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Cytokine IL-36γ Improves CAR T Cell Functionality and Induces Endogenous Anti-Tumor Response

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Abstract

Chimeric antigen receptor (CAR) T cell therapy has shown remarkable responses in B cell malignancies. However, many patients suffer from limited response and tumor relapse due to lack of persisting CAR T cells and immune escape. These clinical challenges have compromised the long-term efficacy of CAR T cell therapy and call for the development of novel CAR designs. We demonstrated that CAR T cells secreting a cytokine interleukin-36γ (IL-36γ) showed significantly improved CAR T cell expansion and persistence, and resulted in superior tumor eradication compared to conventional CAR T cells. The enhanced cellular function by IL-36γ was mediated through an autocrine manner. In addition, activation of endogenous antigen-presenting cells (APCs) and T cells by IL-36γ aided the formation of a secondary anti-tumor response which delayed the progression of antigen-negative tumor challenge. Together, our data provide preclinical evidence to support the translation of this design for an improved CAR T cell–mediated anti-tumor response.

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Introduction

Chimeric antigen receptor (CAR) T cell therapy has reshaped the landscape of immunotherapy after remarkable responses were achieved in treatment of B cell–derived malignancies using CD19-targeted CAR T cells (1-4). Despite encouraging upfront efficacy, an enduring anti-tumor response is still absent in most patients, as they succumb to either $CD19^+$ or $CD19^-$ tumor relapse in the long term (5, 6). This could be explained by the lack of persistent and functional CAR T cells as well as multiple antigen escape mechanisms exploited by tumor cells (7). In addition, response is highly variable among individuals. Correlate studies suggest that many T cell intrinsic factors, including their expansion capacity, enriched central memory phenotype, and polyfunctional status dictate the response in patients, therefore providing a rationale to engineer CAR T cells with desirable features for an improved clinical outcome (6, 8, 9). Efforts to enhance CAR and subsequent T cell function include incorporation of different costimulatory domains and introduction of mutated signaling motifs to calibrate the strength of activation (10, 11). Alternatively, immune-modulating agents such as pro-inflammatory cytokines, costimulatory molecules, and immune checkpoint blockade can be co-engineered with a CAR into T cells to enhance CAR T cell function and modulate tumor microenvironment (12-14). The CAR T cell secreting pro-inflammatory cytokine interleukin-12 has demonstrated improved cytotoxicity and the ability to overcome an immune inhibitory microenvironment in a solid tumor model (15). Interleukin-18 has also been reported to enhance CAR T proliferation and persistence *in vivo*, and to induce a broadened anti-tumor response through endogenous immune effectors (16-18).

Interleukin-36 (IL-36) refers to a novel group of cytokines belonging to the interleukin-1 superfamily. There are three agonistic isoforms α, β and γ and a natural inhibitor IL-36Ra (19). IL-36 cytokines are highly expressed by epithelial cells in skin, lung, and gastrointestinal tract in response to tissue damage (20). Released cytokines act both on IL-36 receptor (IL-36R)–expressing epithelial cells to further boost IL-36 production, as well as on immune cells including dendritic cells (DCs), monocytes, neutrophils, and T cells to initiate and amplify an inflammatory response. Exposure to IL-36 directly promotes expansion and production of pro-inflammatory cytokines of T cells, therefore providing a rationale to locally deliver IL-36 by CAR T cells to enhance their effector function and expansion to improve the overall anti-tumor response (21, 22).

The IL-36 pathway is involved in initiating and modulating endogenous anti-tumor immune response, evidenced by studies using tumor vaccination and therapeutic DC approaches (22, 23). Irradiated B16 cells that overexpress IL-36γ were able to induce tumor immunogenicity and inhibit tumor growth in B16-bearing mice. Local delivery of DCs expressing IL-36γ promoted formation of tertiary lymphoid structure in the tumor microenvironment and induced rapid infiltration and priming of T cells, which suggests a pivotal role of IL-36 signaling in mediating DC–T cell crosstalk during formation of an adaptive anti-tumor immune response. Yet the impact and therapeutic potential of IL-36γ in the context of an antigen-targeted, effector T cell has not been investigated. Utilizing an “armored” CAR platform, we demonstrated that CAR T cells engineered to secrete IL-36γ exhibit superior

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effector function and the capability to activate the endogenous immune system, together leading to an augmented and broadened anti-tumor response.

Materials and Methods

Study Design

Sample sizes were chosen based on similar previous studies and were not predetermined by power test. A sample size of three to five animals was chosen for animal studies. The specific numbers of animals and experiment repeats are indicated in figure legends. Animals were randomized after tumor inoculation, prior to CAR T cell injection. No blinding was applied when assessing the outcome. No samples or animals were excluded unless not analyzable.

Animals

Wild-type C57BL/6, C57BL/6 Thy1.1^{+} (B6.PL-Thy1a/CyJ), Rag1$^{-/-}$ (B6.129S7-Rag1tm1Mom/J), Myd88$^{-/-}$ (B6.129P2(SJL)-Myd88tm1.1Defr/J), and BALB/c CD45.1^{+} (CByJ.SJL(B6)-Ptprca/J) mice were purchased from the Jackson Laboratory. IL-36R$^{-/-}$ mice (C57BL/6-Il1rl2<tm1Hblu>) were provided by Amgen under an approved material transfer agreement. Wild-type BALB/c and NCG mice (NOD-Prkdcem26Cd52Il2rgem26Cd22/NjuCrl) were purchased from Charles River. Gender-matched, 8-12 week-old mice were used for CAR T cell generation and *in vivo* experiments. All mice were housed under specific pathogen-free conditions in the animal facility of Memorial Sloan Kettering Cancer Center (MSK). Experiments were conducted in accordance with MSK Institutional Animal Care and Use Committee (IACUC)-approved protocols and guidelines.

Cell lines

EL4 cells (catalog number: 87020408) were purchased from Sigma. Murine CD19 was transduced into EL4 via viral transduction. Phoenix-ECO (catalog number: CRL-3214) and A20 cells (TIB-208) were purchased from ATCC. A20-MHC I and MHCII knockout lines were generated using CRISPR as described (12). Tumor cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin. Retroviral producing cell lines (Phoenix-ECO and 293 Glv9) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin. All cell lines are regularly tested for mycoplasma contamination.

Generation of retrovirus constructs

To generate stable retrovirus-producing packaging lines, SFG γ-retroviral vector encoding CAR sequence was transiently transfected into H29 with the ProFection Mammalian Transfection System (Promega) according to manufacturer’s instructions. Virus-containing supernatant from H29 was then collected and used to transduce Phoenix-ECO or 293 Glv9 cells to generate stable retroviral producing cell lines. A self-cleaving peptide 2A sequence and murine IL-36γ–encoding gene was synthesized (Genewiz) in tandem and cloned into

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SFG-m19m28mz vector through Gibson Assembly (New England BioLabs). A similar approach was used to generate human IL-36γ–secreting CAR.

