Chimeric Antigen Receptors With Mutated IgG4 Fc Spacer Avoid Fc Receptor Binding and Improve T Cell Persistence and Antitumor Efficacy

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The success of adoptive therapy using chimeric antigen receptor (CAR)–expressing T cells partly depends on optimal CAR design. CARs frequently incorporate a spacer/linker region based on the constant region of either IgG1 or IgG4 to connect extracellular ligand-binding with intracellular signaling domains. Here, we evaluated the potential for the IgG4-Fc linker to result in off-target interactions with Fc gamma receptors (FcγRs). As proof-of-principle, we focused on a CD19-specific scFv-IgG4-CD28-zeta CAR and found that, in contrast to CAR-negative cells, CAR+ T cells bound soluble FcγRs *in vitro* and did not engraft in NSG mice. We hypothesized that mutations to avoid FcγR binding would improve CAR+ T cell engraftment and antitumor efficacy. Thus, we generated CD19-specific CARs with IgG4-Fc spacers that had either been mutated at two sites (L235E; N297Q) within the CH2 region (CD19R(EQ)) or incorporated a CH2 deletion (CD19Rch2Δ). These mutations reduced binding to soluble FcγRs without altering the ability of the CAR to mediate antigen-specific lysis. Importantly, CD19R(EQ) and CD19Rch2Δ T cells exhibited improved persistence and more potent CD19-specific antilymphoma efficacy in NSG mice. Together, these studies suggest that optimal CAR function may require the elimination of cellular FcγR interactions to improve T cell persistence and antitumor responses.

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INTRODUCTION

Adoptive immunotherapy using chimeric antigen receptor (CAR)–expressing T cells is a promising cancer treatment, because these cells can directly recognize and kill antigen-expressing tumor cells in a human leukocyte antigen–independent manner. However, besides a careful choice of the target tumor-associated antigen, this therapeutic approach is highly dependent on the optimal molecular design of the CAR. For example, several groups have demonstrated that including one or more intracellular costimulatory domains improves CAR T cell potency both *in vitro* and *in vivo*.[1–3] Other groups have also suggested that where the CAR binds the target antigen (*i.e.*, membrane proximal versus distal epitopes)[4] and/or the length of the linker sequence[5–7] are important considerations in optimizing CAR design. Here, our attention has also recently been drawn to the spacer or hinge sequences that are used to link the ligand-binding domain to transmembrane and intracellular-signaling domains of the CAR—specifically the use of immunoglobulin Fc spacers commonly applied to CAR design.[8–16]

The constant domain, or Fc, of immunoglobulins is known to direct binding to Fc receptors as a potential effector function.[17] There are several amino acid sequences within the Fc CH2 domain that are important for recognition and binding by Fc receptors (FcRs) (reviewed in ref. 18). FcRs, such as FcγRI, are integral membrane proteins located on immune cells including natural killer cells, neutrophils and macrophages, which then use this Fc-targeting ability to carry out various immune functions such as antibody-dependent cell-mediated cytotoxicity and phagocytosis. Thus, we hypothesized that this potential for FcR recognition might play a role in the immunological rejection and clearance of adoptively transferred T cells expressing CARs that contain such Ig Fc spacers.

To evaluate whether FcR-mediated interactions play a role in the efficacy of adoptively transferred CAR-expressing T cells, we have generated a CD19-specific CAR that has been mutated at one or two sites within the CH2 region (L235E and/or N297Q) of its IgG4 Fc spacer—here called CD19R(L235E), CD19R(N297Q), or CD19R(EQ)—as well as a CD19-specific CAR that has a CH2 deletion in its IgG4 Fc spacer—here called CD19Rch2Δ. T cells expressing these mutated CARs were then compared to T cells expressing only a truncated epidermal growth factor receptor molecule (EGFRt) as a tracking marker,[19] or a nonmutated CAR (CD19R) for *in vitro* FcγR binding and CAR-mediated cytolytic activity, as well as *in vivo* engraftment and therapeutic efficacy.

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These studies expand on previous findings demonstrating that mutations in the IgG1 spacer can help reduce the off-target *in vitro* activation of CAR-expressing T cells and FcR-expressing cells.[20] Overall, our results provide evidence that elimination of FcγR interactions can improve the persistence and antitumor responses of adoptively transferred CAR-expressing T cells.

RESULTS

CAR+ T cells fail to engraft in NSG mice

In the process of characterizing central memory T cells (T_{CM}) as a T cell subpopulation that might have superior engraftment potential, and thus therapeutic efficacy, after adoptive transfer,[21] we found evidence that CAR expression on the T_{CM}-derived cells seemed to correlate with decreased *in vivo* persistence in our *in vivo* xenograft model using NSG mice. This was exemplified most clearly in an experiment comparing the engraftment of nontransduced T_{CM}-derived cells to those that had been lentivirally transduced to express either a truncated EGFR (EGFRt) as a tracking marker alone or both a CD19-specific scFv-IgG4-CD28-zeta CAR (CD19R) and the EGFRt tracking marker on the cell surface (**Figure 1**). Upon co-staining for the EGFRt tracking marker to detect gene-modified cells, it was apparent that, despite the similar level of transduction and/or EGFRt expression of the input cells (**Figure 1b**, 78–79% positive), there was significantly less engraftment of cells in the peripheral blood of mice that received CD19R/EGFRt$^+$ T_{CM} compared to those that received EGFRt$^+$ T_{CM} (**Figure 1c**, $P < 0.0001$ comparing percentages of huCD45/EGFRt$^+$ cells in each group at either day 7 or day 14 using unpaired Student's *t*-tests). Indeed, although low levels of T cells were detected for the CD19R/EGFRt+ T_{CM}-treated mice, all of the persistent T cells at days 7 and 14 were CAR negative. This impaired *in vivo* persistence is not associated with lentiviral transduction of the T cells, as it is specific to cells transduced to express the CAR transgene and not the EGFRt transgene. Furthermore, the lack of CD19 antigen in these NSG mice and the fact that we have seen a similar phenomenon with T cells expressing CARs of different antigen specificity (data not shown) suggest that the lack of engraftment/persistence in the peripheral blood is antigen independent. Together, these data led us to investigate whether there was something inherent in the CAR design that could be mediating the impaired persistence of these cells.

Soluble FcγR binds CAR+ T cells

Our CD19R construct consists of a CD19-specific scFv derived from mouse monoclonal antibody FMC63, a human IgG4 Fc linker, human CD28 transmembrane and cytoplasmic domains, and a human CD3-zeta cytoplasmic domain. Based on the potential for the IgG4 Fc linker—which was a consistent component of all CARs designed by our group—to interact with FcRs, we speculated that this feature might be responsible for the selective clearance of our CD19R/EGFRt$^+$ but not EGFRt$^+$ cells. Indeed, binding assays using either soluble murine or human FcγR1 revealed that, in contrast to T_{CM}-derived cells that were nontransduced or expressed only the EGFRt, those that expressed the IgG4-linker containing CD19R CAR exhibited binding of the FcγR1 molecules that could be titrated down with higher dilutions (**Figure 2**). Of note, while NSG mice lack mature T cells, B cells, and functional natural killer cells, they are known to still have FcR-expressing innate immune cells including neutrophils and monocytes[22–24]; and our own analysis has revealed the presence of FcR-expressing (*i.e.*, FcγRII- and FcγRIII-expressing) Gr-1, CD11b, CD11c, and F4/80 cells in the blood, bone marrow, and livers of NSG mice (**Supplementary Figure S1**). This provided a potential rationale for the lack of CAR+ T cell persistence observed in our prior engraftment studies.

