CD28 Costimulation Provided through a CD19-Specific Chimeric Antigen Receptor Enhances *In vivo* Persistence and Antitumor Efficacy of Adoptively Transferred T Cells

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Abstract

Chimeric antigen receptors (CAR) combine an antigen-binding domain with a CD3-ζ signaling motif to redirect T-cell specificity to clinically important targets. First-generation CAR, such as the CD19-specific CAR (designated CD19R), may fail to fully engage genetically modified T cells because activation is initiated by antigen-dependent signaling through chimeric CD3-ζ, independent of costimulation through accessory molecules. We show that enforced expression of the full-length costimulatory molecule CD28 in $CD8^+CD19R^+CD28^-$ T cells can restore fully competent antigen-dependent T-cell activation upon binding $CD19^+$ targets expressing CD80/CD86. Thus, to provide costimulation to T cells through a CD19-specific CAR, independent of binding to CD80/CD86, we developed a second-generation CAR (designated CD19RCD28), which includes a modified chimeric CD28 signaling domain fused to chimeric CD3-ζ. $CD19R^+$ and $CD19RCD28^+$ $CD8^+$ T cells specifically lyse $CD19^+$ tumor cells. However, the $CD19RCD28^+$ $CD8^+$ T cells proliferate in absence of exogenous recombinant human interleukin-2, produce interleukin-2, propagate, and up-regulate antiapoptotic Bcl-X_L after stimulation by $CD19^+$ tumor cells. For the first time, we show *in vivo* that adoptively transferred $CD19RCD28^+$ T cells show an improved persistence and antitumor effect compared with $CD19R^+$ T cells. These data imply that modifications to the CAR can result in improved therapeutic potential of CD19-specific T cells expressing this second-generation CAR. (Cancer Res 2006; 66(22): 10995-1004)

Introduction

Adoptive transfer of T cells expressing chimeric antigen receptors (CAR) into oncology patients is a promising approach to eradicate tumor cells. Initially, CARs designed for adoptive immunotherapy were generated by connecting an extracellular antigen-binding domain to a transmembrane domain and a single intracellular signal transduction domain, such as CD3-ζ or FcRγ (1–8). When these first-generation CARs were expressed in primary T cells, the genetically modified T cells showed antigen-specific binding, and target cell lysis *in vitro*, but limited *in vivo* antitumor efficacy (9). One restriction of a CAR that activates T cells solely through chimeric CD3-ζ is that engagement of this signaling motif does not fully activate the genetically modified T cells, resulting in compromised antigen-dependent interleukin-2 (IL-2) production, cell proliferation, and survival. This is in contrast to the physiologic activation of T cells via the endogenous αβ T-cell receptor by antigen-presenting cells (APC), which leads to both a primary signal initiated by the engagement of CD3-ζ and a second signal provided by ligation of costimulatory receptors, such as CD28 (10–12). Binding of CD28 on activated antigen-specific T cells to its ligands CD80/CD86 on APCs or tumor cells is essential for (*a*) producing IL-2 (13–15), (*b*) preventing anergy (16), (*c*) promoting survival through up-regulation of antiapoptotic proteins (17, 18), and (*d*) prevention of replication senescence (19). Because B-lineage malignancies, such as acute lymphoblastic leukemias, vary or lack expression of CD28 ligands (20–22), and because *ex vivo* propagation of our genetically modified T cells typically leads to loss of expression of endogenous CD28 (8), infused T cells expressing first-generation CARs may be prone incomplete T-cell activation, leading to truncated *in vivo* persistence, compromising the therapeutic success of this adoptive immunotherapy.

One target for redirected T-cell specificity is CD19, a cell surface molecule that is restricted to B cells and their malignant counterparts (23). In this study, we investigated whether we could provide CD28-mediated costimulation to genetically modified T cells through a CD19-specific CAR. Using $CD8^+CD28^-$ T cells expressing the first-generation CD19-specific CAR (designated CD19R), we show that enforced expression of full-length CD28 transgene in $CD19R^+$ T cells could restore CD19-dependent costimulation through introduced CD28. To provide fully competent T-cell activation through the CD19-specific CAR, which could be used to target tumors lacking CD80/CD86, we generated a second-generation CD19-specific CAR (designated CD19RCD28), by fusing a CD19-specific scFv and modified human Fc region to the transmembrane and modified intracellular signaling domains of CD28 and CD3-ζ. We compared $CD19R^+$ and $CD19RCD28^+$ T cells *in vitro* and *in vivo* and show augmented persistence of adoptively transferred $CD19RCD28^+$ T cells in a nonobese diabetic, severe combined immunodeficient (NOD/*scid*) mouse model, which results in an increased clearance of established $CD19^+$ tumor.

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Materials and Methods

DNA constructs. *CD19R* transgene has been previously described (8). The *CD19RCD28* transgene was assembled by PCR using splicing by overlap extension. It was composed of a CD19-specific single-chain variable fragment (scFv) derived from the murine FMC63 monoclonal antibody (23) fused to a modified (CPSC → CPPC; ref. 24) human IgG4 hinge and Fc region fused to residues 153 to 179 of intracellular human CD28 molecule, which included the transmembrane and a modified (RLLH → RGGH; ref. 25) intracellular domain, fused to residues 31 to 142 of the human cytoplasmic CD3-ζ chain (Fig. 2*A*). The *CD19RCD28* and *CD19R* transgenes were inserted into the plasmid ffLucHyTK-pMG (26) to create the plasmids CD19RCD28/ffLucHyTK-pMG (Fig. 2*B*) and CD19R/ffLucHyTK-pMG, respectively. A bifunctional *hRluc-Zeocin* fusion gene that coexpressed the *Renilla* luciferase (*hRluc*) and zeomycin resistance genes (*zeo*) was cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) to generate plasmid hRluc:Zeocin-pcDNA3.1.

Cell culture and genetic modification. T cells were isolated from primary peripheral blood mononuclear cells (PBMC), numerically expanded, and genetically modified by electroporation as previously described (8, 27). Briefly, expansion cultures were established using 10^6 T cells, 30 ng/mL anti-CD3 (OKT3; Ortho Biotech, Raritan, NJ), 5×10^7 γ-irradiated allogeneic PBMC (3,500 cGy), and 10^7 γ-irradiated allogeneic lymphoblastoid cell lines (LCL; 8,000 cGy) in 50 mL culture medium containing 10% FCS (Hyclone, Logan, UT) or 10% human AB serum. Recombinant human IL-2 (rhIL-2; Chiron, Emeryville, CA) was added at 25 to 50 units/mL every 48 hours, beginning on day 1 of each 14-day expansion cycle. T-cell clones were obtained by plating at limiting dilution in 96-well plates as previously described (8). Transduction of a $CD8^+CD28^-CD19R^+$ T-cell clone was accomplished on day 2 after OKT3-mediated stimulation and 24 hours of culturing in phosphate-deficient medium (Invitrogen, Carlsbad, CA) supplemented with 50 units/mL rhIL-2. The washed T cells were resuspended in retroviral supernatant (collected from puromycin-resistant Phoenix GALV packaging cells) encoding the full-length human transgene (28), supplemented with 50 units/mL rhIL-2 and 8 μg/mL polybrene, spun at 1,000 × *g* for 1 hour at 32°C, and incubated for 23 hours. T cells were then washed and cultured in culture medium containing 50 units/mL rhIL-2. The leukemia cell lines SUP-B15, RS4, JM-1, Daudi, and $CD19^-CD80^-CD86^-$ K562 cells were obtained from the American Type Culture Collection (Manassas, VA). The EBV-transformed LCL was kindly provided by Drs. Phillip Greenberg and Stanley Riddell (Fred Hutchinson Cancer Research Center, Seattle, WA). These lines were cultured as previously described (8). Daudi cells were electroporated with the linearized plasmid hRLuc:Zeocin-pcDNA3.1 as previously described (27). Three days after electroporation, zeocin (InvivoGen) was added to the culture at a cytocidal concentration of 0.4 mg/mL.

Flow cytometry. $F(ab')_2$ fragment of FITC-conjugated goat anti-human Fcγ (Jackson ImmunoResearch, West Grove, PA) was used at a 1:20 dilution to detect cell surface expression of CD19R and CD19RCD28. The other FITC-, phycoerythrin-, and CyChrome-conjugated reagents were obtained from BD Biosciences (San Jose, CA). Data acquisition was done on a FACScan and FACSCalibur (BD Biosciences). The median fluorescent intensity, coefficient of variation (CV), and percentage of cells in a region of interest was calculated using CellQuest version 3.3 (BD Biosciences) and FCS Express version 3 (De Novo Software, Thornhill, Ontario, Canada). Fluorescence-activated cell sorting was done on an Epics Altra (Beckman Coulter, Fullerton, CA).

