacer CAR for tumor recognition *in vitro* may inadvertently be deemed suboptimal based on poor *in vivo* antitumor efficacy in NSG mice, and should be revisited with alternative spacer designs. It is important to note that NSG mice lack endogenous immunoglobulins, and FcγRs are unoccupied, which may accentuate interactions between $Fc\gamma R^+$ cells and CAR-T cells. The administration of human Ig to NSG mice before infusion of CAR-T cells reduced T-cell activation and improved persistence, although not to the level achieved with a short spacer CAR that lacks the CH2/CH3 domains (data not shown).

The extent to which FcγR binding of CAR-T cells would occur *in vivo* in patients is unknown. Our data demonstrate that K562 cells transfected with human CD64 efficiently activate CAR-T cells *in vitro*, and it is likely that off-target activation of CAR-T cells would occur in patients with low Ig levels, or in the lungs, spleens, and bone marrow, where a high frequency of cells that express FcγRs reside. This could result in AICD of the CAR-T cells as demonstrated in NSG mice, and lead to cytokine release contributing to toxicity of CAR-T cells. It is intriguing that clinical trials that have used CAR-T cells with an unmodified IgG1 Fc spacer have reported inconsistent persistence of CAR-T cells, although this could also be explained by variation in cell products or patient conditioning (17).

We demonstrate multiple solutions to the problem of FcγR binding that can be applied to CAR design. One strategy is to remove the entire CH2 domain that is responsible for FcγR binding to create a spacer with an intermediate length containing only the CH3 domain. This approach provided functional CD19-CAR-T cells, which did not bind to cells expressing CD64 *in vitro* and were effective *in vivo* in the tumor model. However,