T cell isolation and retroviral transduction

To generate mouse CAR T cells, splenocytes were harvested and resuspended at $8x10^6$ cells/ml in RPMI-1640 supplemented with 10% FBS, nonessential amino acids, 1 mM sodium pyruvate, 10 nM HEPES, 2 mM L-glutamine, 1% penicillin/streptomycin, 11 mM glucose, and 2 μM 2-mercaptoethanol. Recombinant human IL-2 (100 IU/ml, Proleukin) and concanavalin A (1 μg/ml, Sigma) were also supplemented for T cell activation and expansion. After 24 and 48 hours of culture, cells were spinoculated with viral supernatant collected from Phoenix-ECO packaging cells as described previously (24). When examining the anti-tumor efficacy of CAR T cells in exposure to IL-36γ in culture prior to injection, m19m28mz CAR T cells were cultured with recombinant murine IL-36γ (Biolegend) at 100 ng/ml from day 2 to day 4. Cytokine was supplemented in virus-containing media and added to the culture plate every day. On day 5, CAR T cell transduction efficiency and concentration were examined using flow cytometry and counting beads (Ebioscience). To generate human CAR T cells, peripheral blood mononuclear cells (PBMCs) from healthy blood donors were separated using density gradient centrifugation with Accu-prep (axis-Shield PoC AS, Oslo, Norway), followed by T cell isolation using the EasySep human T cell isolation kit (StemCell). Isolated T cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 IU/ml human recombinant IL-2 (Proleukin) and human anti-CD3/CD28 Dynabeads (Life Technologies) at a bead-to-cell ratio of 1:2 for 48 hours, followed by spinoculation on day 3 and day 4 with viral supernatant collected from 293 Glv9 cells, and resting with fresh media supplemented on day 5 and day 6. On day 7, CAR T cell transduction efficiency and concentration were determined by flow cytometry.

Western blot

Supernatant from Phoenix-ECO packaging cells was collected, filtered, loaded onto protean TGX gels (Biorad) and transferred to polyvinylidene difluoride (PVDF) membranes (Biorad). IL-36γ was probed with rabbit-anti-mouse IL-36γ primary antibody (ABClonal) and secondary goat-anti-rabbit HRP antibody (Invitrogen), followed by development using Pierce ECL Western blot substrate (Thermo Scientific).

In vitro cytotoxicity assay

$2x10^4$ EL4-$CD19^+$ tumor cells expressing firefly luciferase were co-cultured with CD19-targeting CAR T cells in 200 μl of media at different effector-to-target ratios in triplicates. Twenty-four hours after co-culture, tumor bioluminescence signal was read using a Spark plate reader (Tecan) from each well after adding 75 ng of D-Luciferin (Gold Biotechnology) dissolved in 50 ul phosphate-buffered saline (PBS). Percent lysis was determined as described (12).

In vitro cytokine secretion analysis

For *in vitro* IL-2 secretion, mouse T cells were harvested from spleen and isolated using EasySep mouse T cell isolation kit (StemCell). $2x10^5$ T cells were cultured in a 96-well

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plate pre-coated with 1 μg/ml anti-CD3 antibody (Biolegend), and supplemented with media of different conditions as the experiment indicated. Supernatant was collected after 24 hours and analyzed using MILLIPLEX MAP Mouse Cytokine/Chemokine premixed 13 Plex kit (Millipore) and the FLEXMAP 3D system (Luminex). To examine the *in vitro* cytokine profile, $2x10^5$ CAR T cells were co-cultured with EL4-CD19$^+$ cells at 1:1 ratio or alone in 200 μl of media for 24 hours. Collected supernatant was analyzed using the Luminex system.

In vitro proliferation analysis

$1x10^6$ CAR T cells were cultured with $1x10^6$ EL4-CD19$^+$ cells in a 6-well plate with a total volume of 3 ml media. CAR T cell number was determined at days 2, 4, and 7 by flow cytometry using 123count eBeads Counting Beads (Thermo Fisher).

Mice and *in vivo* models

Syngeneic mice experiments—CAR T cells with transduction efficiency between 50-70% were obtained and used for *in vivo* experiment. Total T cell numbers were adjusted based on CAR transduction efficiency. C57BL/6 mice were intravenously (i.v.) inoculated with $1x10^6$ EL4-CD19$^+$ cells on day 0, randomized, and i.v. treated with $2.5x10^6$ CAR T cells the following day. Mice were euthanized when showing symptoms of hind limb paralysis, hydrocephalus, distended abdomen or big tumor burden that significantly interfered with animals' capability to move. Mice that survived past 90 days were considered long-term survivors and were i.v. inoculated with $2x10^5$ EL4-CD19$^+$ tumor or $1x10^5$ EL4-CD19$^-$ tumor for antigen-positive and antigen-negative tumor re-challenge studies, respectively. For pre-conditioning studies, mice were intraperitoneally (i.p.) treated with 250 mg/kg cyclophosphamide (Sigma), then i.v. inoculated with $1x10^6$ EL4-CD19$^+$ cells 3 days later, followed by i.v. injection of $2.5\ x10^6$ CAR T cells the next day.

Xenograft mice experiments—NCG mice were i.v. inoculated with $1x10^6$ Nalm6 tumor cells transduced with GFP/luciferase on day 0, randomized, followed by treatment with $1x10^5$ human CAR T cells on day 4. At indicated time points, tumor-bearing mice were i.p. injected with D-Luciferin (Gold Biotechnology) (150 mg/kg) and imaged using the IVIS Imaging System (PerkinElmer) with Living Image software (PerkinElmer), under the settings of 22.6 cm field of view, medium binning level, and 30-second exposure time. Data were analyzed using Living Image software (Perkin Elmer). Tumor burden was quantified as described (25).

Ex vivo CAR T cell analysis

Mice were i.v. inoculated with $1x10^6$ EL4-CD19$^+$ cells on day 0 and i.v. treated with $9x10^6$ vexGFP-CAR T cells or $2.5x10^6$ Thy1.1$^+$ CAR T cells the following day as the experiment indicated. Spleen and bone marrow cells were harvested, lysed with ammonium-chloride-potassium (ACK) red blood cell lysing buffer (Lonza) and resuspended in media for subsequent cell counting, phenotypical, and functional analysis.