IL-2 production. Secretion of IL-2 was assessed following stimulation with Daudi cells (10^5 per well) of a CD19-specific T-cell clone or CD28 modified CD19-specific T-cell clone (2×10^5 per well). CTLA-4 Ig (10 μg/mL), kindly provided by Dr. George Georges (Fred Hutchinson Cancer Research Center), was added to selected wells; supernatants were harvested after 36 hours; and IL-2 production was analyzed by ELISA (R&D Systems, Minneapolis, MN).

Chromium release assay. A 4-hour chromium release assay (CRA) was used to determine the cytolytic activity of T cells. The assay was carried out and analyzed as previously described using effector to target (*E/T*) cell ratios of 50:1, 25:1, 5:1, and 1:1 (8). Data are reported as mean ± 1 SD.

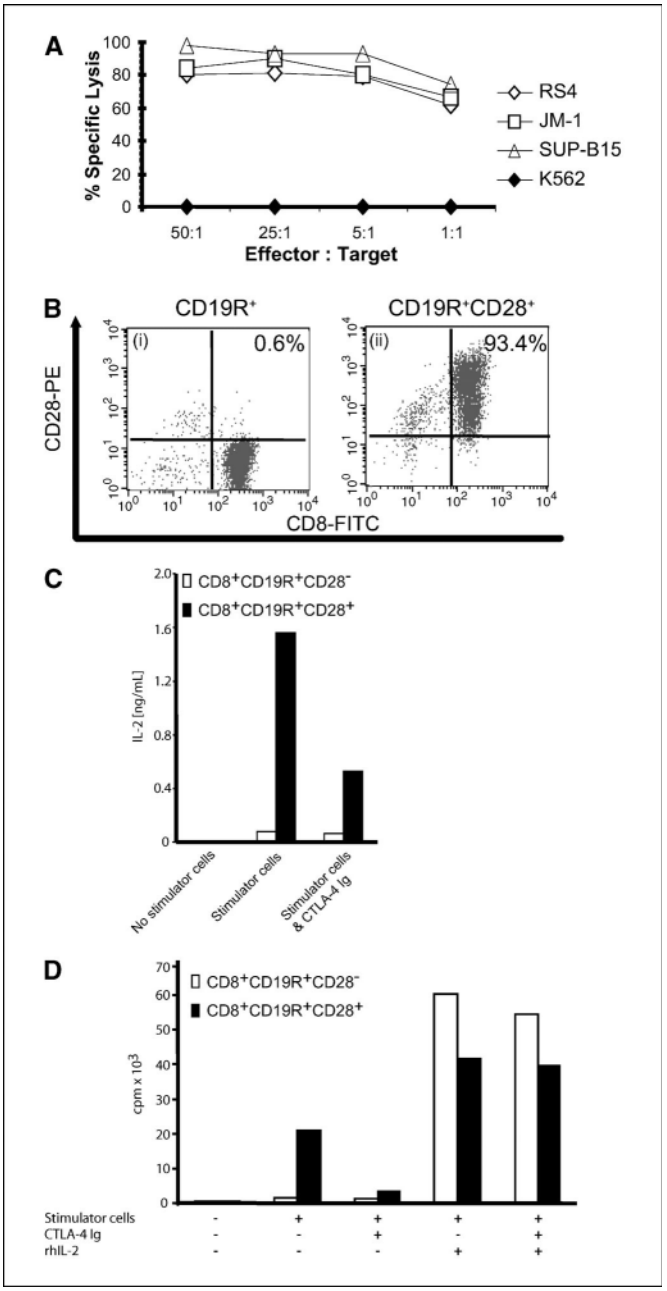

Figure 1. Generation and characterization of $CD19R^+CD28^+$ T cells. *A,* lysis of $CD19^+$ leukemia lines (RS4, JM-1, and SUP-B15) by $CD19R^+CD28^-$ T-cell clone by 4-hour CRA. Absence of lysis of $CD19^-$ K562 cells shows specificity of killing through CD19R CAR. *B,* cell surface expression by flow cytometry of CD28 on $CD19R^+CD8^+$ T-cell clone (*i*) before and (*ii*) after transduction with retrovirus expressing full-length *CD28* transgene. *C,* IL-2 in culture medium was analyzed after 36 hours of coculture of 2×10^5 T cells and Daudi responder tumor cells at 1:1 ratio. *D,* T-cell proliferation by ^3H-thymidine incorporation measured at 90 hours after coculture at 1:1 ratio with 2×10^5 mitomycin C–treated Daudi stimulator cells supplemented with 10 units/mL rhIL-2 and 10 μg/mL CTLA-4 Ig as described.

Quantitative reverse transcription-PCR. T cells were cocultured in a 12-well plate with irradiated (1 Gy) Daudi or SUP-B15 cells at a ratio of 1:5 (responder/stimulator). The cells were harvested after 0, 2, and 24 hours, and RNA for quantitative PCR assays was isolated using the RNeasy kit (Qiagen, Valencia, CA). The SuperScript III Platinum SYBR green One-step qRT-PCR kit (Invitrogen) was used to reverse transcribe and amplify 100 ng of total RNA. Samples were reverse transcribed for 30 minutes at 50°C and

then subjected to 45 rounds of amplification for 10 seconds at 95°C and 30 seconds at 60°C with the Bio-Rad iCycler iQ Multiple-Color Real-time PCR Detection System (Hercules, CA) using real-time PCR tested primer pairs for IL-2, IFN-γ, Bcl-X_L, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Biosource, Camarillo, CA). To confirm accuracy and reproducibility, the real-time PCR was carried out in duplicates for each sample within one iCycler run. Amplification of the GAPDH mRNA was done on all tested samples as a control for variations in amounts of RNA. The quantitative PCR results were analyzed using the comparative C_t method (29).

Proliferation assay. $CD19R^+$ or $CD19RCD28^+$ T cells were cocultured for 72 to 90 hours at a 1:1 ratio with mitomycin C–treated (10% mitomycin C for 90 minutes) Daudi, LCL, SUP-B15, or K562 cells. CTLA-4 Ig (10 μg/mL) and/or rhIL-2 (10 units/mL) was added to selected wells as indicated. During the last 18 hours, the cultures were pulsed with 1 μCi/well (0.037 MBq/well) methyl-[^{3}H]thymidine (ICN, Costa Mesa, CA). Cell-associated radioactivity was measured by scintillation counting (TopCount, Perkin-Elmer, Shelton, CT). Data are reported as mean ± 1 SD.

Xenograft tumor model. On day 0, 24 to 48 hours after irradiation [γ-irradiated to 2.5 Gy using an external ^{137}Cs-source (JL Shepherd Mark I Irradiator, San Fernando, CA), 6- to 10-week-old female NOD/*scid* (NOD/LtSz-*Prkdc*^{scid}/J) mice (Jackson Laboratory, Bar Harbor, ME)] were injected in the peritoneum with 10^6 $hRluc^+$ Daudi cells. Beginning on day 3, tumor engraftment was evaluated by serial biophotonic imaging (see below). Mice with progressively growing tumors were segregated into treatment groups bearing comparable tumor loads. The mice were subsequently injected in the peritoneum with CAR^+ffluc^+ T cells and imaged 2 hours later to record the initial ffLuc-derived optical signal.

Biophotonic imaging. The *in vivo* luciferase activity was noninvasively detected in groups of four NOD/*scid* mice using an intensified charge-coupled device camera (bioluminescence), based on previously published methods (27). The T-cell–derived ffLuc activity and tumor-derived hRluc activity were measured using an IVIS100 series system (Xenogen, Alameda, CA) beginning ~15 minutes after i.p. injection of the respective substrates: 150 μL (4.29 mg/mouse) of D-luciferin potassium salt (Xenogen), 100 μL (64 μg/mouse) of Enduren (Promega, Madison, WI). One unmanipulated mouse in each imaging group was injected with the same doses of D-luciferin or Enduren as the experimental mice to determine the background bioluminescence signal. Photons were quantified using the software program "Living Image" version 2.20 (Xenogen). The bioluminescence signal was measured as total photon flux normalized for exposure time and surface area and expressed in units of photons/s/cm^2/steradian (p/s/cm^2/sr). The statistical difference between treatment groups was determined by comparing the area under the curve using the two-sided Wilcoxon rank sum test. For anatomic localization, a pseudocolor image representing light intensity (blue, least intense; red, most intense) was superimposed over a digital grayscale body surface reference image.