Generation of CARs with mutated IgG4 spacer

To specifically address the significance of potential FcR-mediated effects for CAR-expressing T cells, we mutated our CD19-specific CAR at two sites within the IgG4 CH2 domain that are known to be important for FcR binding (using the IgG4 sequence reviewed in ref. 18)—L235E, which has been shown to reduce FcγR1 binding,[25] and/or N297Q, an aglycosylation motif which has been shown to prevent binding to FcγRIIA, FcγIIB, and FcγIIIA.[26] We also created a CAR with a deletion of the IgG4 CH2 domain, thus eliminating the region involved in FcR interaction (*i.e.*, deleting the domain that contains residues 235 and 297) (**Figure 3a**). The resulting single mutants, CD19R(L235E) and CD19R(N297Q), double mutant CD19R(EQ), and deletion CD19Rch2Δ sequences were incorporated into separate lentiviral constructs, where they were each coordinately expressed with EGFRt from a single transcript, using the T2A ribosome skip sequence in a design similar to that described in **Figure 1a** for the nonmutated CD19R. After lentiviral transduction, immunomagnetic enrichment of EGFRt-expressing cells, and a single round of rapid expansion, each of the T_{CM}-derived lines were 92–99% positive for the expected transgenes (**Figure 3b**), demonstrating that the mutations do not adversely affect CAR expression. Furthermore, none of these mutations altered the CD19-specific cytolytic potential of these T_{CM}-derived cells in 4-hour ^{51}Cr-release assays (**Figure 3c**).

FcγR binding to CARs with mutated IgG4 spacer is impaired

To determine the ability of the different mutations in the CAR to affect FcR binding, we next performed flow cytometric analyses using various human and murine biotinylated soluble FcγRs, and PE-streptavidin (SA-PE) to detect the binding of the FcγRs to the different cell populations. As expected, T cells that expressed only EGFRt were not bound by these FcγRs, while T cells that expressed the nonmutated CD19R were bound by human FcγR1, FcγR2a and FcγR2b, as well as murine FcγR1 and FcγR2b (**Figure 4**). In contrast, T cells that expressed either the CD19R(N297Q), CD19R(L235E), or CD19R(EQ) mutants or the CD19Rch2Δ deletion all displayed significantly reduced binding to these FcγRs. Because the physiological significance of these binding levels was not known, we continued to analyze all four CD19R mutants for T cell persistence in NSG mice.

T cells with CAR mutants exhibit improved *in vivo* engraftment and persistence

To see if the CD19R mutations, which impaired soluble FcγR binding, would then translate to increased *in vivo* persistence upon adoptive transfer, 10^7 EGFRt-enriched and expanded T cells expressing either the EGFRt marker alone,

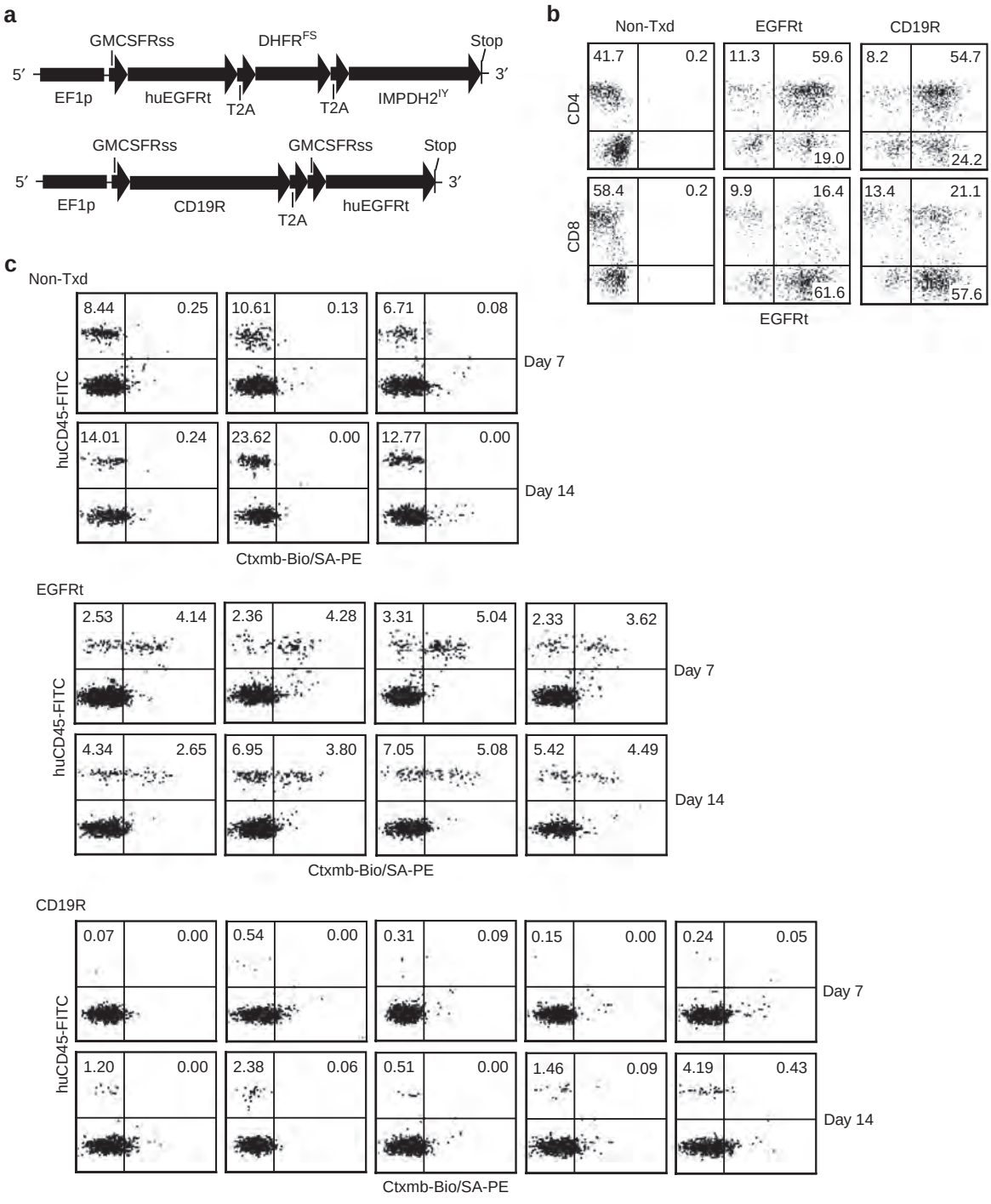

Figure 1 CD19-specific CAR-expressing T cells do not efficiently engraft in NSG mice. (**a**) Schematics of the EGFRt (top) and CD19R/EGFRt (bottom) expression constructs that were used to gene modify T cells for engraftment studies. The CD19-specific, CD28-costimulatory CAR (CD19R), the self-cleavable T2A, the huEGFRt, and the drug resistance DHFR^{FS} and IMPDH2^{IY} genes are indicated, along with the elongation factor 1 promoter sequences (EF-1p), the GM-CSF receptor alpha chain signal sequences (GMCSFRss), and the three nucleotide stop codons. (**b**) Flow cytometric analysis of input T cells administered to NSG mice for engraftment studies. T_{CM}-derived cells were either nontransduced (Non-Txd) or transduced with lentiviral vectors containing the EGFRt or CD19R/EGFRt (CD19R) constructs described in **a**, immunomagnetically selected for EGFRt-expression, and underwent a single round of rapid expansion after which they were analyzed for cell surface phenotype on day 19. Percentages of cells staining with antibodies specific for CD4 (top) or CD8 (bottom) versus EGFRt are indicated in each histogram, using quadrants that were created based on negative control staining. (**c**) 10^7 T_{CM}-derived cells as described in **b** were administered i.v. to NSG mice with irradiated NS0-IL15 support. Day 7 and 14 peripheral blood leukocytes harvested from each group (n = 3–5 mice) were stained using FITC-conjugated antihuman CD45, and biotinylated-cetuximab followed by PE-conjugated streptavidin. Percentages of lymphocyte-gated, huCD45$^+$, and huCD45$^+$EGFRt$^+$ cells are indicated in each histogram, using quadrants that were created based on negative control staining. Data are representative of four different experiments performed with T_{CM}-derived cells from multiple donors.