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Flow cytometry and FACS sorting

Flow cytometry was used to determine the transduction efficiency of CAR T cells or immune phenotype cells. Antibodies used for murine cell analysis included: anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD19 (eBio1D3), anti-CD44 (IM7), anti-CD45 (30-F11), anti-CD62L (MEL-14), anti-CD86 (GL1), anti-myc (9B11), anti-Ly-6G/Ly-6C (RB6-8C5), anti-F4/80 (BM8), anti-MHC II (M5/114.15.2), anti-IL-2 (JES6-5H4), anti-TNF-α (MP6-XT22), anti-IFN-γ (XMG1.2), anti-Ki67 (SolA15), anti-Thy1.1 (HIS51), and anti-Thy1.2 (30-H12). Human CD19–targeting CAR T cells were stained with Alexa Fluor 647-conjugated 19E3 antibody (MSK antibody core facility) to determine CAR expression. For intracellular staining of IFN-γ and TNF-α, splenocytes harvested from CAR-treated mice were prepared in single cell suspension and stimulated with Cell Stimulation Cocktail (PMA, ionomycin) (Thermo Fisher) for 4 hours, followed by intracellular staining using Cytofix/Cytoperm Plus kit (BD Bioscience) according to manufacturer's instructions. For staining of Ki67, *ex vivo* harvested cells were first stained with extracellular antigens, followed by treatment with Foxp3/Transcription factor staining buffer set (eBioscience). Samples were analyzed on a Beckman Coulter Gallios or a Thermo Fisher Attune NxT flow cytometer and data were analyzed using FlowJo (Three Star). Cell sorting was performed using a BD FACS Aria sorter with the assistance of the MSK flow cytometry core facility.

Bone marrow–derived dendritic cells isolation and culture

BMDCs were generated as described (26). Briefly, bone marrow cells were harvested and seeded at $1x10^7$ cells/well in a 6-well tissue culture–treated plate in 4 ml of RPMI-1640 media supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin, and GM-CSF (20 ng/ml, Biolegend). Every two days, half of the media were discarded and new media were replenished. On day3, cells were resuspended in fresh media containing GM-CSF. On day 6, non-adherent cells were harvested and examined for CD11c and MHCII expression. To set up the experiment, $1x10^6$ $CD11c^+$ BMDCs were co-cultured with $1x10^6$ CAR T cells in 2 ml media. BMDCs and CAR T cells alone were served as controls. Supernatant was collected after 24 hours and examined for cytokine production by Luminex.

ELISpot assay

For the EL4 tumor model, C57BL/6 mice were i.v. inoculated with $1x10^6$ EL4-$CD19^+$ cells on day 0 and i.v. treated with $2.5x10^6$ $Thy1.1^+$ CAR T cells the following day. Seven days after CAR transfer, splenocytes were harvested and sorted for endogenous $CD4^+$ and $CD8^+$ T cells (pre-gated on $CD3^+$, $Thy1.2^+$ population). $1x10^5$ $CD4^+$ or $CD8^+$ T cells and EL4 cells were co-cultured in the presence or absence of MHC I or MHCII blocking antibody (1 μg/ml) (Thermo Fisher) for 24 hours. For the A20 tumor model, Balb/c mice were i.v. inoculated with $1x10^6$ A20 cells on day 0 and i.v. treated with $3x10^6$ $CD45.1^+$ CAR T cells on day 7. On day 14 after tumor inoculation, splenocytes were harvested and sorted for endogenous $CD4^+$ and $CD8^+$ T cells (pre-gated on $CD3^+$, $CD45.2^+$ population). $1x10^5$ T cells were cultured with $1x10^5$ tumor cells (A20, A20-MHC I KO, or A20-MHC II KO) for 24 hours. Secretion of IFN-γ was detected using Millipore ELISpot kit (Mabtech) according to the manufacturer's instructions.

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Statistical analysis

All statistical analyses were performed using GraphPad Prism software (GraphPad). Significance analyses include unpaired two-tailed Student's t-test, unpaired t-test with Welch's correction, one-way ANOVA, two-way ANOVA with Tukey's and Sidak's multiple comparison test, and log-rank test, as indicated in the figure legends. Variance are similar between groups with exceptions of Fig. 2D and 2E, Fig. 4C, Fig 5A, fig. S3A (IFN-γ, TNF-α and IL-6), fig. S5C and fig. S7C.

Results

Generation of mouse CAR T cells to secrete IL-36γ

To assess the role of constitutive exposure of IL-36γ to CAR T cells in a syngeneic context, we generated bicistronic retroviral vectors containing the CD19-targeting CAR– and the IL-36γ–coding sequences. CARs lacking intracellular signaling domains served as negative controls (Fig. 1A). Flow cytometry analysis suggested comparable expression of CAR by m19m28mz and m19m28mz-IL-36γ CAR T cells (Fig. 1B). Secreted IL-36γ was detected in the supernatant of m19m28mz-IL-36γ packaging cells by Western blot (fig. S1A). To validate the functionality of the secreted IL-36γ, we collected supernatant from packaging cells and incubated it with mouse T cells isolated from spleen in the presence of CD3 stimulation. Cytokine analysis showed enhanced IL-2 in the co-culture containing IL-36γ, substantiating the functionality of the cytokine (fig. S1B).

To validate the function of engineered CAR T cells and assess the impact of IL-36γ, we examined the tumor lytic capacity, cytokine secretion profile, and proliferation of CAR T cells in response to antigen stimulation. Bioluminescence-based killing assay demonstrated comparable tumor lysis by IL-36γ–secreting CAR T cells and conventional CAR T cells against CD19-expressing EL4 tumor cells (Fig. 1C). Compared to conventional CAR T cells, IL-36γ–secreting CAR T cells produced similar levels of interferon-γ (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF), decreased level of tumor necrosis factor-α (TNF-α) (Fig. 1D). Surprisingly, despite the fact that IL-36γ increases IL-2 secretion by CD3-stimulated T cells, we did not detect IL-2 production in the context of tumor-stimulated CAR T cells. We speculate this is likely due to crosslinking antibody inducing higher levels of T cell activation and IL-2 secretion than CAR stimulation via cell-based surface antigen. IL-36γ–secreting CAR T cells and control CAR T cells expanded to a similar extent in response to antigen stimulation (Fig. 1E). In addition, we did not observe significant differences in the composition of CD4 and CD8 subsets or in the central memory and effector memory subsets in IL-36γ–secreting CAR T cells (fig. S1C-S1D). Collectively, the data indicate that IL-36γ–secreting CAR T cells are functional and do not exhibit major differences in function and phenotype compared to conventional CAR T cells *in vitro*.