Results

Generation and immunobiology of $CD19R^+$ full-length $CD28^+$ T cells. Our genetically modified T cells recursively expanded every 14 days with OKT3 and rhIL-2 typically lose cell surface expression of endogenous CD28. To evaluate whether CD28-mediated signaling $CD19R^+$ T cells could be reestablished in these T cells, a $CD8^+CD19R^+CD28^-$ T-cell clone, which could lyse $CD19^+$ tumor targets (Fig. 1*A*), was transduced with retrovirus (because re-electroporation of $CD19R^+$ T-cell clones has been unsuccessful) to enforce expression of full-length CD28 transgene. After transduction, the $CD19R^+$ T-cell clone expressed 93% CD28, compared with 0.6% endogenous CD28 expression before genetic modification (Fig. 1*B*). Secretion of IL-2 was used as a surrogate end point for CD28-mediated T-cell activation. As expected, the $CD8^+CD19R^+CD28^-$ T-cell clone failed to produce IL-2 in response to CD19 antigen. However, the CD19-dependent production of IL-2 was restored in transduced $CD8^+CD19R^+CD28^+$ T cells, and IL-2 secretion could be partially blocked with CTLA-4 Ig (Fig. 1*C*). This endogenous production of IL-2 led to the CD19-dependent proliferation of $CD8^+CD19R^+CD28^+$ T cells in the absence of exogenous rhIL-2, which could mostly be blocked by the addition of CTLA-4 Ig (Fig. 1*D*). The $CD8^+CD19R^+CD28^-$ T-cell clone did not proliferate in absence of rhIL-2, but proliferation resulted when rhIL-2 was provided. Addition of exogenous rhIL-2 further enhanced [^{3}H]thymidine uptake by $CD8^+CD19R^+CD28^+$ T cells. This was not significantly blocked by CTLA-4 Ig, which is consistent with T-cell activation for proliferation through the IL-2 receptor beyond activation achieved after binding of introduced CD28 to CD80/CD86 (Fig. 1*D*). As previously shown for $CD8^+$ cytomegalovirus-specific T cells, our results suggest that $CD8^+$ CD19-specific effector T cells do not have an intrinsic block to IL-2 production; rather, the lack of costimulation is due to a failure of endogenous CD28 expression (28).

Generation of $CD19RCD28^+$ T cells. Having shown that enforced expression of full-length CD28 could reestablish fully competent CD19-dependent signaling, we investigated whether the CD19-specific CAR itself could be modified to provide CD28-mediated signaling. This is desirable because some $CD19^+$ malignant targets that lack CD80/CD86 expression will not provide a costimulatory signal to T cells through CD28. Therefore, a second-generation CD19-specific CAR that included a fusion of both chimeric CD28 and CD3-ζ intracellular domains was generated (Fig. 2*A*), and the cDNA was cloned into the expression plasmid ffLucHyTK-pMG (Fig. 2*B*; ref. 26). This plasmid encodes the *ffluc*HyTK transgene, a trifunctional reporter/selection/suicide fusion gene consisting of (*a*) enzymatically active ffLuc, (*b*) hygromycin phosphotransferase (Hy), and (*c*) herpes simplex virus-1 thymidine kinase (TK), which permits *in vitro* selection of the genetically modified T cells with hygromycin B, potential *in vivo* TK-mediated ablation using ganciclovir, and ability to noninvasively measure persistence by bioluminescent imaging (BLI) *in vivo*. To compare the contribution of chimeric CD28 in CD19-specific T-cell activation, we cloned *CD19R* transgene into the same expression plasmid (26). Our *in vitro* tissue culture conditions typically result in the preferential expansion of $CD8^+CD19R^+$ T cells (Fig. 2*C*; refs. 8, 27). However, expression of *CD19RCD28* in primary T cells led to numerical expansion of $CD4^+$ as well as $CD8^+$ T cells (data not shown). Because we wished to evaluate the role of chimeric CD28 in $CD8^+$ T cells, we used flow cytometry sorting to obtain two populations of genetically modified CD19-specific T cells, which were $CD8^+$ and expressed minimal levels of endogenous CD28 (≤4%; Fig. 2*C*). CAR expression was verified by staining with an Fc-specific antibody, and flow cytometry revealed that 58% and 43% of the CD19R- and CD19RCD28-modified T cells were CAR^+ (Fc positive), respectively (Fig. 2*C*). The relative density of the two CARs on the cell surface was nearly identical for $CD19R^+$ and $CD19RCD28^+$ T cells as measured by the median fluorescent intensity from flow cytometry data (13.4 and 12.8 with a CV of 57 and 55, respectively). These results show that the percentage of T cells expressing *CD19R* was slightly higher than T cells expressing *CD19RCD28*, and that the numbers of CAR molecules per T cell were similar.

CD19-specific lysis of tumor cells. The cytolytic abilities of $CD8^+$ T cells expressing either *CD19R* or *CD19RCD28* transgenes were evaluated using a 4-hour CRA. Genetically modified $CD19R^+$ and $CD19RCD28^+$ T cells were able to specifically lyse $CD19^+$ human Daudi lymphoma cells (Fig. 3*A*) and SUP-B15 leukemia

cells (Fig. 3*B*). Lysis of CD19$^-$ K562 cells, a natural killer–sensitive target (30), was not detected, showing the antigen specificity of the cytolysis and apparent lack of natural killer activity. We noted that the CD19RCD28$^+$ T cells had a slightly reduced ability to lyse CD19$^+$ tumor cells compared with CD19R$^+$ T cells. This difference is attributed to an altered molecular distance of the chimeric CD3-ζ domain between the two CARs and/or the slightly decreased expression of the CD19RCD28 CAR relative to CD19R CAR. Taken together, these data suggest that including the chimeric CD28 signaling domain in a CD19-specific CAR did not significantly diminish the cytolytic ability of the genetically modified T cells.

Antigen-specific IL-2 and IFN-γ production by genetically modified T cells. Because T cells increase IFN-γ production upon stimulation with cognate antigen, we measured the production of IFN-γ in the modified T cells using quantitative reverse transcription-PCR (RT-PCR). The results show that both CD19RCD28$^+$ and CD19R$^+$ T cells increased IFN-γ expression after a 2-hour stimulation with CD19$^+$ tumor cells. IFN-γ was up-regulated 164- and 96-fold after stimulation of CD19RCD28$^+$ T cells with CD19$^+$CD80$^+$CD86$^+$ Daudi or CD19$^+$CD80$^-$CD86$^-$ SUP-B15 tumor cells, respectively (Fig. 3*C*). Stimulation of CD19R$^+$ T cells with Daudi or SUP-B15 tumor cells led to a 65- and 57-fold up-regulation of IFN-γ, respectively. No up-regulation of IFN-γ expression was detected in cultures of T cells or tumor cells alone or irradiated T-cell/tumor cocultures. This shows that T-cell activation through CD19RCD28 could lead to a greater induction of CD19-specific IFN-γ production compared with CD19R. These data are consistent with published data that show increased IFN-γ secretion from a second-generation CAR (composed of the CD28 and CD3-ζ signaling domains) compared with a first-generation CAR (31, 32). Because antigen-specific T-cell costimulation through endogenous CD28 can lead to IL-2 production, we used quantitative RT-PCR to evaluate whether the CAR$^+$ T cells were able to up-regulate IL-2 upon stimulation. Our results show that CD19RCD28$^+$ T cells increased IL-2 expression 33- and 9-fold, respectively, when stimulated by CD19$^+$ Daudi or SUP-B15 tumor cells (Fig. 3*D*). No up-regulation of IL-2 could be detected in CD19R$^+$ T cells when stimulated with either tumor cell line. Negative controls that included tumor cells or T cells alone and irradiated T-cell/tumor cocultures did not show a detectable up-regulation of IL-2 expression. The up-regulation of IFN-γ expression as detected in both CD19R$^+$ and CD19RCD28$^+$ T cells is a result of activation through chimeric CD3-ζ. However, the detection of IL-2 expression in only CD19RCD28$^+$ T cells is consistent with stimulation through chimeric CD28. This observation is further supported by the fact that SUP-B15 cells lack cell surface expression of CD80/CD86.