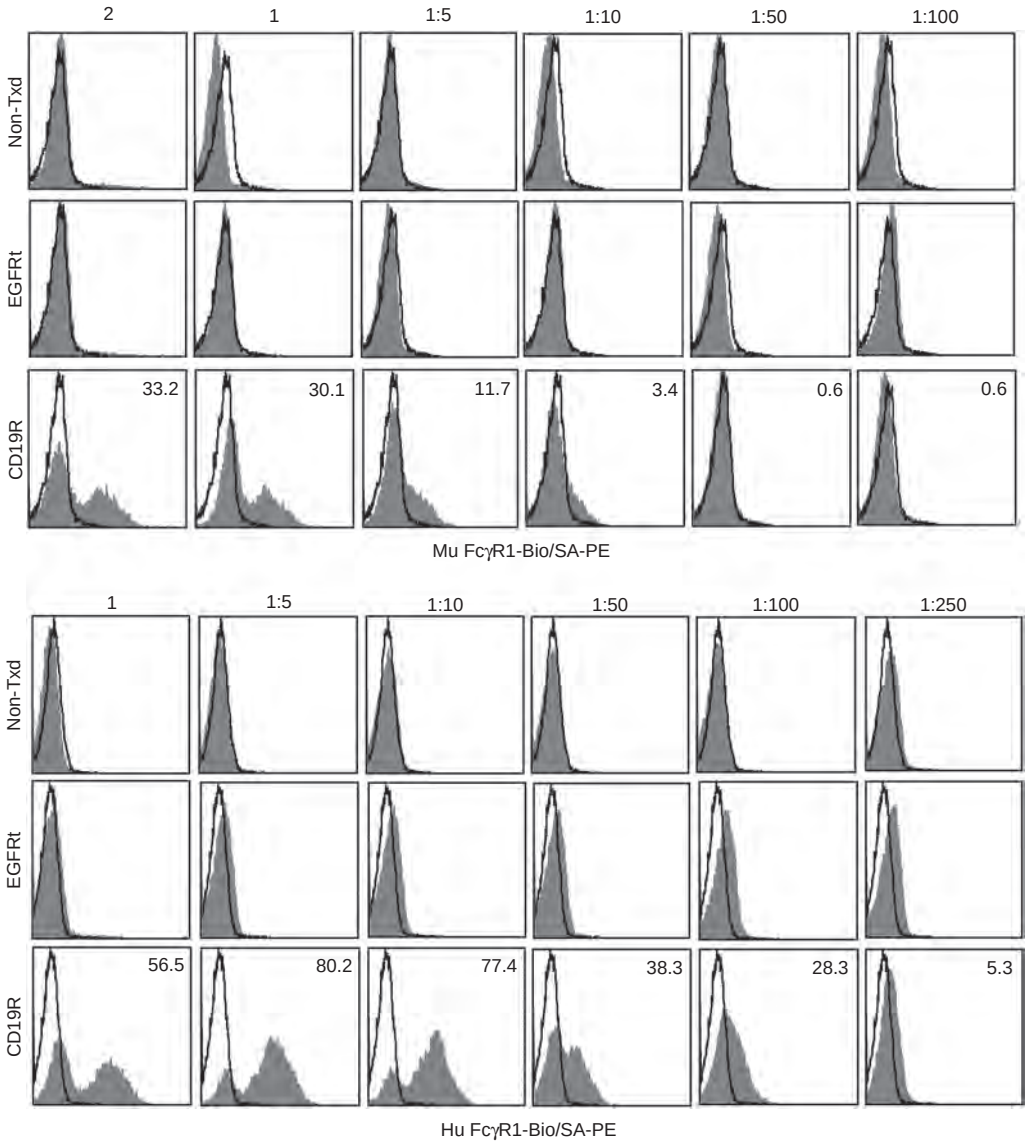

Figure 2 CD19-specific CAR-expressing T cells bind soluble FcγR1. The same T cells used in **Figure 1** were stained with the indicated volume titration of biotinylated soluble mouse (top) or human (bottom) FcγR1 followed by PE-conjugated streptavidin (SA-PE, gray histogram). For CD19R-expressing cells, percentages of immune reactive cells are indicated in each histogram, and based on an M1 gate set to detect ≤1% of that stained with SA-PE alone (black line).

the parental CD19R, the single point-mutated CD19R(L235E) or CD19R(N297Q), the double point-mutated CD19R(EQ), or the CH2-deleted CD19Rch2Δ were infused i.v. into NSG mice. Seven and fourteen days later, peripheral blood was assayed for CD45/EGFRt$^+$ cell engraftment (**Figure 5**). Interestingly, similar to that seen with the nonmutated CD19R, only low/undetectable engraftment of EGFRt$^+$ cells was observed when the T cells expressed the single point-mutated CD19R(L235E) or CD19R(N297Q). However, expression of the double point-mutated CD19R(EQ) or CH2-deleted CD19Rch2Δ rescued T cell engraftment, as levels of huCD45/EGFRt$^+$ cells observed in these groups of mice were similar to that seen when EGFRt alone was expressed. This rescued engraftment and persistence of gene-modified cells was also observed using T_{CM}-derived cells that were not EGFRt-enriched prior to adoptive transfer (**Supplementary Figure S2**).

To provide further evidence that improved CAR T cell persistence can be achieved by blocking interactions *in vivo* with FcRs, we evaluated the use of intravenous immunoglobulin (IVIG) to compete for FcR-mediated effects. For this experiment, engraftment of T cells expressing either the nonmutated CD19R (47.2% CAR$^+$) or the CD19R(EQ) (37.9% CAR$^+$) was monitored 1 day after T cell administration to assess early effects of IgG-FcR interactions on CAR T cell persistence (**Figure 6**). As expected, when the peripheral blood was examined 1 day after T cell administration, only low levels of EGFRt$^+$ human (*i.e.*, huCD45-gated) cells are observed in the peripheral blood of mice that received nonmutated CD19R-expressing T cells (12–16% EGFRt$^+$ cells), which represented a significant reduction as compared to the input cells (45.9% EGFRt$^+$; **Figure 6**). This is in contrast to mice that received the CD19R(EQ)-expressing T cells, which show similar EGFRt$^+$ human T cell levels in the