Low dose of IL-36γ–secreting CAR T cells induces long-term remission in tumor-bearing mice following pre-conditioning chemotherapy

To determine the efficacy of modified CAR T cells *in vivo*, we used a clinically relevant model where lymphodepletion is achieved by intraperitoneal injection of cyclophosphamide (Cy). Three days after lymphodepletion, C57BL/6 mice were inoculated with EL4-CD19^{+}

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cells, followed by intravenous injection of CAR T cells the next day (Fig. 2A). At a dose level of $1x10^6$ CAR T cells, both conventional CAR and IL-36γ–secreting CAR T cells exhibited equivalent tumor-control capability (Fig. 2B). Notably, IL-36γ–secreting CAR T cells retained anti-tumor efficacy at a log lower dose, where conventional CAR T cells failed to eradicate the disease (Fig. 2C). Consistent with the survival benefit, the low dose of IL-36γ–secreting CAR T cells was able to induce B cell aplasia and significantly elevate serum IFN-γ levels in mice (Fig. 2D-2E). No increase of serum IL-6 was observed (Fig. 2F). Collectively, these results suggest IL-36γ significantly enhances the therapeutic efficacy of CAR T cells in the context of lymphodepletion.

IL-36γ–secreting CAR T cells exhibit superior anti-tumor efficacy and induce prolonged B cell aplasia without pre-conditioning

Although lymphodepletion enhances the efficacy of adoptively transferred T cells, the resulting ablation of functional endogenous immune effectors may hamper the ability to initiate or enhance an endogenous anti-tumor immune response recognizing other tumor antigens. Therefore, we sought to determine whether IL-36γ–secreting CAR T cells could eliminate the requirement for lymphodepletion. Using the same syngeneic tumor model in which a higher dose of conventional CAR T cells did not confer any survival benefit in the absence of lymphodepletion, IL-36γ–secreting CAR T cells were able to induce long-term remission in tumor-bearing mice (Fig. 2G-2H). To further examine whether the enhanced CAR T cell function requires continuous secretion of IL-36γ, we compared the anti-tumor efficacy of CAR T cells in exposure to exogenous IL-36γ prior to injection to those constitutively secreting the cytokine. We chose a concentration of IL-36γ in the pre-incubation experiment where the dose-responsive curve reaches to plateau and theoretically should saturate the receptor. Exposure to exogenous or secreted IL-36γ both increased CAR T cell size, which reflected a more activated status (fig. S2A). However, exposure to IL-36γ in culture failed to induce long-term remission in mice, as opposed to cytokine-secreting CAR T cells. This result suggests that the anti-tumor efficacy requires continuous exposure of CAR T cells to IL-36γ *in vivo* (fig. S2B). As a control, CAR T cells with cytokine secretion but lacking signaling domains did not show any survival improvement (fig. S2C). The improved function of IL-36γ–secreting CAR T cells was further supported by the enhanced production of pro-inflammatory cytokines in the serum at day 7 and the profound and persistent B cell aplasia detected in mice up to 250 days, a surrogate marker indicating the longevity of the CAR T cells (fig. S3A, Fig. 2I-2J). Although IL-6 was elevated in mice treated with IL-36γ–secreting CAR T cells, no significant weight loss or acute toxicity was observed in these animals (fig. S3B). Given that antigen–positive tumor relapse often occurs as a result of short CAR T cell persistence, we asked whether the prolonged CAR T cell persistence by IL-36γ was able to protect animals from antigen-positive tumor re-challenge. As expected, mice that were treated with m19m28mz-IL-36γ CAR T cells and developed long-term B cell aplasia were able to reject or delay the progression of a low-dose tumor re-challenge compared to naïve mice, further substantiating the functionality of long-persisting IL-36γ–secreting CAR T cells (Fig. 2K-2L). We also compared the anti-tumor efficacy to another “armored” CAR which secretes interleukin IL-18 (m19m28mz-IL18). In the same tumor model, both CAR T cells were able to induce long-term remission and deep B cell

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aplasia in mice. Nevertheless, IL-36γ–secreting CAR T cells induced higher serum levels of IFN-γ and TNF-α, potentially indicating a more robust immune response (fig. S4).

IL-36γ enhances CAR T cell proliferation and cytokine secretion ex vivo

Given that high peak CAR T cell expansion-to-tumor ratio and CAR T cell polyfunctionality are correlated with good response in patients (6, 9), we hypothesized that IL-36γ improves CAR T cell expansion and functionality *in vivo*. To identify and isolate CAR T cells *ex vivo*, we generated a CAR construct co-expressing a fluorescent protein vexGFP which demonstrated similar activity *in vivo* (fig. S5A-S5C). IL-36γ–secreting CAR T cells exhibited 10-fold higher expansion than conventional CAR T cells in both the spleen and bone marrow at day 3 (Fig. 3A-3B). Concomitantly, the percentage of $CD19^+$ cells in IL-36γ–secreting CAR T cell–treated group decreased at day 3 and were eliminated in bone marrow at day 7 (Fig. 3C). The expanded IL-36γ–secreting CAR T cells exhibited enriched effector memory phenotype (fig. S5D). To further confirm that the improved expansion was due to superior proliferative capability, we examined the expression of a proliferation marker Ki67 using CAR T cells derived from $Thy1.1^+$ donors. In this model, IL-36γ–secreting CAR T cells demonstrated superior expansion at day 7 post-transfer (fig. S6). Interestingly, IL-36γ–secreting CAR T cells maintained a high-level expression of the proliferation marker Ki67 in the bone marrow compared to conventional CAR T cells, while both CAR T cells exhibited similar expression level of Ki67 in the spleen (Figure 3D). This result indicates that IL-36γ promotes CAR T cell proliferation in the bone marrow, which is a disease relevant site.

The ability to rapidly secrete inflammatory cytokines in response to stimulation is a hallmark of effector T cell functionality. Under chronic antigen stimulation, T cells upregulate PD-1 and gradually lose their capacity to secrete IFN-γ and TNF-α (29). To understand whether IL-36γ enhances the effector function of CAR T cells, we harvested cells from mice treated with $vexGFP^+$ CAR T cells and assessed the production of IFN-γ and TNF-α by CAR T cells in the presence or absence of PMA and ionomycin stimulation *ex vivo*. In absence of stimulation, IL-36γ–secreting CAR T cells showed higher production of IFN-γ *ex vivo*, which may be caused by non-specific IFN-γ secretion induced by IL-36γ. Upon stimulation, IL-36γ–secreting CAR T cells had a more robust cytokine production profile, demonstrated by a significantly higher percentage of cells secreting both cytokines ($IFN\text{-}\gamma^+$, $TNF\text{-}\alpha^+$), and fewer cells not responsive to the stimulation ($IFN\text{-}\gamma^-$, $TNF\text{-}\alpha^-$) (Fig. 3E). Collectively, the data suggest IL-36γ improves the functionality of CAR T cells on a per-cell basis through enhanced proliferation and cytokine production.