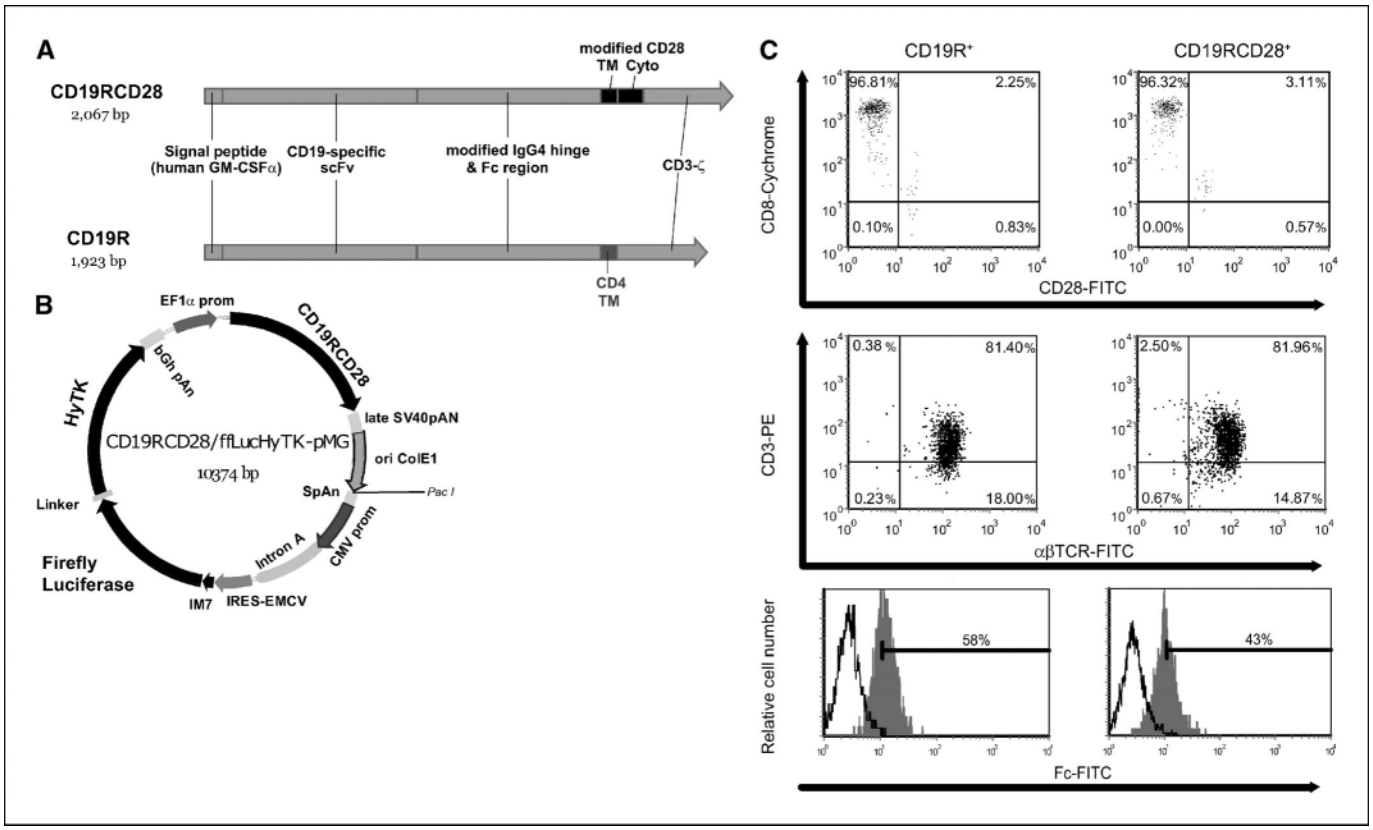

Figure 2. Generation and characterization of CD19R$^+$ and CD19RCD28$^+$ T cells. *A,* schematics of the *CD19RCD28* and *CD19R* transgenes. The CD19RCD28 cDNA contains several modifications. Briefly, CD19RCD28 contains a modified IgG4 hinge (CPSC → CPPC; ref. 24) and a modified transmembrane/intracellular domain from CD28 (RLLH → RGGH; ref. 25). The CD19R cDNA has been described previously (8). *B,* schematic of the expression plasmid CD19RCD28/ffLucHyTK-pMG. EF1α promoter, human elongation factor-1α promoter; CMV promoter, cytomegalovirus (*CMV*) enhancer/promoter; late SV40pAN, polyadenylation signals from SV40; bGh pAn, polyadenylation signal from bovine growth hormone; IM7, synthetic prokaryotic promoter; SpAn, synthetic pause. *C,* phenotype of CD19R$^+$ and CD19RCD28$^+$ T cells by flow cytometry. The genetically modified T cells were stained with CD3-, TCR-, CD8-, and CD28-specific monoclonal antibodies for flow cytometric analysis. To verify expression of the chimeric receptor, the modified T cells were also stained with a goat-derived polyclonal FITC-conjugated Fc-specific antibody and nonspecific control antibody. The percentage of positive cells is indicated.

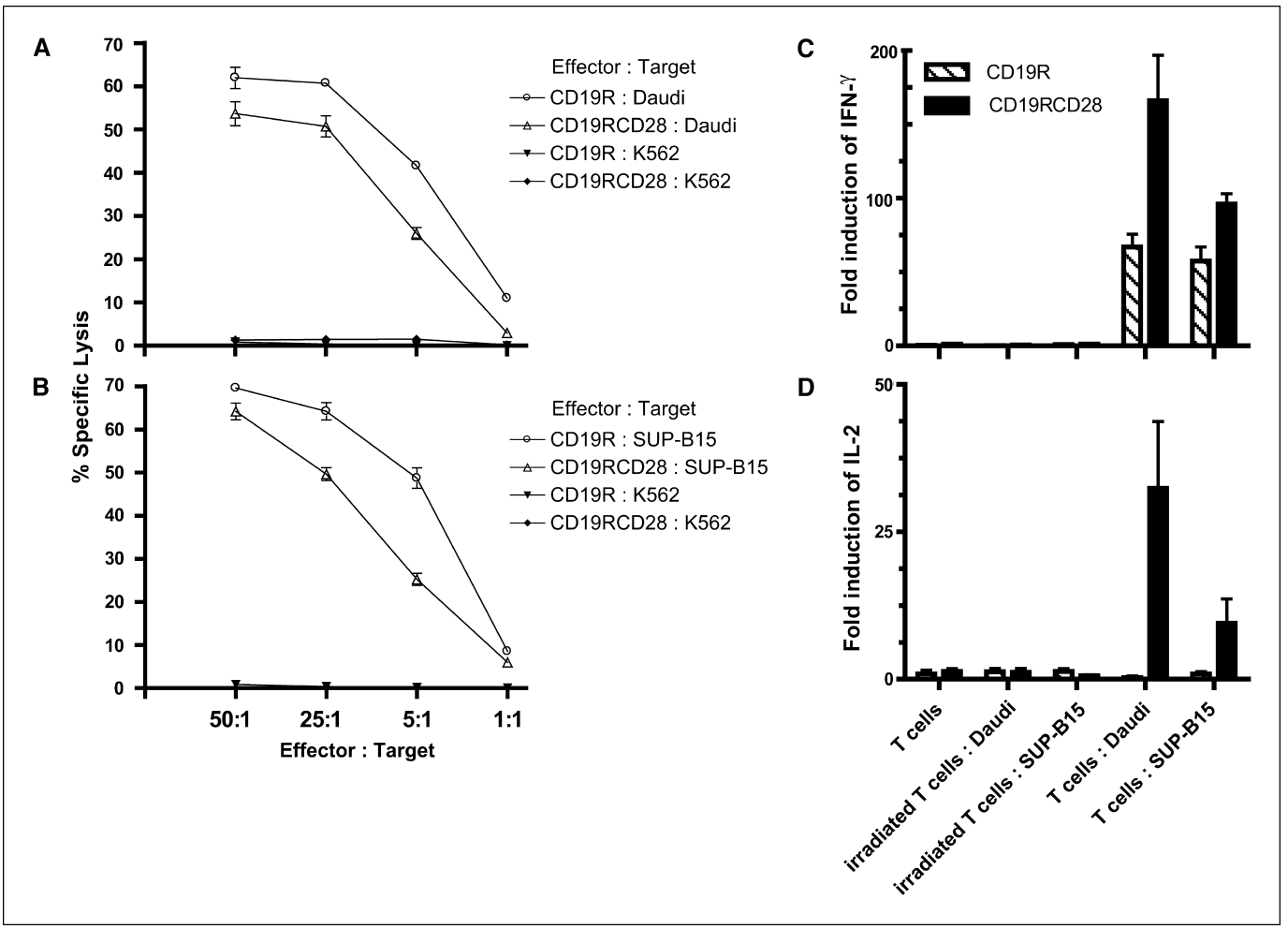

Figure 3. CD19-specific killing of tumor cells and cytokine production by CAR$^+$ T cells. Killing of (*A*) CD19$^+$ Daudi and (*B*) SUP-B15 target cells by CD19R$^+$ and CD19RCD28$^+$ T cells. Lytic activity of the genetically modified T cells was calculated by measuring chromium release after 4 hours. Spontaneous release for each target was <10%. CD19$^-$ K562 target cells were used as a negative control. *Points,* mean CRA results of specific lysis of triplicate wells; *bars,* SE. The ability of CD19R$^+$ and CD19RCD28$^+$ T cells to up-regulate (*C*) IFN-γ and (*D*) IL-2 production in response to antigen stimulation was investigated by quantitative RT-PCR. CD19R$^+$ and CD19RCD28$^+$ T cells were cocultured with irradiated CD19$^+$ Daudi or SUP-B15 tumor cells at a ratio of 1:5. Aliquots were taken at 0 and 2 hours. Total RNA was isolated and used for quantitative RT-PCR. *GAPDH* served as a reference gene. Controls included cultures of tumor or T cells alone as well as irradiated tumor and T-cell cocultures. The fold change of expression was calculated using the comparative C_t method (29).