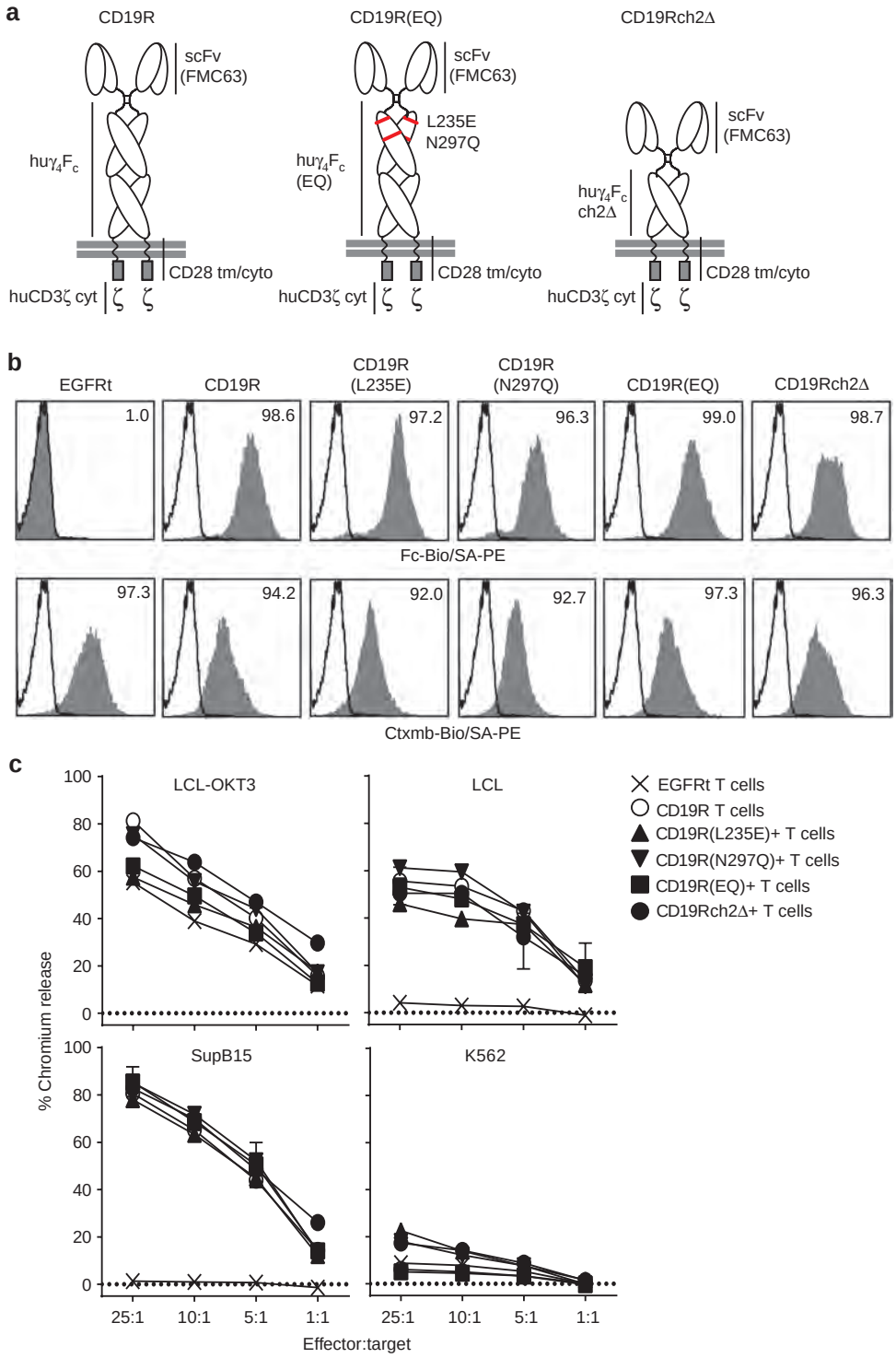

Figure 3 Mutated IgG4 spacer does not affect CD19-specific cytolytic function of CAR-expressing T cells. (**a**) Schematics of the parental CD19-specific CAR (CD19R), the CD19-specific CAR that contains the two point mutations, L235E and N297Q, in the CH2 portion of the IgG4 spacer (CD19R(EQ)), and the CD19-specific CAR that contains a truncated IgG4 spacer, where the whole CH2 domain (amino acid 231–340) is removed (CD19Rch2Δ). The ligand-binding scFv domain derived from the FMC63 mAb, the transmembrane and cytoplasmic signaling domains derived from huCD28, and the cytoplasmic signaling domain of huCD3ζ are also depicted. (**b**) T_{CM}-derived, EGFRt-enriched, and expanded cells expressing either the EGFRt marker alone, the parental CD19R, the CD19R containing a single IgG4 point mutation at either amino acid 235 (CD19R(L235E)) or amino acid 297 (CD19R(N297Q)), the double-mutated CD19R(EQ), or the CH2-deleted CD19Rch2Δ were analyzed for transgene expression. Percentages of cells staining with antibodies specific for the Fc-containing CAR (top) or EGFRt (bottom) are indicated in each histogram, and based on an M1 gate set to detect ≤1% of that stained with SA-PE alone (black line). (**c**) The same cells as in **b** were used as effectors in a 4-hour chromium release assay against ^{51}Cr-labeled CD19^{+} LCL or SupB15 targets. LCL expressing the CD3 agonist OKT3 (LCL-OKT3) and CD19-negative K562 cells were used as positive- and negative-control targets, respectively. Mean percent chromium release ± SD of triplicate wells at the indicated E:T ratios are depicted.

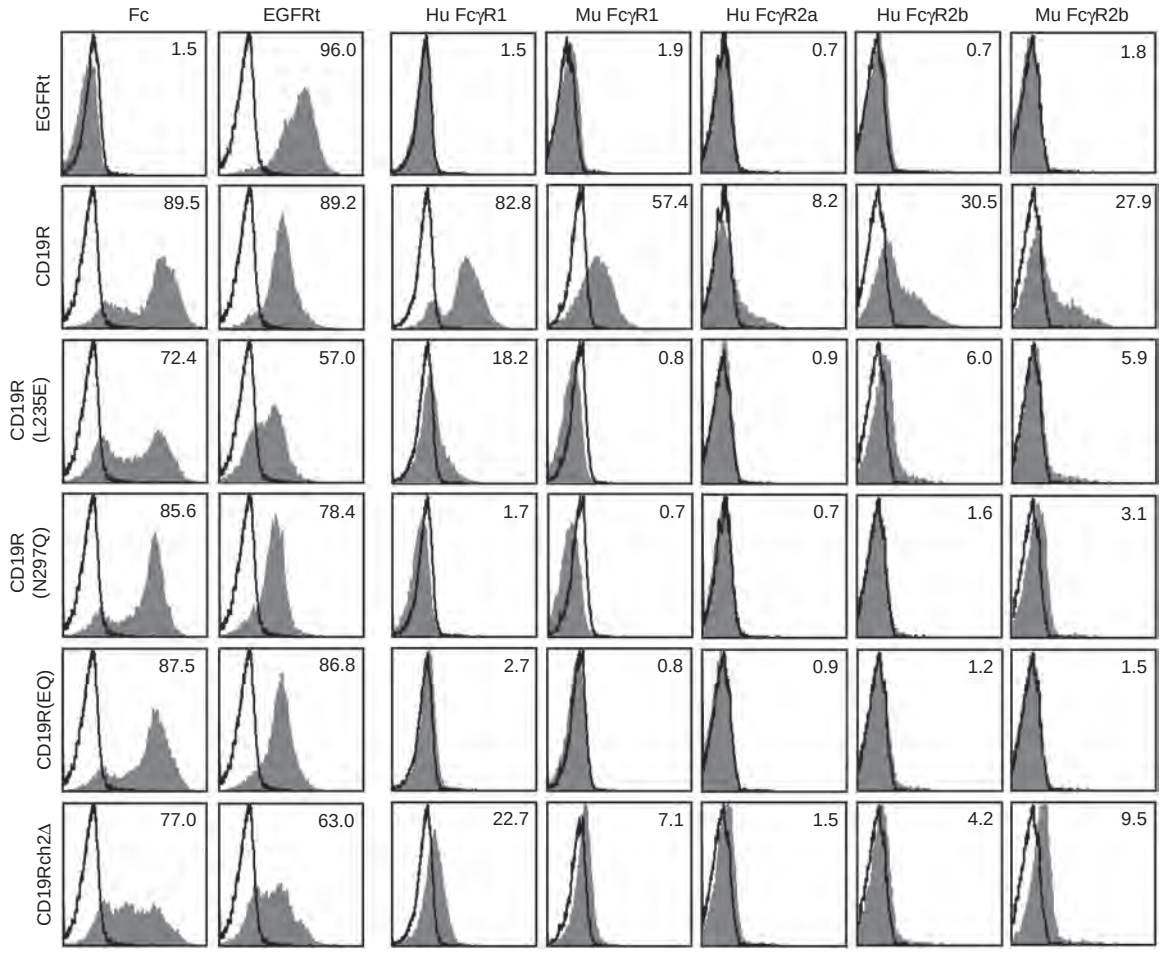

Figure 4 CARs with mutated IgG4 spacers exhibit inhibited FcγR binding. T_{CM}-derived, EGFRt-enriched, expanded cell lines expressing either the EGFRt marker alone, the parental CD19R, the CD19R(L235E), the CD19R(N297Q), the CD19R(EQ), or the CD19Rch2Δ were stained with the following biotinylated reagents: anti-Fc antibody (to detect the CAR), cetuximab (to detect EGFRt), or the indicated human (Hu) or murine (Mu) soluble Fc receptors (FcγR1, R2a, or R2b), followed by PE-conjugated streptavidin (SA-PE, gray histogram). Percentages of immune reactive cells are indicated in each histogram, and based on an M1 gate set to detect ≤1% of that stained with SA-PE alone (black line).

peripheral blood (22–33% $EGFRt^+$ cells) as compared to the input cells (36.6% $EGFRt^+$; **Figure 6**). Importantly, administration of human IVIG at clinically relevant levels (600 mg/kg, or 12 mg per 20 g mouse over 2 days) prior to administration of the T cells resulted in an approximate twofold increase in engraftment for the nonmutated CD19R-expressing T cells, to levels similar to that observed for the CD19R(EQ)-expressing T cells (**Figure 6**). Taken together, our data suggests that reducing interactions with FcRs through specific mutations or FcR blocking with IVIG improves persistence of IgG-containing CAR T cells.