Anti-tumor efficacy requires the $CD8^+$ CAR T cell subset, and is mediated by autocrine IL-36 signaling via the Myd88 pathway

The composition of $CD4^+$ and $CD8^+$ T cells can impact the outcome of adoptive T cell therapy (30, 31). In our study, we noticed an enrichment of $CD8^+$ CAR T cells in mice treated with IL-36γ–secreting CAR T cells compared to prior injection (Fig. 4A). Given the distinctive cellular function and molecular profile of $CD8^+$ and $CD4^+$ CAR T cells, we postulated that the enhanced efficacy of IL-36γ–secreting CAR T cells was due in part to the expansion and enrichment of the $CD8^+$ population, which is known for a superior cytotoxic

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effect. To test this hypothesis, we injected the same number of isolated $CD4^+$, $CD8^+$ or bulk CAR T cells into tumor-bearing mice and monitored B cell levels at day 7 and overall survival. Interestingly, $CD8^+$ CAR T cells alone were able to induce long-term remission and absolute B cell aplasia in mice as well as bulk CAR T cells, in contrast to a more modest tumor protection and relative B cell aplasia conferred by $CD4^+$ CAR T cells (Fig. 4B-4C). These results suggest that the IL-36γ–secreting CAR-mediated anti-tumor effect is dependent on $CD8^+$ CAR T cells.

The development of B cell aplasia, a surrogate marker of CAR T cell functionality, suggests the anti-tumor effect is likely attributed to improved CAR T cell function. However, endogenous immune effectors can also be activated by IL-36γ and contribute to tumor eradication. To distinguish these two possibilities, we inoculated EL4-$CD19^+$ tumor into wild-type (WT) and $Rag1^{-/-}$ mice which lack functional T and B cells. There was no significant difference in survival between WT and $Rag1^{-/-}$ recipients, suggesting that endogenous T cells are dispensable for upfront tumor clearance (Fig. 4D). Notably, some $Rag1^{-/-}$ mice displayed inflammation-associated tissue damage and lymphoproliferation in secondary lymphoid organs, likely attributed to the expansion of adoptively transferred T cells. To further confirm that IL-36γ directly enhances CAR T cell function in an autocrine manner, we generated IL-36γ–secreting CAR T cells derived from WT and $IL\text{-}36R^{-/-}$ donors and compared their anti-tumor efficacy. No impairment in CAR T cell cytotoxicity and cytokine production was observed in $IL\text{-}36R^{-/-}$ CAR T cells (fig. S7A-S7B). Nevertheless, $IL\text{-}36R^{-/-}$ CAR T cells failed to induce B cell aplasia (fig. S7C) and protect mice from tumor compared to CAR T cells generated from WT mice (Fig. 4E), suggesting the autocrine IL-36γ signaling is critical for the improved CAR T cell function. The intracellular signaling of IL-36γ was transduced via the adaptor protein Myd88. To further demonstrate the autocrine dependency, we generated IL-36γ–secreting CAR T cells from WT and $Myd88^{-/-}$ mice, and observed similar results where IL-36γ–secreting CAR T cells from WT but not $Myd88^{-/-}$ mice were able to eradicate the disease in mice (Fig. 4F). We also tested the dependency of the IL-36 pathway in the lymphodepletion model. In the high-dose model, where the secretion of IL-36γ is not necessary, absence of Myd88-mediated pathway did not impair the anti-tumor efficacy of CAR T cells (fig. S7D). In contrast, in the low-dose model, where IL-36γ–secreting CAR T cells, but not the second-generation control CAR T cells, were able to eradicate tumor, the disruption of IL-36 pathway through Myd88 knockout on CAR T cells completely abrogated their anti-tumor efficacy (Fig. 4G). Together, the results suggest that the IL-36 pathway is not required for the baseline function of CAR T cell, but it will significantly augment CAR T cell function and improve the anti-tumor response through an autocrine fashion.

IL-36γ activates myeloid cells and exposure to IL-36γ–secreting CAR T cells promotes tumor recognition by endogenous T cells

Previous studies suggest that IL-36R is abundantly expressed on myeloid cells, including DCs, macrophages and monocytes, and the activation IL-36 pathway on DCs contributes to the formation of T cell–mediated anti-tumor response (23, 32, 33). We hypothesized that IL-36γ produced by CAR T cells would promote the activation and maturation of myeloid-derived APCs and facilitate T cell priming. To test the hypothesis, we first examined the

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impact of IL-36γ on bone marrow–derived dendritic cells (BMDCs) *in vitro*. BMDCs co-cultured with IL-36γ–secreting CAR T cells showed significant increase of IL-6 production, a cytokine typically produced by activated myeloid cells (Fig. 5A). Interestingly, we also observed an increase of IFN-γ in the co-culture compared to CAR T cell or BMDCs alone, and the presence of IL-36γ in the co-culture further boosted the secretion of IFN-γ in a synergistic manner.

Next, we asked whether IL-36γ–secreting CAR T cells were able to promote the maturation of APCs in spleen, where T cell and APC interaction and immune responses develop. Three days after CAR T cell transfer, the percentages of $CD86^+$ MHC II^+ DCs and macrophages were significantly increased in mice treated with IL-36γ–secreting CAR T cells (Fig. 5B-5C). In addition, the effector function of endogenous T cells was also augmented as a higher percentage of cells were poised to produce IFN-γ and TNF-α in response to stimulation (Fig. 5D).

Given the immune modulatory effects, we then examined whether the activation of APCs and endogenous T cells could translate into a meaningful anti-tumor response mediated through endogenous T cell receptor (TCR) repertoire. To test this hypothesis, CAR T cells derived from $Thy1.1^+$ strain were injected into EL4 tumor-bearing, $Thy1.2^+$ congenic hosts. Seven days after CAR T cell transfer, the endogenous, $Thy1.2^+$ T cells in the spleen were flow sorted, co-cultured with EL4 tumor and examined for IFN-γ release by ELISpot assay. Endogenous $CD8^+$ T cell from IL-36γ–secreting CAR T cell–treated mice were able to mount tumor-specific responses, while regular CAR T cell–-treated animals failed to do so. The recognition was effectively blocked by major histocompatibility complex (MHC) I antibody, indicating that the T cell–mediated response was dependent on TCR-MHC interactions. As for $CD4^+$ T cells, the response in IL-36γ–secreting CAR T cell-treated mice was not tumor-specific due to the high basal level of cytokine production. (Fig. 5E). To further evaluate the significance of the endogenous immune response, we challenged surviving mice with EL4-$CD19^-$ tumor to avoid any tumor eradication from persistent CAR at this point. Compared to the naïve group, mice previously treated with IL-36γ–secreting CAR T cells showed a significant delay in tumor progression and improved survival, indicating the formation of an immunological memory against antigen-negative tumors (Fig. 5F). Lastly, to confirm that the development of endogenous immune response is not restricted to a specific tumor or a mouse strain, we conducted a similar experiment in the BALB/c strain where CAR T cells derived from $CD45.2^+$ donors were injected into A20 tumor-bearing, $CD45.1^+$ hosts. In this model, endogenous T cells from both cohorts were able to develop tumor-specific response compared to T cells alone — this is potentially due to a higher immunogenicity of A20 cells. However, the magnitude of response was significantly enhanced in IL-36γ–secreting CAR T cell–treated animals and was abrogated when T cells were co-cultured with A20 tumor cells knocked out of MHC II or MHC I expression, despite a higher basal level of cytokine production (Fig. 5G). Together, the data suggest that exposure to IL-36γ–secreting CAR T cells were capable of inducing and enhancing an endogenous T cell–mediated anti-tumor response.