Stimulation with CD19 antigen leads to proliferation and propagation of CD19RCD28$^+$ T cells. The ability of the genetically modified CD8$^+$CAR$^+$ T cells to proliferate in response to stimulation with CD19$^+$ target cells was investigated by measuring the incorporation of [^{3}H]thymidine. CD19RCD28$^+$ T cells cocultured with CD19$^+$ Daudi, SUP-B15, or CD19$^+$CD80$^+$CD86$^+$ LCL cells in absence of exogenous rhIL-2 showed ~10-fold higher stimulation indexes compared with CD19R$^+$ T cells cocultured with these same stimulator cells (Fig. 4*A*). The near-background stimulation index observed when the modified T cells were cocultured with CD19$^-$ K562 shows that proliferation of CD19RCD28$^+$ T cells is dependent on CD19. No significant incorporation of thymidine by CD19R$^+$ T cells cocultured with CD19$^+$ stimulator cells could be detected, indicating that CD19R$^+$ T cells were not able to significantly proliferate in the absence of exogenous IL-2. These data imply that CD19-dependent T-cell activation through the CD19RCD28 CAR results in more robust proliferation compared with the first-generation CAR.

We then tested whether irradiated CD19$^+$ LCL cells could act as artificial APCs to support propagation of the genetically modified T cells. Both CD19R$^+$ and CD19RCD28$^+$ T cells failed to robustly expand over 2 weeks in the absence of exogenous rhIL-2 when cocultured with LCL (Fig. 4*B*). However, the addition of low-dose (25 units/mL) IL-2 to the cocultures led to a 43-fold expansion of CD19RCD28$^+$ T cells over a 2-week period, whereas CD19R$^+$ T cells expanded only 8-fold over the same time period (Fig. 4*B*). This latter result is consistent with our previous report showing a ~6-fold expansion over 2 weeks of CD19R$^+$ T cells cocultured with PBMC, LCL, and exogenous IL-2 (8). The CD19RCD28$^+$ T cells could be restimulated after 14 days with irradiated LCL, resulting in an additional 30-fold growth after 2 weeks (data not shown). These proliferation and numerical expansion data in response to stimulation with a CD19$^+$ artificial APC suggest that the CD19RCD28 CAR was able to deliver a sustained positive growth signal to genetically modified T cells.

Bcl-X_L up-regulation in CD19RCD28$^+$ T cells upon stimulation with CD19$^+$ antigen. Because T-cell costimulation through endogenous CD28 has been shown to lead to increased production of the antiapoptotic protein Bcl-X_L (17, 18, 33), we investigated whether CD19RCD28$^+$ T cells up-regulate Bcl-X_L in response to

CD19-antigen stimulation using quantitative RT-PCR. Our results show that CD19RCD28$^+$ T cells increase Bcl-X_L expression 15-fold after stimulation with Daudi tumor cells for 24 hours (Fig. 4*C*). A lack of up-regulation of Bcl-X_L was observed in CD19R$^+$ T cells after 24 hours. Controls using T cells or tumors alone or irradiated T-cell/tumor cocultures also showed no detectable up-regulation of this antiapoptotic gene. These data suggest that CD19RCD28$^+$ T cells, and not CD19R$^+$ T cells, can receive a costimulatory signal through CD28 that leads to increased Bcl-X_L expression.

Improved CD19-dependent *in vivo* persistence of adoptively transferred CD19RCD28$^+$ T cells compared with CD19R$^+$ T cells. We investigated whether the presence of a chimeric CD28 signaling domain in the second-generation CD19-specific CAR could result in improved *in vivo* survival of adoptively transferred genetically modified T cells. Using a noninvasive biophotonic imaging system, we tracked the survival of ffLuc$^+$ T cells in NOD/*scid* mice bearing Daudi tumor. Our results show that the ffLuc-derived BLI signal from CD19RCD28$^+$ T cells persisted for at least 50 days *in vivo*, whereas the ffLuc-derived BLI signal decreased to the background level after day 6 in mice receiving an equivalent number of CD19R$^+$ T cells (Fig. 5*A* and *B*). The background flux level, as assessed by simultaneously imaging mice that did not receive T cells after D-luciferin injection, was $\sim 10^6$ p/s/cm^2/sr, which is similar to previously reported data (26). These data reveal that CD19RCD28$^+$ T cells persisted significantly longer *in vivo* compared with CD19R$^+$ T cells ($P < 0.05$, Wilcoxon rank sum test). As both CD19R$^+$ and CD19RCD28$^+$ T cells express very low levels of endogenous CD28, it is unlikely that the binding of wild-type CD28 to CD80 and CD86 on Daudi tumor cells accounts for the improved *in vivo* persistence of T cells bearing the second-generation CAR. When mice without tumors were injected with CD19RCD28$^+$ or CD19R$^+$ T cells, the genetically modified T cells could not be detected beyond 5 to 10 days (Fig. 5*A, inset*), indicating that the survival of the modified T cells depends on the presence of CD19$^+$ tumor cells. In aggregate, these data show that the CD28 signaling domain in the new CAR led to improved CD19-dependent survival of the adoptively transferred genetically modified T cells *in vivo*.

Enhanced *in vivo* antitumor efficacy of adoptively transferred CD19RCD28$^+$ T cells compared with CD19R$^+$ T cells. We evaluated whether the enhanced persistence of CD19RCD28$^+$ T cells, relative to the CD19R$^+$ T cells, could lead to improved antitumor efficacy *in vivo*. Using the biophotonic imaging system, we longitudinally tracked the tumor growth as well as the T-cell persistence in NOD/*scid* mice with established hRluc$^+$ Daudi tumor before and after adoptive immunotherapy. The number of CAR$^+$ T cells to be infused was based on a single dose of CD19R$^+$ T cells that led to partial clearance of established Daudi tumor. This allowed us to determine if there was added antitumor effect to infusing CD19RCD28$^+$ T cells. Initial T-cell–derived ffLuc activities were comparable in mice injected with CD19R$^+$ and CD19RCD28$^+$ T cells. On day 24, however, the T cell–derived ffLuc signal could only be detected above background in mice treated with CD19RCD28$^+$ T cells but not in mice injected with CD19R$^+$ T cells (data not shown). Thus, treatment with CD19R$^+$ T cells led to an initial reduction in the hRluc-derived biophotonic signal but failed to control tumor growth beyond 4 to 5 days after the T-cell injection (experiment days 11-12), as indicated by the logarithmic increase in the tumor-derived hRluc signal (Fig. 6*A*). The hRluc-mediated signal in mice treated with CD19RCD28$^+$ T cells decreased slowly and was near background level ~ 2 weeks after a single T-cell injection, without the addition of exogenous IL-2 (Fig. 6*A* and *B*). A control group of