T cells with CAR mutants exhibit improved therapeutic efficacy

We next tested the impact of improved T cell engraftment on therapeutic efficacy by comparing antitumor responses of T_{CM}-derived cells engineered to express either the nonmutated CD19R, the double point-mutated CD19R(EQ), or the CH2-deleted CD19Rch2Δ. NSG mice were injected i.v. with a CD19-expressing EBV-transformed lymphoblastoid cell line (LCL) that had been transduced to express firefly luciferase (ffLuc) to allow for bioluminescent monitoring of *in vivo* tumor growth. Following $ffLuc^+$ LCL engraftment, the mice were treated i.v. with either phosphate-buffered saline as a control or 5×10^6 CAR+ T cells expressing either the EGFRt marker alone, CD19R, CD19R(EQ), or CD19Rch2Δ. Expression of either the CD19R(EQ) and CD19Rch2Δ on the T_{CM}-derived cells resulted in significant control of tumor growth as monitored by Xenogen imaging and improved survival ($P = 0.0009$; **Figure 7**). This efficacy correlated with the presence/persistence of the gene-modified cells in the peripheral blood at day 21 (**Figure 7d**). Indeed, while the phosphate-buffered saline, CD19R, and EGFRt control groups all had to be euthanized at day 21, all of the mice in the CD19R(EQ) and CD19Rch2Δ groups survived past 100 days (**Figure 7e**). While these engraftment and efficacy studies focused on the T_{CM} subset of T cells, our findings suggest that the positive benefit of IgG4-mutations for eliminating FcR interaction are independent of the T cell population that is engineered. Indeed, expression of the CD19R(EQ) in bulk peripheral blood mononuclear cell–derived T cells, instead of T_{CM}-derived lines, also resulted in improved antitumor efficacy and long-term survival ($P = 0.0295$; **Figure 8**).

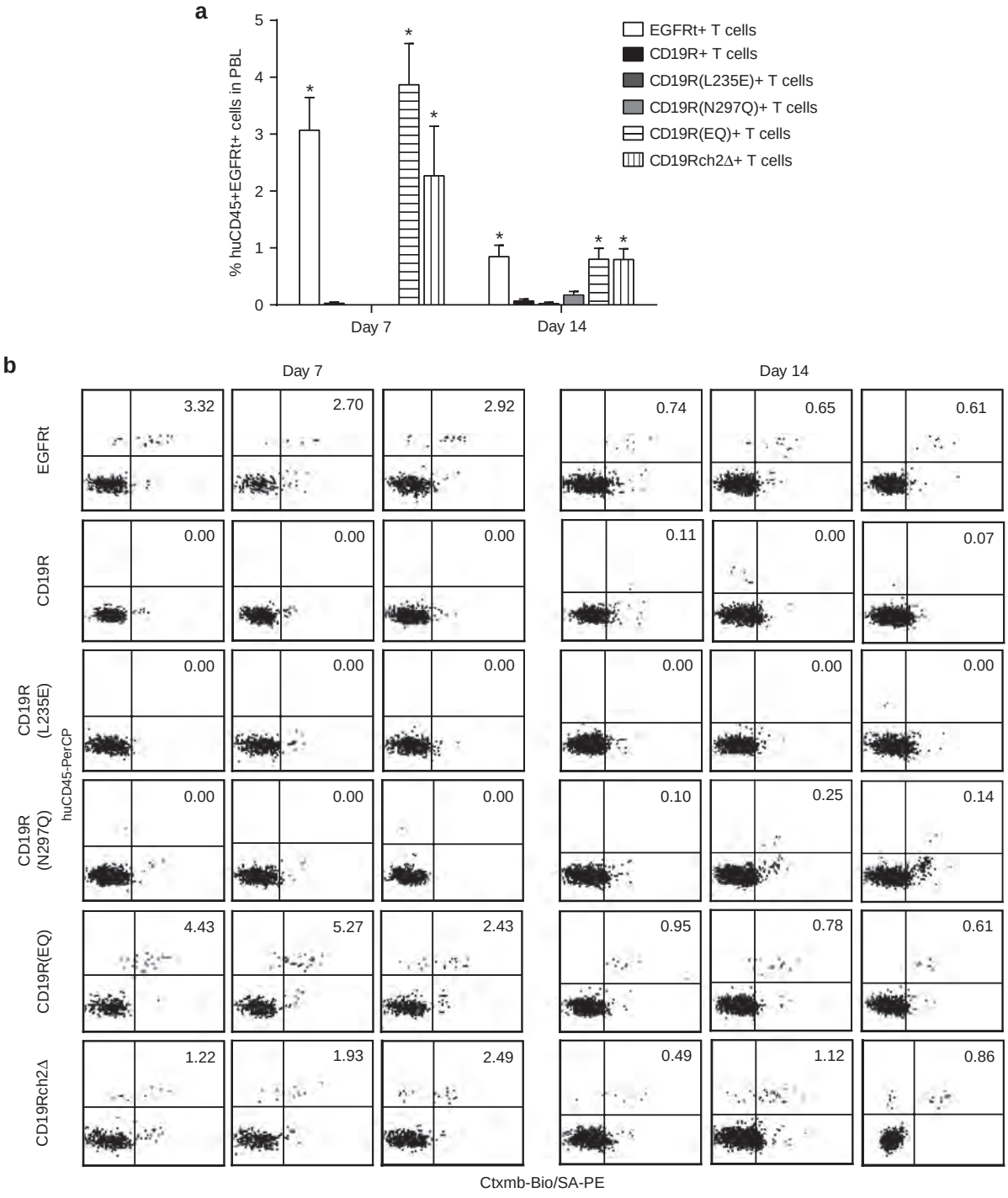

Figure 5 T_{CM}-derived cells expressing CARs with mutated IgG4 spacers exhibit enhanced *in vivo* engraftment. 10^7 T_{CM}-derived, EGFRt-enriched, and expanded cells expressing either the EGFRt marker alone, the parental CD19R, the CD19R(L235E), the CD19R(N297Q), the CD19R(EQ), or the CD19Rch2Δ (see phenotype in **Figure 3b**) were infused i.v. into NSG mice on day 0 with irradiated NS0-IL15 support. Day 7 and 14 peripheral blood leukocytes harvested from each group ($n = 5$ mice) were stained using PerCP-conjugated antihuman CD45, and biotinylated-cetuximab followed by PE-conjugated streptavidin. (**a**) Mean percentages (+SEM) of $CD45^+$ $EGFRt^+$ cells in the viable lymphocyte-gated population of peripheral blood are indicated. *$P < 0.034$ when compared to mice given CD19R-expressing cells using an unpaired Student's *t*-test. (**b**) Representative histograms (*i.e.*, median three of each group of five mice) are depicted with quadrants created based on control staining. Percentages of $huCD45^+EGFRt^+$ cells are indicated in each histogram.