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IL-36γ–secreting CAR T cell enhances tumor eradication in a xenograft model

Lastly, to demonstrate the translational value of our finding, we engineered human CD19-targeting CARs with or without IL-36γ secretion (Fig. 6A) and evaluated their *in vivo* anti-tumor efficacy. NCG mice were intravenously injected with luciferase-expressing Nalm6 tumor at day 0, and treated with $1x10^5$ CAR T cells at day 4 (Fig. 6B). Mice treated with 1928z-IL-36γ–secreting CAR T cells demonstrated a significantly improved tumor eradication compared to mice treated with conventional CAR T cells, as assessed by both bioluminescence imaging and survival (Fig. 6C-6E). These results suggest that the enhanced anti-tumor efficacy conferred by IL-36γ can be extended to the human system; they further strengthen the rationale to integrate IL-36γ into CAR T cells for the treatment of malignancies.

Discussion

In this study, we demonstrated that IL-36γ enhances the functionality of CAR T cells both quantitatively and qualitatively, evidenced by improved expansion and persistence of CAR T cells *in vivo*, and more robust production of IFN-γ and TNF-α upon stimulation on a per-cell basis. Together, these effects led to a superior tumor-control capacity and long-term immune protection against antigen-positive tumor re-challenge. In the pre-conditioned model, the “armored” CAR T cells required 10-fold lower cell numbers to achieve comparable efficacy compared to the conventional CAR T cells. Given that CAR T cell peak expansion and persistence correlate with an effective and sustainable response (6, 8, 34), this approach will be particularly meaningful for patients who do not respond to the therapy or succumb to antigen-positive tumor relapse due to poor CAR T cell expansion and persistence. Interestingly, despite a higher frequency of IL-36γ–secreting CAR T cells in the spleen, we did not observe a difference in the expression of the proliferation marker Ki67 in those CAR T cells, suggesting alternative mechanisms, such as enhanced survival or cell homing, may be accountable for the increased cell number. Indeed, IL-36γ has been reported to upregulate chemokine expression on dendritic cells, monocytes and epithelial cells, which may help to recruit T cells to the inflamed site (32, 35). Further transcriptional profiling to assess the expression of relevant genes and pathways associated with cell survival and homing are undergoing. Notably, IL-36γ–secreting CAR T cells maintained the level of Ki67 expression in the bone marrow, in contrast to a diminished expression in conventional CAR T cells. It is not known yet whether the CAR T cells in the bone marrow are migrated from spleen and gradually lose the proliferative capacity. Given that IL-36γ has been reported to promote T cell proliferation through activation on DCs (33), our data prompt an interesting hypothesis that IL-36γ may interact with factors present in the bone marrow microenvironment to maintain CAR T cell proliferation there. This can translate into a better CAR T cell expansion and persistency in the bone marrow and improves the efficacy for treating some hematological malignancies where bone marrow is a major disease site.

Our data suggest that enhanced CAR T cell function requires continuous exposure to IL-36γ, as pre-incubation of CAR T cells with exogenous cytokine failed to recapitulate the anti-tumor efficacy. The lack of response with prior incubation is likely due to the short half-life of the cytokine, as oxidation of IL-1 family members in the extracellular environment

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and the resulting loss of biological activity has been reported (36). This observation highlights the necessity of utilizing CAR T cell as a continuous source of the cytokine, as it not only enriches the cytokine to the local environment and avoids the potential toxicity, but also overcomes the issue of short biological activity.

The comparison of IL-36γ–secreting CAR T cells to other reported "armored" CAR T cells, such as IL-12 and IL-18–secreting CAR T cells also revealed interesting insights. We have previously reported that IL-12–secreting CAR T cells were only able to induce transient and relative B cell aplasia, in contrast to a deep and persistent B cell aplasia seen in IL-36γ and IL-18–secreting CAR T cells (13, 37). This suggests a differential impact of cytokines on CAR T cell function in the long term. Indeed, IL-12 has been suggested to upregulate FoxO1 expression in T cells, which leads to T cell dysfunction (17). The fact that IL-36γ and IL-18 both belong to the IL-1 superfamily and share very similar downstream signal transduction indicates that the conserved Myd88-dependent transcriptional outputs are critical to enhance and sustain CAR T cell function. Compared to IL-18, mice treated with IL-36γ–secreting CAR T cells showed more robust cytokine production *in vivo*. We speculated the modest level of cytokines secreted by IL-18–secreting CAR T cells may be partially explained by the regulation of IL-18 binding protein, an antagonist to IL-18 receptor, which is pre-existing in the peripheral blood and can be upregulated in response to IFN-γ to counteract the effect of IL-18. In addition, unlike IL-36γ, IL-18 has also been suggested to have both tumor-inhibitory and tumor-stimulatory effects in different tumor contexts, which could further complicate the overall immune response. These observations suggest that in-depth understanding of the biology of cytokines and their dynamic impacts within the tumor microenvironment is required to make the best assessment of CAR T cells.

Importantly, IL-36γ–secreting CAR T cells also abrogate the need for pre-conditioning chemotherapy, which could deplete the endogenous lymphocytes and therefore prevent the development of T cell–mediated secondary immune responses against other tumor antigens through endogenous TCR. A broadened anti-tumor immunity beyond CAR-defined specificity is important for the prevention of $CD19^{-}$ tumor relapse, which is commonly seen in 7-25% of initially responding patients (7), and can extend the application of CAR T cell therapy to solid tumors where a ubiquitous tumor antigen that spares healthy tissues is usually lacking. The fact that IL-36γ–secreting CAR T cells were capable of activating endogenous immune effectors and inducing endogenous anti-tumor response to delay the progression of antigen-negative tumor highlights the potential of utilizing this approach to address antigen heterogeneity and antigen-negative relapse. However, it is not clear yet whether the endogenous T cell–mediated anti-tumor response is directly mediated through IL-36 pathways on host immune system, or via other intermediaries secondary to the curative immune response induced by IL-36γ–secreting CAR T cells. Alternatively, the prolonged survival of mice treated with IL-36–secreting CAR T cells may simply allow time for tumors to induce an endogenous anti-tumor response independent of any effect induced by IL-36γ–secreting CAR T cells. Therefore, delineating the necessity of the IL-36 pathway on host immune cells will be critical for understanding the molecular mechanism of this process. Other studies using tumor cells and therapeutic DCs engineered to express IL-36γ also substantiate the effect of IL-36γ on promoting a tumor-specific response (22, 23). However, tumor vaccine and therapeutic DCs are administered intratumorally, which greatly

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restricts the application potential. In addition, limited efficacy has been observed, potentially due to the lack of upfront tumor debulking for sufficient antigen presentation. Therefore, the rationale of integrating IL-36 signaling into a cytotoxic cell, such as a CAR T cell, overcomes these limitations and embraces parallel anti-tumor mechanisms that allows for optimal efficacy.