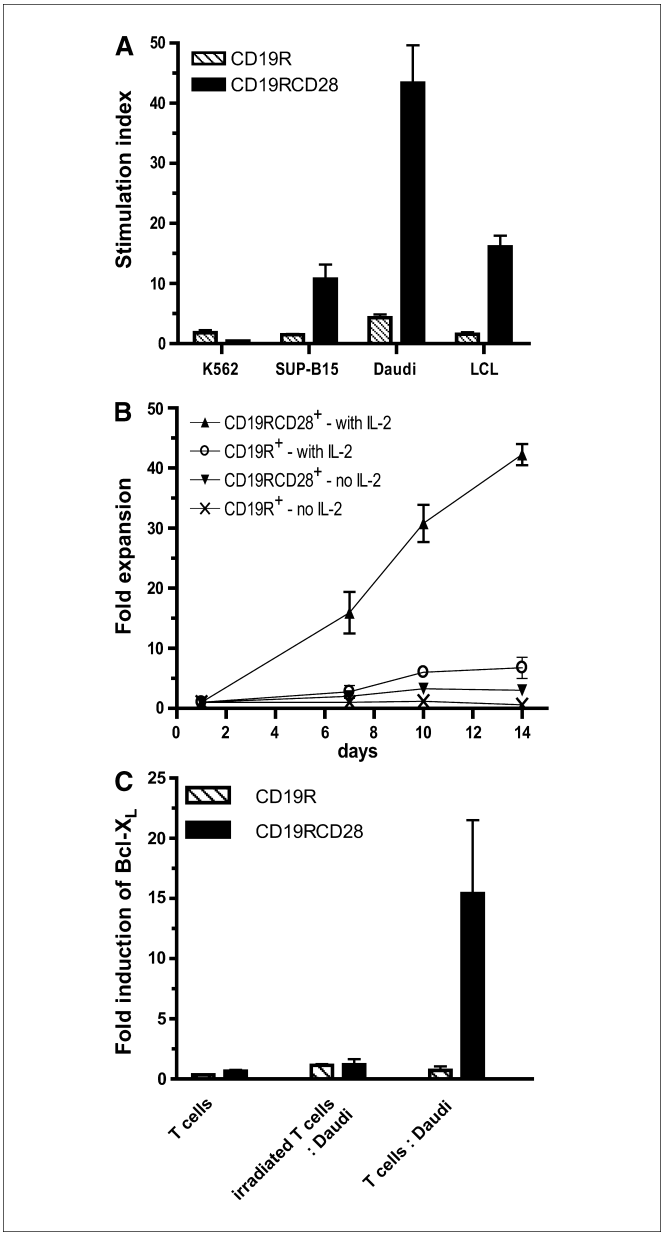

Figure 4. Proliferation and Bcl X_L up-regulation by CAR$^+$ T cells in response to antigen stimulation with CD19$^+$ tumor cells. *A,* thymidine proliferation assay in absence of exogenous rhIL-2. Genetically modified T cells (10^4) were cocultured with mitomycin C–treated CD19$^+$ Daudi, SUP-B15, LCL cells, and CD19$^-$ K562 cells at a 1:1 ratio. [^{3}H]thymidine was added after 54 hours, and the incorporated counts were measured 18 hours later. *Columns,* stimulation index from triplicates; *bars,* SD. The T-cell stimulation index on the three CD19$^+$ stimulator cells is significantly different between the CD19RCD28$^+$ and CD19R$^+$ responder cells ($P < 0.05$, Student's *t* test). *B,* T-cell numerical expansion on irradiated LCL (artificial APC). *Points,* mean; *bars,* SD. CD19RCD28$^+$ and CD19R$^+$ were stimulated with irradiated LCL at a T cell/LCL ratio of 1:50. Some cultures were supplemented with 25 units/mL of recombinant human IL-2 every other day. *C,* Bcl-X_L induction by CAR$^+$ T cells in response to antigen stimulation. The ability of CD19R$^+$ and CD19RCD28$^+$ T cells to up-regulate Bcl-X_L production in response to antigen stimulation was investigated by quantitative RT-PCR. CD19R$^+$ and CD19RCD28$^+$ T cells were cocultured with irradiated CD19$^+$ Daudi tumor cells at a ratio of 1:5. Aliquots were taken at 0 and 24 hours. Controls included cultures of T cells alone as well as irradiated T cell and tumor cocultures. The fold change of expression was calculated using the comparative C_t method (29).

mice that did not receive any T cells showed progressively increasing hRluc-mediated biophotonic signals (Fig. 6*A* and *B*). Although we detected a statistically significant difference between groups treated with CD19RCD28$^+$ T cells and the control group

($P < 0.05$, Wilcoxon rank sum test), there was no statistically significant difference between the groups receiving CD19R$^+$ T cells and no T-cell treatment ($P > 0.05$, Wilcoxon rank sum test). These data show that CD19RCD28$^+$ T cells were able to exert better control of tumor growth compared with CD19R$^+$ T cells. Taken together, these results suggest that the improved persistence of the adoptively transferred CD19RCD28$^+$ T cells can lead to enhanced antitumor efficacy *in vivo*.

Discussion

Genetic modification of T cells to express CARs for adoptive immunotherapy is a method to redirect the specificity of T cells for desired cell surface tumor antigens, which are not readily recognized by the endogenous αβ T-cell receptor. However, a potential disadvantage of using CAR$^+$ T cells is that the design of the CAR may limit a T cell's ability to receive a fully competent activation signal. Because our *ex vivo* expanded genetically modified CD8$^+$ cytolytic T cells generally lack expression of endogenous CD28, the CD19-specific CAR$^+$ T cells must rely on enforced expression of full-length CD28 (28) or chimeric CD28 (32, 34–42), 4-1BB (43), or OX40 (44) to achieve a fully competent T-cell activation signal. Thus, the development of next-generation CARs, which include one or more T-cell cosignaling domains, may be crucial for the therapeutic success of adoptive immunotherapy particularly to target tumors, such as B-cell acute lymphoblastic leukemia, which may not express CD80/CD86.

In this study, we directly compared the *in vitro* and *in vivo* immunobiology of a first- and a second-generation CD19-specific CAR, which differ in their ability to provide costimulation through chimeric CD28. Our data suggest that *ex vivo* propagated CD8$^+$CD19R$^+$ T cells are capable of CD28-mediated signaling, for

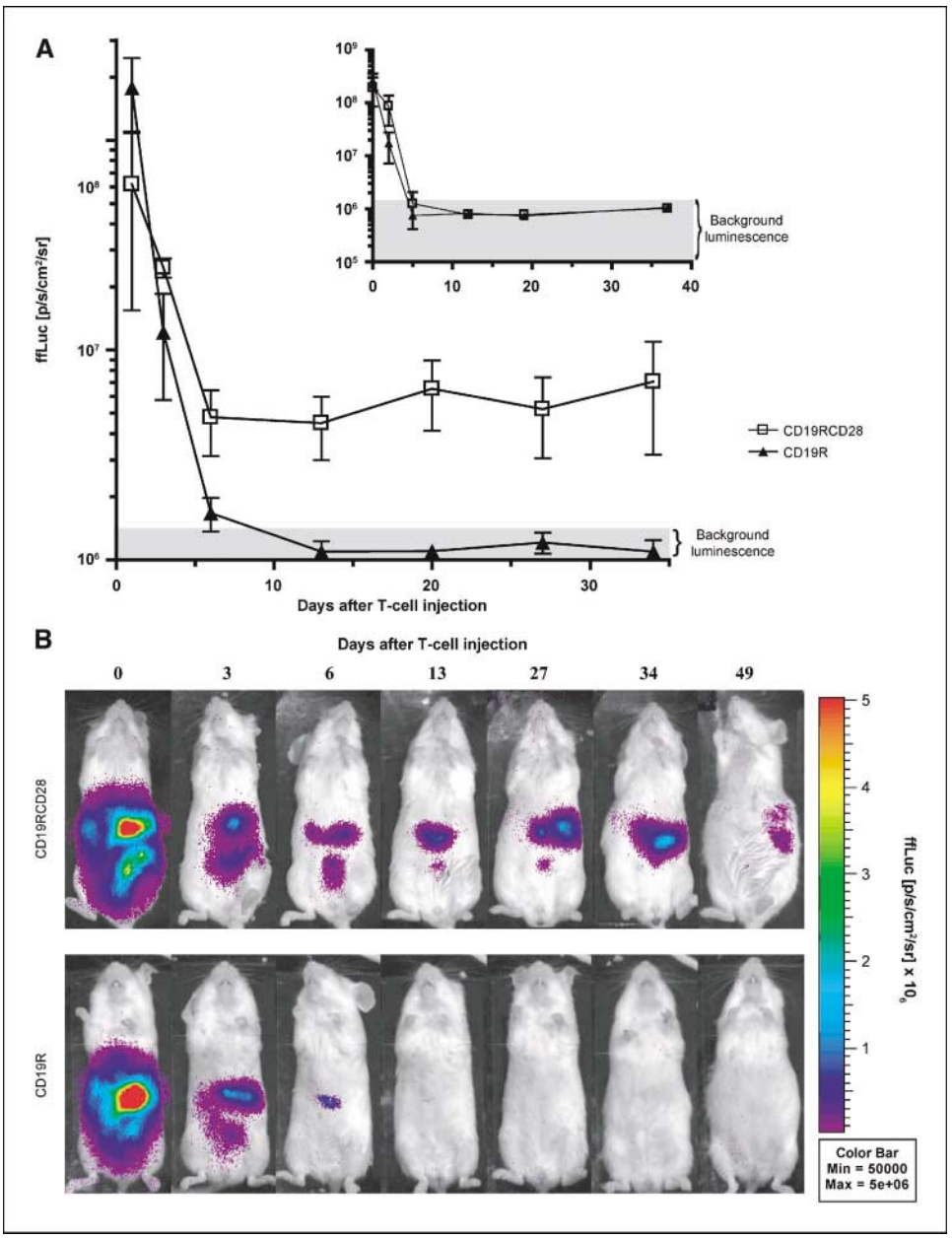

Figure 5. *In vivo* persistence of CAR$^+$ T cells. *A,* longitudinal monitoring of the bioluminescent signals of 5×10^6 ffLuc$^+$ CD19RCD28$^+$ or CD19R$^+$ T cells injected into groups of four NOD/*scid* mice bearing Daudi tumor cells. Initial T-cell signal was measured 3 hours after injection. *Points,* mean photon flux (in p/s/cm^2/sr); *bars,* SD. *Gray area,* average background ffLuc-derived flux for a group of mice that did not receive injections of tumor or T cells. *Inset,* T-cell survival after adoptive transfer in NOD/*scid* mice that did not receive tumor. *B,* pseudocolor image representing light intensity and anatomic localization of the ffLuc-derived T-cell signal in two representative mice. The color bar displays the ffLuc activity in p/s/cm^2/sr.