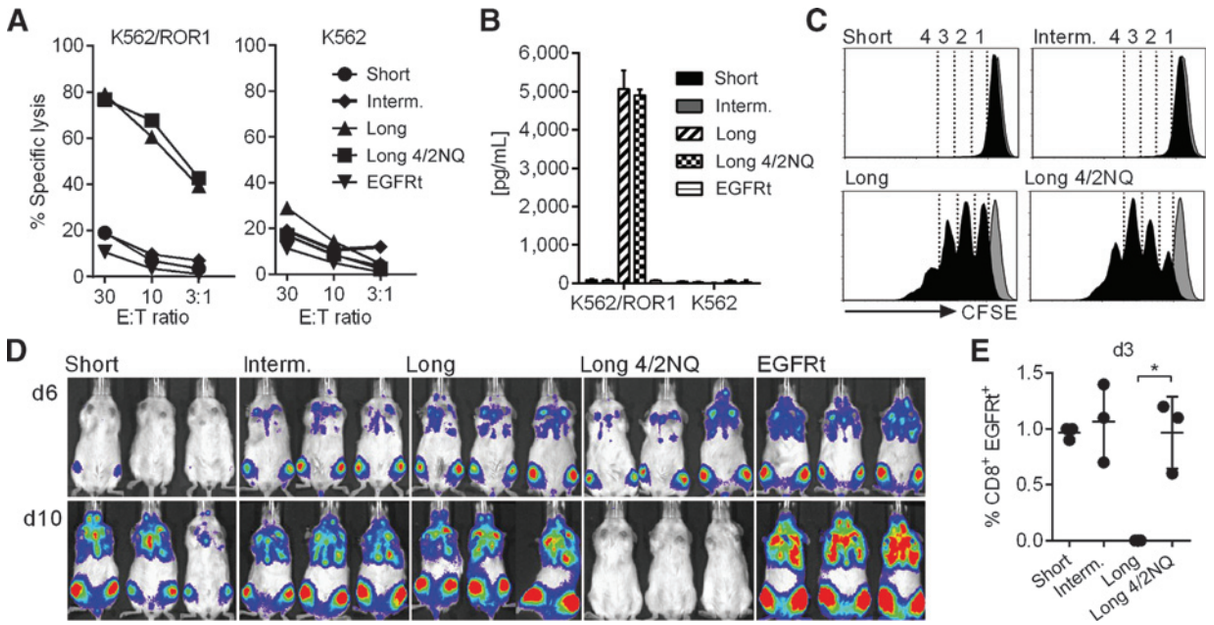

Figure 7.
T cells with a ROR1-specific CAR eliminate tumor cells *in vivo* only with a modified long spacer. Cytotoxicity (A), IFNγ release (B), and proliferation (C) of $CD8^+$ T_{CM} transduced with ROR1-specific R11-CARs with short, intermediate (interm.), long, and long 4/2NQ spacers after coculture with K562 and K562/ROR1 cells. D, antitumor activity of 5×10^6 R11-CAR-T cells with different spacers in NSG mice engrafted with 5×10^5 JeKo-1-ffluc 7 days before T-cell infusion (3 mice per group). Images obtained 1 day before (day 6) and 3 days after (day 10) T-cell injection are shown. E, persistence of ROR1-CAR-T cells in blood 3 days after T-cell transfer. *, statistically significant differences between groups. Data in A to C are representative of experiments with CAR-T cells derived from at least two different donors.

the R11 ROR1-CAR that recognizes a membrane-proximal epitope of ROR1 requires a full-length Fc spacer for recognition of tumor cells, and R11-CARs designed with a hinge-CH3 spacer were nonfunctional. Here, a second strategy was used in which the CH2 sequences of IgG4 were swapped with those of IgG2, which eliminated binding to CD64 *in vitro* and provided functional CARs as shown previously for CD30 (27). However, this CH2 modification alone was not sufficient to restore antitumor activity and improve CAR-T-cell persistence in NSG mice, presumably due to activation of the CAR-T cells by binding to other FcγRs (28). Crystal structures of the complex between Ig Fc regions and FcγRs revealed a contribution of glycosylation at Asn^{297}, which is conserved in all Ig CH2 domains, to FcR binding (42). Therefore, in addition to swapping IgG4 CH2 sequences with those of IgG2, we replaced Asn^{297} with a conserved residue that is not a site for N-linked glycosylation. This modified R11-CAR was functional *in vitro* and mediated antitumor activity against $ROR1^+$ lymphoma in NSG mice. Collectively, the studies show that the spacer domains of synthetic CARs that lack intrinsic signaling properties have dramatic effects on *in vivo* antitumor activity independent of costimulatory signaling. The length and composition of this region are additional critical variables to be considered in designing optimal CARs for clinical applications.

Disclosure of Potential Conflicts of Interest

M. Hudecek, M.C. Jensen, and S.R. Riddell are inventors on a patent application (PCT/US2013/055862) related to this work that has been filed by the Fred Hutchinson Cancer Research Center and licensed by Juno Therapeutics, Inc. C. Rader is inventor on a patent application (PCT/US2011/062670) that claims anti-ROR1 mAb R11 and has been filed by the NIH. M.C. Jensen reports receiving a commercial research grant from, and is a consultant/advisory board member for, Juno Therapeutics, Inc. S.R. Riddell reports receiving a commercial research grant from Juno Therapeutics, Inc., and is a consultant/advisory board member for Cell Medica and Juno Therapeutics, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: M. Hudecek, D. Sommermeyer, A. Silva-Benedict, C. Rader, M.C. Jensen, S.R. Riddell

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Hudecek, D. Sommermeyer, L. Liu, S.R. Riddell

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Hudecek, D. Sommermeyer, P.L. Kosasih, L. Liu, M.C. Jensen, S.R. Riddell

Writing, review, and/or revision of the manuscript: M. Hudecek, D. Sommermeyer, C. Rader, M.C. Jensen, S.R. Riddell

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Rader, M.C. Jensen, S.R. Riddell

Study supervision: S.R. Riddell

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