DISCUSSION

Clinically, the *in vivo* therapeutic efficacy of adoptive T cell strategies is known to directly correlate with their engraftment and persistence upon adoptive transfer (reviewed in refs. 27,28). Various approaches have been suggested to improve transferred T cell persistence, including lymphodepletion of the host prior to cell transfer,[29] cytokine support after cell transfer (most recently reviewed in ref. 30), and use of the optimal T cell population(s) for

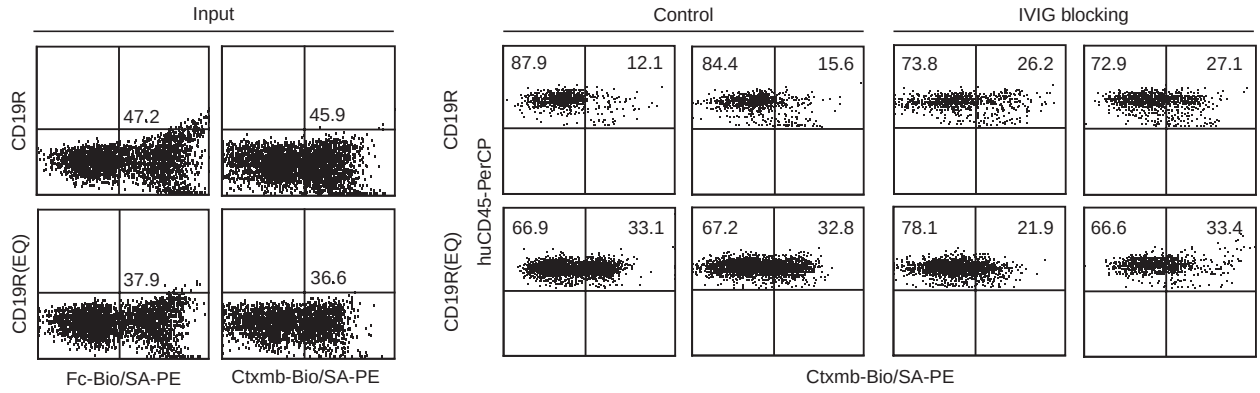

Figure 6 IVIG administration rescues engraftment of T_{CM}-derived cells expressing the nonmutated CAR. 10^7 T_{CM}-derived cells expressing either the parental CD19R or the CD19R(EQ) were infused i.v. into either untreated NSG mice or NSG mice that had received intravenous human immunoglobulin pretreatment (IVIG). Histograms of viable (*i.e.*, DAPI-negative) input cells that were stained with biotinylated anti-Fc (Fc-Bio, to detect CAR) or biotinylated-cetuximab (Ctxmb-Bio, to detect EGFRt) followed by PE-conjugated streptavidin (SA-PE) are depicted on the left, with percentages of CAR^+ or $EGFRt^+$ cells indicated. One day after these cells were administered to the mice, peripheral blood leukocytes were harvested from each group ($n = 2$ mice) and stained using DAPI as a viability dye, PerCP-conjugated antihuman CD45, and Ctxmb-Bio followed by SA-PE. Representative histograms of viable human CD45-gated cells are depicted on the right, with quadrants created based on control staining. Percentages of $huCD45^+$ and $huCD45^+EGFRt^+$ cells are indicated in each histogram.

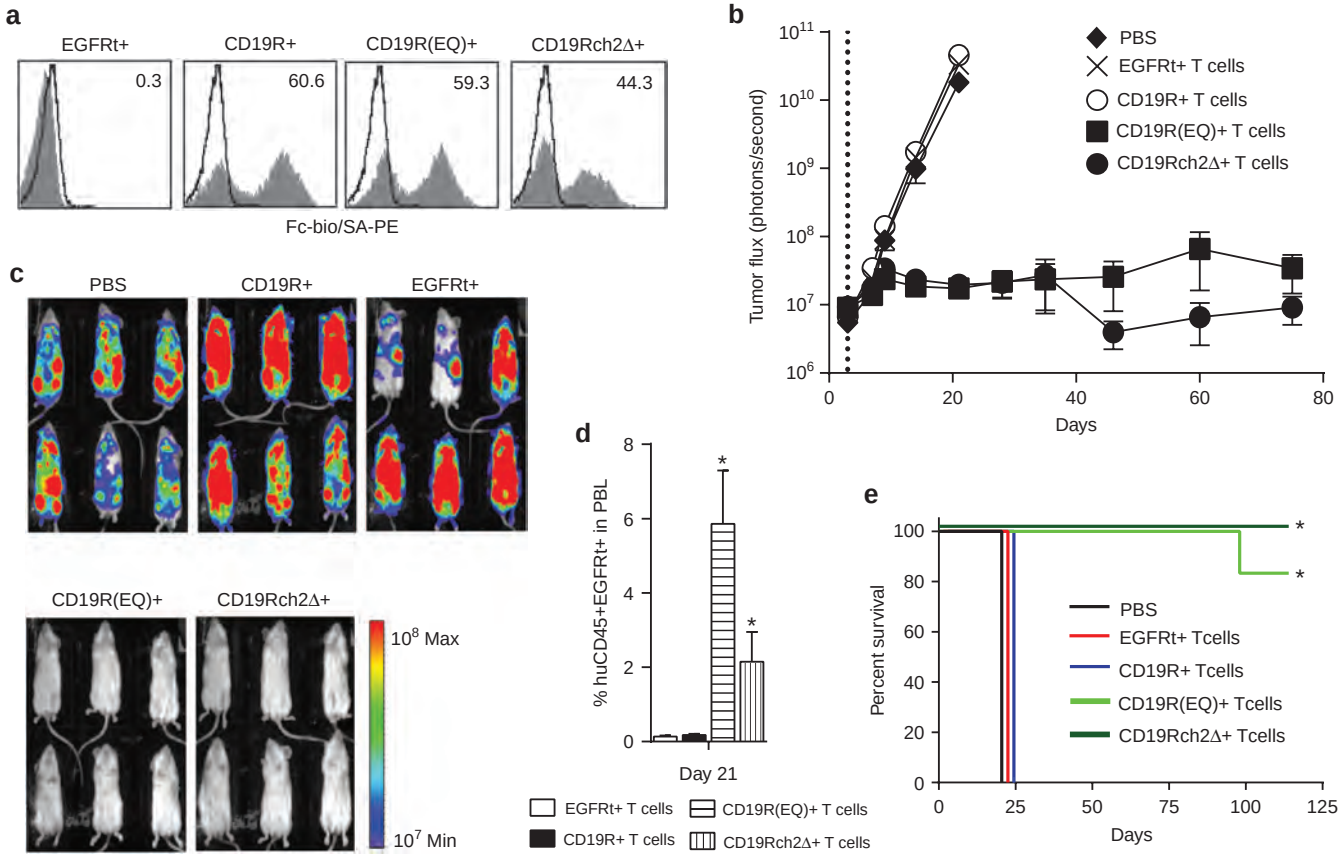

Figure 7 T_{CM}-derived cells expressing CARs with mutated IgG4 spacers exhibit enhanced therapeutic efficacy. 1.5×10^6 $ffLuc^+$ LCL cells were administered i.v. into NSG mice on day 0, and then 5×10^6 CAR^+ T_{CM}-derived cells (10^7 cells total) expressing either the EGFRt marker alone, the parental CD19R, the double point-mutated CD19R(EQ), or the CH2-deleted CD19Rch2Δ were infused i.v. into NSG mice on day 3. LCL tumor growth was then monitored by Xenogen imaging. (**a**) Flow cytometric analysis depicting the CAR profiles of the input T_{CM}-derived cells (used at day 23 after bead stimulation and lentitransduction). Percentages of immunoreactive cells are indicated in each histogram, and based on an M1 gate set to detect ≤1% of that stained with SA-PE alone (black line). (**b**) Mean flux levels (±SEM) of luciferase activity are depicted for each group ($n = 6$). (**c**) Representative bioluminescence images of NSG mice at day 21 are depicted for each group. (**d**) Mean percentages (+SEM) of $CD45^+$ $EGFRt^+$ cells in the viable lymphocyte-gated population of peripheral blood at day 21 are indicated. *$P < 0.035$ when compared to mice given CD19R-expressing cells using an unpaired Student's *t*-test. (**e**) Kaplan–Meier analysis of survival for each group. Log-rank (Mantel-COX) tests were used to perform statistical analyses of survival between groups. *$P = 0.0009$ when compared to mice that received T cells expressing the parental CD19R.