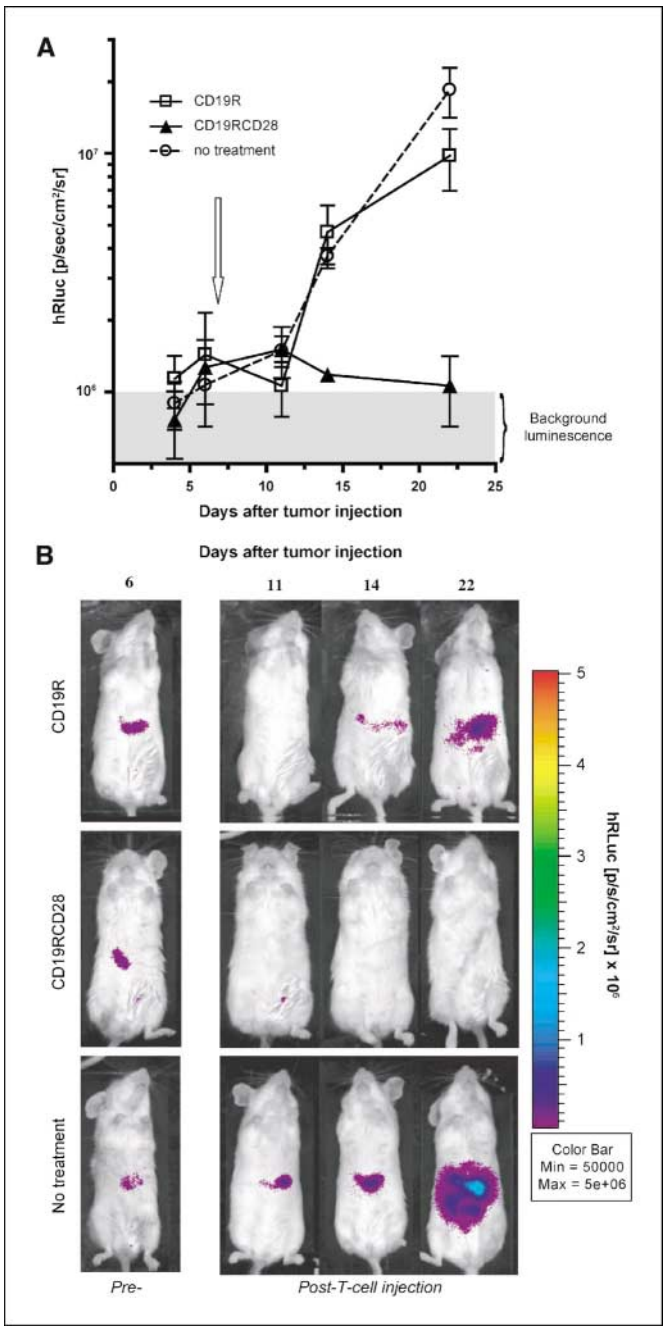

Figure 6. *In vivo* antitumor activity of CAR$^+$ T cells. *A,* longitudinal monitoring of the bioluminescent signals of hRluc$^+$ Daudi cells in NOD/*scid* mice. Mice with progressively growing tumors were separated into three groups (four mice per group) bearing comparable tumor loads. Seven days later (*arrow*), the mice were injected with 20×10^6 ffLuc$^+$ CD19R$^+$ or CD19RCD28$^+$ T cells. A control group received no T-cell treatment. *Points,* mean photon flux (in p/s/cm^2/sr); *bars,* SD. Using the Wilcoxon rank sum test, we detected a statistically significant difference between groups treated with CD19RCD28$^+$ T cells and the control group ($P < 0.05$), no statistically significant difference between the groups receiving CD19R$^+$ T cells and no treatment ($P > 0.05$). *Gray area,* average background hRluc-derived flux for a group of mice that did not receive injections of tumor or T cells. *B,* pseudocolor image representing light intensity and anatomic localization of the hRluc-derived Daudi flux signals in three representative mice. The color bar displays the relative hRluc activity in p/s/cm^2/sr.

enforced expression of full-length CD28 led to CD19-dependent IL-2 secretion and proliferation of T cells expressing the first-generation CAR. Because there seemed to be no intrinsic block to achieve CD28-mediated fully competent activation of CD8$^+$ genetically modified T cells, and given that some CD19$^+$ tumor targets fail to express CD80/CD86, we developed a new CAR capable of delivering a signal through chimeric CD28. Our results suggest that CD8$^+$ T cells expressing the *CD19RCD28* transgene can receive a CD28-mediated costimulatory signal upon binding to CD19, as indicated by the ability to up-regulate IL-2 and the antiapoptotic protein Bcl-X_L. Both IL-2 and Bcl-X_L production are hallmarks of CD28-mediated costimulation of activated T cells (15, 18). Because IL-2 up-regulation occurred in cocultures with CD80/CD86$^-$ SUP-B15 tumor cells, we conclude that CD28-mediated signaling in CD8$^+$ T cells can be provided through the second-generation CAR. However, it is not known to what extent T-cell signaling through chimeric CD28 can recapitulate the signaling mediated by endogenous CD28. We suggest that the autocrine or paracrine IL-2 production from the CD19RCD28$^+$ T cells upon stimulation with CD19$^+$ tumor cells was sufficient to drive T-cell proliferation. However, exogenous rhIL-2 was needed to continuously propagate CD19RCD28$^+$ T cells over a long (weeks) period of time. Indeed, the addition of exogenous rhIL-2 also led to improved proliferation (measured over hours) of CD19R$^+$ T cells with enforced expression of CD28. In aggregate, these data imply that exogenous rhIL-2 binding through cytokine receptor can further improve proliferation/propagation of CD19-specific T cells expressing full-length or chimeric CD28 T cells.

The need for supplemental rhIL-2 to efficiently propagate genetically modified T cells on CD19 antigen *in vitro* suggests that the design of our second-generation CD19-specific CAR might be further modified to produce T cells that do not rely on exogenous IL-2 to efficiently numerically expand. Indeed, a recent study by Pule et al. (44) showed that incorporation of signaling domains from both CD28 and OX40 in a GD_2-specific CAR resulted in sustained T-cell numerical expansion when compared with CARs lacking OX40. Another approach to generating genetically modified T cells, which might have an enhanced ability to propagate, involves removing three COOH-terminal amino acids from the CD28 signaling domain in the *CD19RCD28* transgene. This modification would potentially improve IL-2 production by the genetically modified T cells because this motif has been shown to interact with the negative regulator mitogen-activated protein kinase phosphatase-6 (45). However, further modifications of CARs to enhance costimulation, must be balanced by the safety considerations of infusing T cells, which may be capable of autonomous propagation and may not be governed by regulatory molecules to help prevent antigen-independent outgrowth of adoptively transferred cells.

The design of our second-generation CD19-specific CAR differs from some recently described CARs that include extracellular portions of CD28 (35, 38, 39). For example, incorporation of extracellular CD28 domain that apparently contains the binding site (46, 47) for CD80/CD86, in series with chimeric CD3-ζ, may have the potential for unwanted CD28-mediated activation of the genetically modified T cells. A CAR described by Friedmann-Morvinski et al. (39) circumvents this problem by excluding the CD80/CD86 binding site located in the CD28 extracellular domain. A second-generation CAR designed for human trials, which completely lacks the CD28 extracellular domain, would further minimize concerns regarding inappropriate T-cell binding to CD28 ligands. Therefore, our *CD19RCD28* transgene does not include the CD28 ectodomain.

A major finding of this study is that adoptively transferred human T cells expressing a CAR with inherent CD28-mediated costimulatory activity led to improved persistence of the infused T cells and subsequently enhanced the antitumor efficacy *in vivo*,

compared with the first-generation CAR$^+$ T cells. This was despite a slightly lower expression of CD19RCD28 CAR and lytic ability, compared with CD19R$^+$ T cells. The improved survival is predicted from our finding that the CD19RCD28$^+$ T cells were able to up-regulate the antiapoptotic factor Bcl-X_L. The improved antitumor effect is consistent with *in vivo* studies in which murine T cells expressing a CAR, constructed with CD28 and CD3-ζ signaling domains, show a robust antitumor effect (48). Thus, for the first time, we show that improved persistence of adoptively transferred CD19-specific T cells expressing a second-generation CAR results in an augmented antitumor effect. Future experiments using noninvasive imaging to detect two types of engrafted cells expressing *ffLuc* and *hRluc* transgenes will help determine T-cell trafficking to tumor sites and the temporal and spatial distribution of adoptively transferred T cells and tumor deposits.

In summary, our data show that combining chimeric CD28 and CD3-ζ in a second-generation CAR led to an improved *in vitro* and *in vivo* phenotype for CD19-specific T cells, compared with the first-generation CD19-specific CAR. Clinical trials are needed to determine whether this new CAR design may improve the immunobiology of adoptively transferred CD19-specific T cells and their therapeutic efficacy.