We also demonstrated that the enhanced CAR T cell efficacy was mediated via an autocrine, Myd88-dependent pathway. The impact of the Myd88 pathway on CAR T cell function has also been demonstrated in other approaches. For example, a CAR T cell including the ζ chain, Myd88, and CD40 signaling domains showed improved effector function and resistance to exhaustion after CAR engagement (38, 39). However, direct incorporation of Myd88 signaling domain into CAR was reported to hamper CAR expression level and reduce the *in vivo* anti-tumor efficacy (39). In comparison, a cytokine-secreting CAR not only induces efficient signaling transduction within CAR, but allows for activation of bystander immune effectors to collaboratively amplify and broaden the anti-tumor immune response.

A potential limitation of cytokine-armored CAR T cells is the uncontrolled secretion of cytokines and possible toxicities due to the pleiotropic effects of cytokines on multiple immune cells. Activation of myeloid cells and secretion of IL-6 has been implicated in the development of cytokine release syndrome (40, 41). In the non-preconditioning model, we observed an elevated IL-6 in the serum in mice treated with IL-36γ–secreting CAR T cells, although we did not observe apparent weight loss or acute toxicity–related death in any of the mice, suggesting the tolerance of IL-36γ–secreting CAR T cells in this model (fig. S3). Nevertheless, in $Rag1^{-/-}$ animals where engraftment of adoptively transferred T cells can be greatly enhanced due to no competition from endogenous T cells, inflammation-associated tissue damage and abnormal expansion of lymphoid organs were observed in some animals. More work to delineate the conditions and mechanisms contributing to the development of the treatment-related toxicity is undergoing. This observation does highlight the need for a careful examination of safety using this approach, including comprehensively profiling the immune cell composition in patients under treatment regimen and understanding the kinetics of markers associated with cytokine-associated toxicities. Moreover, inducible cytokine expression systems, such as nuclear factor of activated T cell–responsive expression cassette or a synNotch module, that regulate cytokine secretion in a controlled manner can be incorporated into CAR T cell design to mitigate the concern of toxicity (42-44).

To conclude, IL-36γ–secreting CAR T cells demonstrate effective and long–lasting anti-tumor efficacy and are able to engage endogenous immune cells to develop secondary anti-tumor response. Our strategy provides a potential solution to overcome the current challenges of limited CAR T cell-mediated efficacy due to poor expansion and tumor relapse. The improved anti-tumor efficacy observed in the human system further substantiates the rationale to translate this discovery for clinical applications.

Supplementary Material

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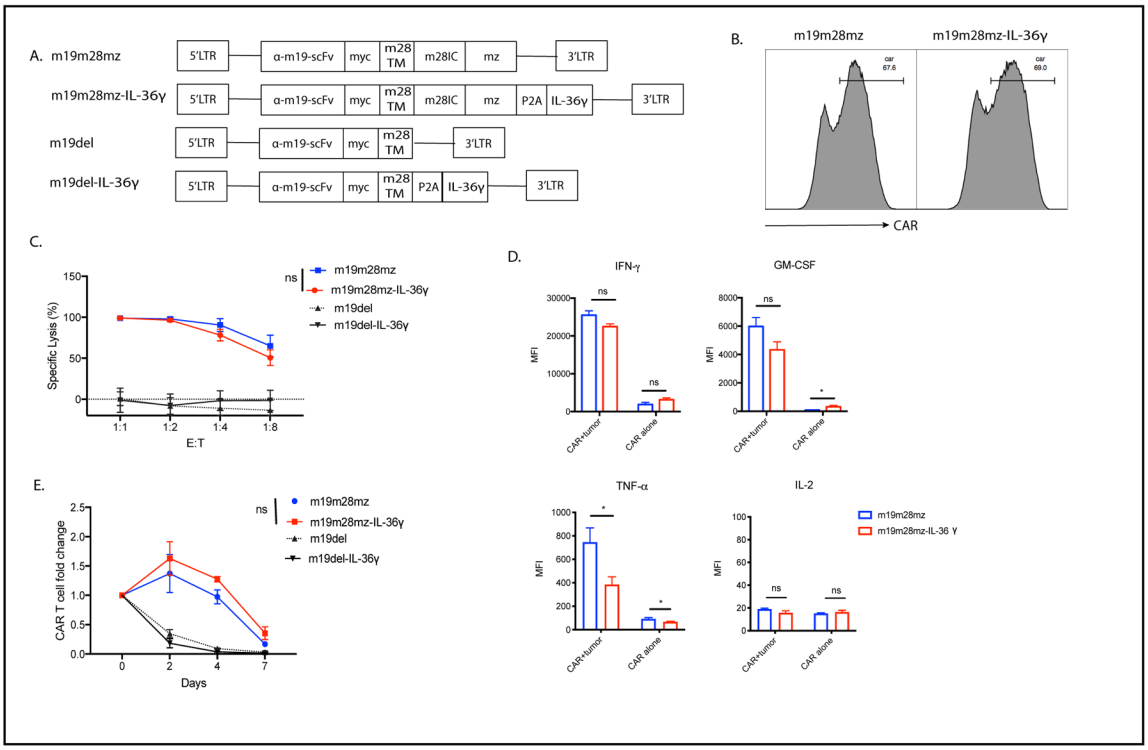

Fig. 1. CAR T cell construct validation and *in vitro* characterization.