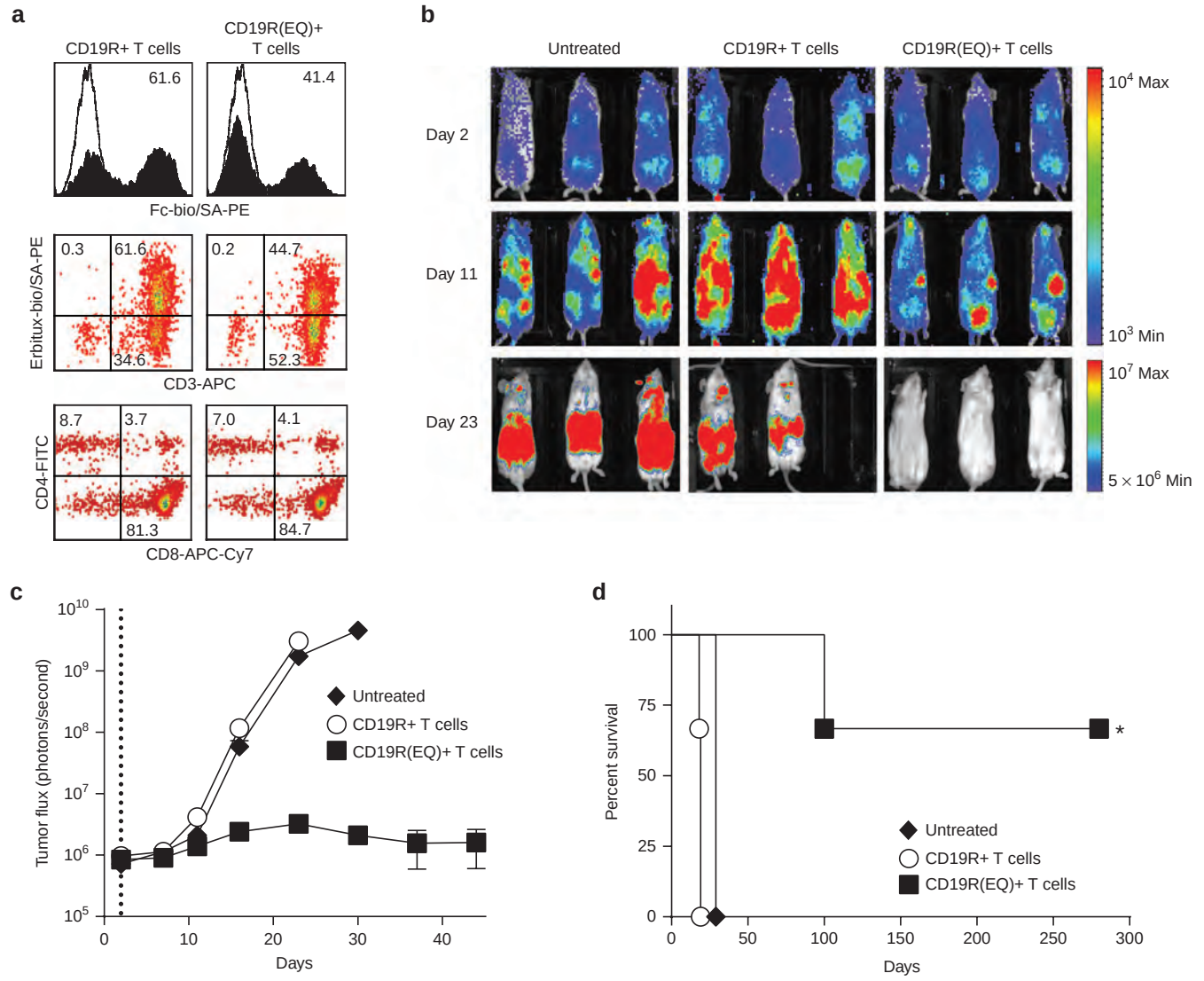

Figure 8 Bulk T cells expressing CD19R(EQ) exhibit enhanced therapeutic efficacy. 1.5×10^6 ffLuc$^+$ LCL cells were administered i.v. into NSG mice on day 0, and then 5×10^6 CAR$^+$ T cells expressing either the parental CD19R or the double point-mutated CD19R(EQ) were infused i.v. into NSG mice on day 2. LCL tumor growth was then monitored by Xenogen imaging. (**a**) Flow cytometric analysis of the CAR (top), EGFRt versus CD3 (middle), and CD4 versus CD8 (bottom) profiles of the input T cells (used at day 21 after bead stimulation and lentitransduction). Percentages of immunoreactive cells as determined by histogram subtraction (top), or based on quadrants that were drawn according to the staining of mock-transduced cells and isotype control staining (middle, bottom) are depicted in each histogram. (**b**) Representative bioluminescence images of NSG mice at days 2, 11, and 23 are depicted for each group. (**c**) Mean flux levels (±SE) of luciferase activity are depicted for each group ($n = 3$). (**d**) Kaplan–Meier analysis of survival for each group. Log-rank (Mantel-COX) tests were used to perform statistical analyses of survival between groups. *$P = 0.0295$ when compared to mice that received T cells expressing the parental CD19R.

transfer.[21,31–33] Here, we provide further evidence that CAR design plays a significant role in directing the engraftment and persistence of therapeutic cells. Indeed, second- and third-generation CARs have shown the benefit of including costimulatory signaling domains within the CAR (reviewed in ref. 34). Our data now also suggest that the sequences that are known as either the spacer, hinge, and/or linker used to connect the ligand-binding domain to the signaling domain(s) of the CAR is of previously unappreciated importance for *in vivo* therapeutic outcome in murine models of malignant disease. Specifically, we have found that the use of an Ig Fc spacer—which has been included in CARs designed by our group and others[8–16]—can potentially inhibit the engraftment and/or persistence of CAR-expressing cells in NSG mouse models in a manner that correlates with FcγR binding. This lack of engraftment can be partially rescued by IVIG administration which is anticipated to compete for FcR-mediated binding. Furthermore, prevention of FcγR binding by either point mutation or deletion of the relevant sequences within the CAR Fc domain can restore the *in vivo* persistence of the adoptively transferred cells to that of cells which do not express a CAR. The increased *in vivo* persistence that is mediated by the spacer-optimized CAR then translates into significantly improved CAR-directed antitumor therapy in our *in vivo* mouse model.

The immunological clearance of adoptively transferred T cells is not a new issue. For example, cellular immune rejection responses against the HyTK and NeoR selection genes that

were coordinately expressed with the CAR have been reported by our group and others.[35,36] However, this work now highlights the importance of FcR-mediated responses against CAR-expressing T cells for *in vivo* T cell persistence and antitumor efficacy. Our studies then also show that there is a relatively easy "fix" to avoid this form of immunogenicity—namely, the incorporation of mutations in the CAR design to prevent FcγR recognition. Indeed, these findings extend those of Hombach *et al.*[20] who demonstrated the utility of modifying an IgG1 Fc linker sequence within the CAR to reduce off-target T cell activation in the presence of FcγR+ cells *in vitro*. While Hombach *et al.*[20] did not directly evaluate either FcR-binding to their CAR$^+$ T cells or the *in vivo* impact of their IgG1 Fc mutations, our studies suggest that such mutations which prevent FcR-interactions would improve persistence of IgG1-containing CAR$^+$ T cells.

Other modifications in CAR design might be just as efficacious as those described in this report in preventing the FcR-mediated clearance of therapeutic cells. One might simply use hinge/spacer sequences that do not originate from Ig Fc domains, such as those from CD8α or CD28.[1,37–39] Although these spacer sequences would alleviate FcR binding, their length may not endow CAR T cells with optimal potency when targeting certain antigens. For instance, when targeting 5T4, NCAM, and MUC1 using CAR T cells, longer linker regions (*i.e.*, longer than those derived from CD8α or CD28) were required for optimal potency.[6,7] Thus, currently, there is no general principle that can be applied to the optimal hinge/spacer/linker to use when designing a CAR. Rather, the optimal sequence to use for a particular antigen will need to be empirically determined. Because several recent studies have examined the use of an IgG spacer in CAR design,[4,5,40] we believe that the mutations presented here will allow investigators to better examine and compare the *in vivo* activity of such Ig Fc-containing CARs without the complications in data interpretation resulting from presumed FcR-mediated rejection/clearance of the CAR-expressing cells.