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References

1. Eshhar Z, Waks T, Gross G, Schindler DG. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. Proc Natl Acad Sci U S A 1993;90:720–4.
2. Hwu P, Shafer GE, Treisman J, et al. Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor gamma chain. J Exp Med 1993;178:361–6.
3. Stancovski I, Schindler DG, Waks T, Yarden Y, Sela M, Eshhar Z. Targeting of T lymphocytes to Neu/HER2-expressing cells using chimeric single chain Fv receptors. J Immunol 1993;151:6577–82.
4. Moritz D, Wels W, Mattern J, Groner B. Cytotoxic T lymphocytes with a grafted recognition specificity for ERBB2-expressing tumor cells. Proc Natl Acad Sci U S A 1994;91:4318–22.
5. Bitton N, Verrier F, Debre P, Gorochov G. Characterization of T cell-expressed chimeric receptors with antibody-type specificity for the CD4 binding site of HIV-1 gp120. Eur J Immunol 1998;28:4177–87.
6. Darcy PK, Kershaw MH, Trapani JA, Smyth MJ. Expression in cytotoxic T lymphocytes of a single-chain anti-carcinoembryonic antigen antibody. Redirected Fas ligand-mediated lysis of colon carcinoma. Eur J Immunol 1998;28:1663–72.
7. Jensen M, Tan G, Forman S, Wu AM, Raubitschek A. CD20 is a molecular target for scFvFc:zeta receptor redirected T cells: implications for cellular immunotherapy of CD20$^+$ malignancy. Biol Blood Marrow Transplant 1998;4:75–83.
8. Cooper LJ, Topp MS, Serrano LM, et al. T-cell clones can be rendered specific for CD19: toward the selective augmentation of the graft-versus-B-lineage leukemia effect. Blood 2003;101:1637–44.
9. Brocker T. Chimeric Fv-zeta or Fv-epsilon receptors are not sufficient to induce activation or cytokine production in peripheral T cells. Blood 2000;96:1999–2001.
10. Schwartz RH. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. Cell 1992;71:1065–8.
11. Greenfield EA, Nguyen KA, Kuchroo VK. CD28/B7 costimulation: a review. Crit Rev Immunol 1998;18:389–418.
12. Frauwirth KA, Thompson CB. Activation and inhibition of lymphocytes by costimulation. J Clin Invest 2002;109:295–9.
13. Fraser JD, Irving BA, Crabtree GR, Weiss A. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. Science 1991;251:313–6.
14. Jenkins MK, Taylor PS, Norton SD, Urdahl KB. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. J Immunol 1991;147:2461–6.
15. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. Annu Rev Immunol 1996;14:233–58.
16. Harding FA, McArthur JG, Gross JA, Raulet DH, Allison JP. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. Nature 1992;356:607–9.
17. Boise LH, Minn AJ, Noel PJ, et al. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. Immunity 1995;3:87–98.
18. Sperling AI, Auger JA, Ehst BD, Rulifson IC, Thompson CB, Bluestone JA. CD28/B7 interactions deliver a unique signal to naive T cells that regulates cell survival but not early proliferation. J Immunol 1996;157:3909–17.
19. Buggins AG, Lea N, Gaken J, et al. Effect of costimulation and the microenvironment on antigen presentation by leukemic cells. Blood 1999;94:3479–90.
20. Cardoso AA, Schultze JL, Boussiotis VA, et al. Pre-B acute lymphoblastic leukemia cells may induce T-cell anergy to alloantigen. Blood 1996;88:41–8.
21. Bruserud O, Ulvestad E. Human acute lymphoblastic leukemia (ALL) blasts as accessory cells during T-cell activation: differences between patients in costimulatory capacity affect proliferative responsiveness and cytokine release by activated T cells. Cancer Immunol Immunother 2003;52:215–25.
22. Brown RD, Pope B, Yuen E, Gibson J, Joshua DE. The expression of T cell related costimulatory molecules in multiple myeloma. Leuk Lymphoma 1998;31:379–84.
23. Nicholson IC, Lenton KA, Little DJ, et al. Construction and characterisation of a functional CD19 specific single chain Fv fragment for immunotherapy of B lineage leukaemia and lymphoma. Mol Immunol 1997;34:1157–65.
24. Reddy MP, Kinney CA, Chaikin MA, et al. Elimination of Fc receptor-dependent effector functions of a modified IgG4 monoclonal antibody to human CD4. J Immunol 2000;164:1925–33.
25. Nguyen P, Moisini I, Geiger TL. Identification of a murine CD28 dileucine motif that suppresses single-chain chimeric T-cell receptor expression and function. Blood 2003;102:4320–5.
26. Serrano LM, Pfeiffer T, Olivares S, et al. Differentiation of naive cord-blood T cells into CD19-specific cytolytic effectors for posttransplantation adoptive immunotherapy. Blood 2006;107:2643–52.
27. Cooper LJ, Al-Kadhimi Z, Serrano LM, et al. Enhanced antilymphoma efficacy of CD19-redirected influenza MP1-specific CTLs by cotransfer of T cells modified to present influenza MP1. Blood 2005;105:1622–31.
28. Topp MS, Riddell SR, Akatsuka Y, Jensen MC, Blattman JN, Greenberg PD. Restoration of CD28 expression in CD28$^-$ CD8$^+$ memory effector T cells reconstitutes antigen-induced IL-2 production. J Exp Med 2003;198:947–55.
29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods 2001;25:402–8.
30. Vitale M, Zamai L, Neri LM, Manzoli L, Facchini A, Papa S. Natural killer function in flow cytometry: identification of human lymphoid subsets able to bind to the NK sensitive target K562. Cytometry 1991;12:717–22.
31. Finney HM, Akbar AN, Lawson AD. Activation of resting human primary T cells with chimeric receptors: costimulation from CD28, inducible costimulator, CD134, and CD137 in series with signals from the TCR zeta chain. J Immunol 2004;172:104–13.
32. Willemsen RA, Ronteltap C, Chames P, Debets R, Bolhuis RL. T cell retargeting with MHC class I-restricted antibodies: the CD28 costimulatory domain enhances antigen-specific cytotoxicity and cytokine production. J Immunol 2005;174:7853–8.
33. Wu LX, La Rose J, Chen L, et al. CD28 regulates the translation of Bcl-x_L via the phosphatidylinositol 3-kinase/mammalian target of rapamycin pathway. J Immunol 2005;174:180–94.
34. Altenschmidt U, Klundt E, Groner B. Adoptive transfer of *in vitro*-targeted, activated T lymphocytes results in total tumor regression. J Immunol 1997;159:5509–15.
35. Krause A, Guo HF, Latouche JB, Tan C, Cheung NK, Sadelain M. Antigen-dependent CD28 signaling selectively enhances survival and proliferation in genetically modified activated human primary T lymphocytes. J Exp Med 1998;188:619–26.
36. Hombach A, Wieczarkowiecz A, Marquardt T, et al. Tumor-specific T cell activation by recombinant immunoreceptors: CD3 zeta signaling and CD28 costimulation are simultaneously required for efficient IL-2 secretion and can be integrated into one combined CD28/CD3 zeta signaling receptor molecule. J Immunol 2001;167:6123–31.
37. Haynes NM, Trapani JA, Teng MW, et al. Single-chain antigen recognition receptors that costimulate potent rejection of established experimental tumors. Blood 2002;100:3155–63.

38. Maher J, Brentjens RJ, Gunset G, Riviere I, Sadelain M. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRzeta/CD28 receptor. Nat Biotechnol 2002;20:70–5.
39. Friedmann-Morvinski D, Bendavid A, Waks T, Schindler D, Eshhar Z. Redirected primary T cells harboring a chimeric receptor require costimulation for their antigen-specific activation. Blood 2005;105: 3087–93.
40. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. Nat Rev Immunol 2001;1:220–8.
41. Rossig C, Pscherer S, Landmeier S, Altvater B, Jurgens H, Vormoor J. Adoptive Cellular Immunotherapy with CD19-Specific T Cells. Klin Padiatr 2005;217:351–6.
42. Simmons A, Robert WP, Kolokoltsov AA, Davey RA. Use of recombinant lentivirus pseudotyped with vesicular stomatitis virus glycoprotein G for efficient generation of human anti-cancer chimeric T cells by transduction of human peripheral blood lymphocytes *in vitro*. Virol J 2006;3:8.
43. Imai C, Mihara K, Andreansky M, et al. Chimeric receptors with 4–1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia. Leukemia 2004;18:676–84.
44. Pule MA, Straathof KC, Dotti G, Heslop HE, Rooney CM, Brenner MK. A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. Mol Ther 2005;12:933–41.
45. Marti F, Krause A, Post NH, et al. Negative-feedback regulation of CD28 costimulation by a novel mitogen-activated protein kinase phosphatase, MKP6. J Immunol 2001;166:197–206.
46. Kariv I, Truneh A, Sweet RW. Analysis of the site of interaction of CD28 with its counter-receptors CD80 and CD86 and correlation with function. J Immunol 1996;157:29–38.
47. Metzler WJ, Bajorath J, Fenderson W, et al. Solution structure of human CTLA-4 and delineation of a CD80/CD86 binding site conserved in CD28. Nat Struct Biol 1997;4:527–31.
48. Moeller M, Haynes NM, Kershaw MH, et al. Adoptive transfer of gene-engineered $CD4^+$ helper T cells induces potent primary and secondary tumor rejection. Blood 2005;106:2995–3003.

CD28 Costimulation Provided through a CD19-Specific Chimeric Antigen Receptor Enhances *In vivo* Persistence and Antitumor Efficacy of Adoptively Transferred T Cells

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