It remains to be seen whether the mutations described here will augment the persistence and therapeutic efficacy of T cells expressing IgG-spacer containing CAR in humans. The discrepancy in CAR T cell engraftment and *in vivo* antitumor efficacy that we have observed is likely impacted by the murine NSG model system. Human IgG4 has been shown to efficiently bind murine FcRs to mediate potent antibody-dependent cell-mediated cytotoxicity.[41,42] In contrast, human FcRs have the strongest affinity toward IgG1 and IgG3 and reduced affinity for IgG4.[17,43] Additionally, given that NSG mice lack serum antibodies, FcRs expressed by their innate immune cells are unoccupied and thus have a greater potential to bind the IgG-Fc spacer within the CAR. With the exception of hypoglobulinemia cases, immunocompetent humans have high serum IgG levels of ~10 mg/ml,[44] which could potentially compete for recognition of IgG-containing CARs as we have shown in our studies (**Figure 6**). Indeed, several groups have administered IgG-Fc bearing CAR T cells to humans, and in some cases, low levels of CAR T cells were detectible by quantitative PCR up to 6 weeks[8] and even 1 year[13] after administration. We propose, however, that incorporation of the mutations described here are not deleterious to CAR T cell function and, importantly, may further improve this CAR T cell persistence in humans.

Overall, the studies reported here provide evidence that CARs containing components of an Ig Fc spacer should incorporate modifications that prevent the FcR-mediated recognition of the cells *in vivo*. Such modifications can involve either point mutations to change the amino acid sequence or sequence deletions such as that seen with our CD19R(EQ) and CD19Rch2Δ constructs. In fact, while this manuscript was under review, Hudecek *et al.*[45] has reported on the efficacy of hinge mutations involving the replacement of IgG4 CH2 amino acids with the corresponding IgG2 amino acids in improving CAR T cell *in vivo* persistence and antitumor responses. Not only will modifications such as these prevent the ability of FcR-expressing cells to recognize the CAR-expressing immunotherapeutic cellular product *in vivo*, but they might also prevent the unintentional activation of the transferred T cells and/or the host immune responses,[20] which could contribute to various unwanted side effects of this immunotherapeutic strategy.

MATERIALS AND METHODS

DNA constructs and lentiviral vectors. The CD19R28Z-T2A-EGFRt_epHIV7 lentiviral construct used to generate the parental CD19R$^+$ T cells contains (i) the CAR sequence consisting of the V_H and V_L gene segments of the CD19-specific FMC63 mAb, an IgG4 hinge-C_{H2}-C_{H3}, the transmembrane and cytoplasmic signaling domains of the costimulatory molecule CD28 that contains gg mutations that enhance CAR expression and function,[46] and the cytoplasmic domain of the CD3ζ chain[47]; (ii) the ribosomal skip T2A sequence,[48] and (iii) the truncated EGFR sequence.[19] The EGFRt-T2A-DHFR^{FS}-T2A-IMPDH2^{IY}_epHIV7 lentiviral vector used to generate EGFRt$^+$ T cells was previously described.[49] The CD19R(L235E)28Z-T2A-EGFRt_epHIV7, CD19R(N297Q)28Z-T2A-EGFRt_epHIV7, and CD19R(EQ)28Z-T2A-EGFRt_epHIV7 vectors used to generate the CD19R(L235E)$^+$, CD19R(N297Q)$^+$, and CD19R(EQ)$^+$ T cells, respectively, were created by site-directed mutagenesis using the QuikChange II XL kit (Agilent Technologies, Santa Clara, CA) of a codon-optimized CD19R28Z_pGA plasmid that had been synthesized by Geneart (Life Technologies, Grand Island, NY), digested with *Nhe*I/*Rsr*II, and ligated with a similarly digested CD19R28Z-T2A-EGFRt_epHIV7. The CD19Rch2Δ28Z-T2A-EGFRt_epHIV7 vector used to make CD19Rch2Δ$^+$ T cells was generated from a codon-optimized CD19R-HL-CH3(CO)_pMK-RQ plasmid (containing a deletion of amino acids 231–340 within the IgG4 hinge sequence) that had been synthesized by Geneart, digested with *Nhe*I/*Rsr*II, and ligated with a similarly digested CD19R28Z-T2A-EGFRt_epHIV7. All construct and construction-associated PCR primer sequences are available upon request.

Cell lines and maintenance. Human peripheral blood mononuclear cells were isolated as described[21] from heparinized peripheral blood obtained from discard kits containing residual blood components of healthy donors that had undergone apheresis at the City of Hope National Medical Center (COHNMC). Because this was de-identified discard blood material, informed consent was waived with the approval of the COHNMC Internal Review Board, and the COHNMC Office of Human Subjects Protection. T_{CM} isolation (using CD14- and CD45RA-depletion followed by CD62L-selection), anti-CD3/CD28 bead stimulation, and lentiviral-mediated transduction were then done as previously described.[10] In some cases, transduced T_{CM} cells were immunomagnetically enriched for EGFRt expression as previously described[19] and/or expanded using a rapid expansion method as previously described.[21] Bulk T cell stimulation, lentiviral-mediated transduction, and expansion were also done as previously described.[16]

EBV-transformed LCL and LCL that expressed OKT3 (LCL-OKT3)[21] or ffLuc$^+$ LCL cells were cultured in RPMI 1640 (Irvine Scientific, Santa

Ana, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT) 2 mmol/l L-glutamine (Irvine Scientific), and 25 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Irvine Scientific). ffLuc$^+$ LCL were generated by transduction with lentiviral vector eGFP-ffluc_epHIV7 at an multiplicity of infection of 20 in the presence of 5 μg/ml polybrene in 500 μl medium, and subsequent purification by sorting GFP$^+$ cells.

Mouse myeloma cells secreting human homeostatic IL-15 cytokine (NS0-IL15) were generated as previously described.[21]. SupB15 and K562 leukemia cell lines (ATCC, Manassas, VA) were grown in the corresponding ATCC recommended media.

Antibodies and flow cytometry. Fluorochrome-conjugated isotype controls, anti-CD3, anti-CD4, anti-CD8, anti-CD45, and streptavidin were obtained from BD Biosciences (San Jose, CA). Biotinylated anti-Fc was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Generation of biotinylated-cetuximab was done as previously described.[19] Biotinylated huFcγR1, muFcγR1, huFcγR2a, huFcγR2b, and muFcγR2b were obtained from Sino Biological (Beijing, China). 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Invitrogen, Life Technologies. The percentages of immunofluorescent cells were analyzed by a FACScalibur system (BD Biosciences) or a MACSQuant Analyzer (Miltenyi Biotec, Auburn, CA), and the percentages of cells in the indicated regions of analysis were calculated using FCS Express V3 (De Novo Software, Glendale, CA).

In vivo *T cell engraftment and therapy.* All mouse experiments were approved by the COHNMC Institute Animal Care and Use Committee. For engraftment studies, 6–10-week-old NOD/*Scid* IL-2RγC ^{null} (NSG) mice were injected i.v. on day 0 with 10^7 of the indicated T_{CM}-derived cells, and i.p. injections three times a week of 2×10^7 irradiated NS0-IL15 to provide a systemic supply of human IL-15 *in vivo*. Peripheral blood was harvested from retro-orbital bleeds, red blood cells were lysed, and cell suspensions were analyzed by flow cytometry. For the IVIG studies, NSG mice were injected i.v. with 6 mg Gamunex-C (Immune Globulin Intravenous – Human, NDC-13533-800-71, COH Pharmacy) on each of days −2 and −1 prior to administration of 10^7 T_{CM}-derived cells on day 0. Mice were euthanized 1 day later, peripheral blood was harvested by cardiac puncture, red blood cells were lysed, and cell suspensions were analyzed by flow cytometry. For the therapeutic studies, 1.5×10^6 ffLuc$^+$ LCL cells were administered i.v. into 6–8-week-old NSG mice on day 0, and then 5×10^6 of the indicated CAR$^+$ T cells were administered i.v. on day 3. Luciferase activity was measured by Xenogen imaging as previously described.[21]

Chromium-release assays. Four-hour ^{51}Cr-release assays were performed as previously described[50] using the indicated effector/target cell ratios.

SUPPLEMENTARY MATERIAL

Figure S1. Phenotype of FcR-expressing cells in NSG mice.
Figure S2. Non-enriched T_{CM}-derived cells expressing CARs with mutated IgG4 spacers exhibit enhanced in vivo engraftment.

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