(**A**) Schematic of vectors encoding a murine CD19–targeting CAR with or without IL-36γ. A myc tag is included for detection of single-chain fragment variable (scFv). (**B**) Representative flow cytometry plot demonstrating CAR expression on transduced mouse T cells. (**C**) CAR T cell cytotoxicity against EL4-CD19^{+} tumor was assessed by 24-hour bioluminescence assay. Data are plotted as mean ± SEM and are pooled from three independent experiments. E:T, ratio between effector and target cells. ns, non-significant by two-way ANOVA test. (**D**) Cytokine production of CAR T cells in presence or absence of tumor stimulation for 24 hours was assessed by Luminex. Data are plotted as mean ± SEM and are pooled from three independent experiments. ns, non-significant, $*P < 0.05$ by Student's t test. (**E**) CAR T cell expansion was assessed after co-culture with EL4-CD19^{+} cells at 1:1 ratio. CAR T cell numbers were counted at days 2, 4, and 7 by flow cytometry. Data are plotted as mean ± SEM and are pooled from three independent experiments. ns, non-significant by two-way ANOVA, Tukey's multiple comparisons test.

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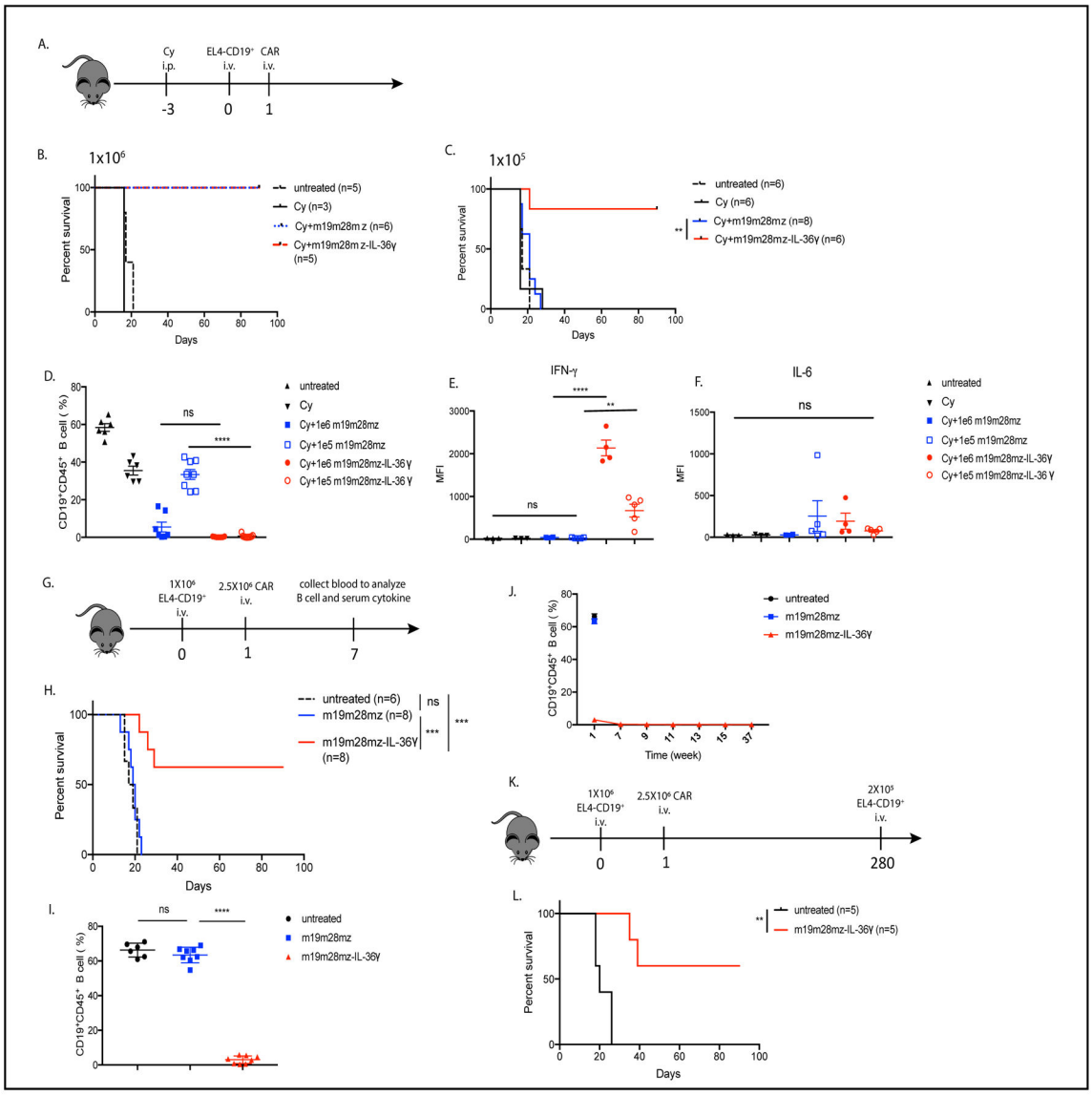

Fig. 2. IL-36γ–secreting CAR T cell exhibited superior anti-tumor efficacy with and without pre-conditioning.

(**A**) Experiment setup for (B-F). C57BL/6 mice were injected with 250 mg/kg cyclophosphamide i.p. 3 days prior to EL4-CD19$^+$ tumor inoculation, followed by intravenous CAR T cell injection the next day. (**B-C**) Survival of mice treated with 1x10^6 (B) and 1x10^5 (C) CAR T cells. Data are pooled from two independent experiments (n=3-8/group). $^{**}P < 0.01$ by log-rank test. (**D**) Mice were bled at day 7 after CAR transfer and peripheral B cell percentage was determined by flow cytometry. Data are plotted as mean ± SEM and are pooled from two independent experiments (n=6-9/group). ns, non-significant, $^{****}P < 0.0001$ by one-way ANOVA test. (**E-F**) Serum cytokine level of IFN-γ (E) and IL-6 (F) was measured by Luminex on day 7 after CAR T cell transfer. Data are plotted as mean ± SEM (n=3-5/group). ns, non-significant, $^{**}P < 0.01$, $^{****}P < 0.0001$ by one-way ANOVA test. (**G**) Experiment setup for (H-J). (**H**) Survival of C57BL/6 mice inoculated with 1x10^6 EL4-CD19$^+$ i.v. at day 0 and 2.5x10^6 CAR i.v. on day 1. Data are pooled from

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two independent experiments (n=6-8/group). ns, non-significant, ***$P < 0.001$ by log-rank test. (**I-J**) Peripheral B cell percentage was determined by flow cytometry on day 7 (I) and across indicated time points (J) after CAR T cell transfer. Data in (I) are plotted as mean ± SEM. (I) and (J) are pooled from two independent experiments (n=6-8/group). ns, non-significant, ****$P < 0.0001$ by one-way ANOVA test. (**K-L**) Long-term surviving mice in the m19m28mz-IL-36γ T cell cohort were re-challenged with $2x10^5$ EL4-$CD19^+$ cells at day 280. Kaplan-Meier survival plot is shown (n=5/group). **$P < 0.01$ by log-rank test.

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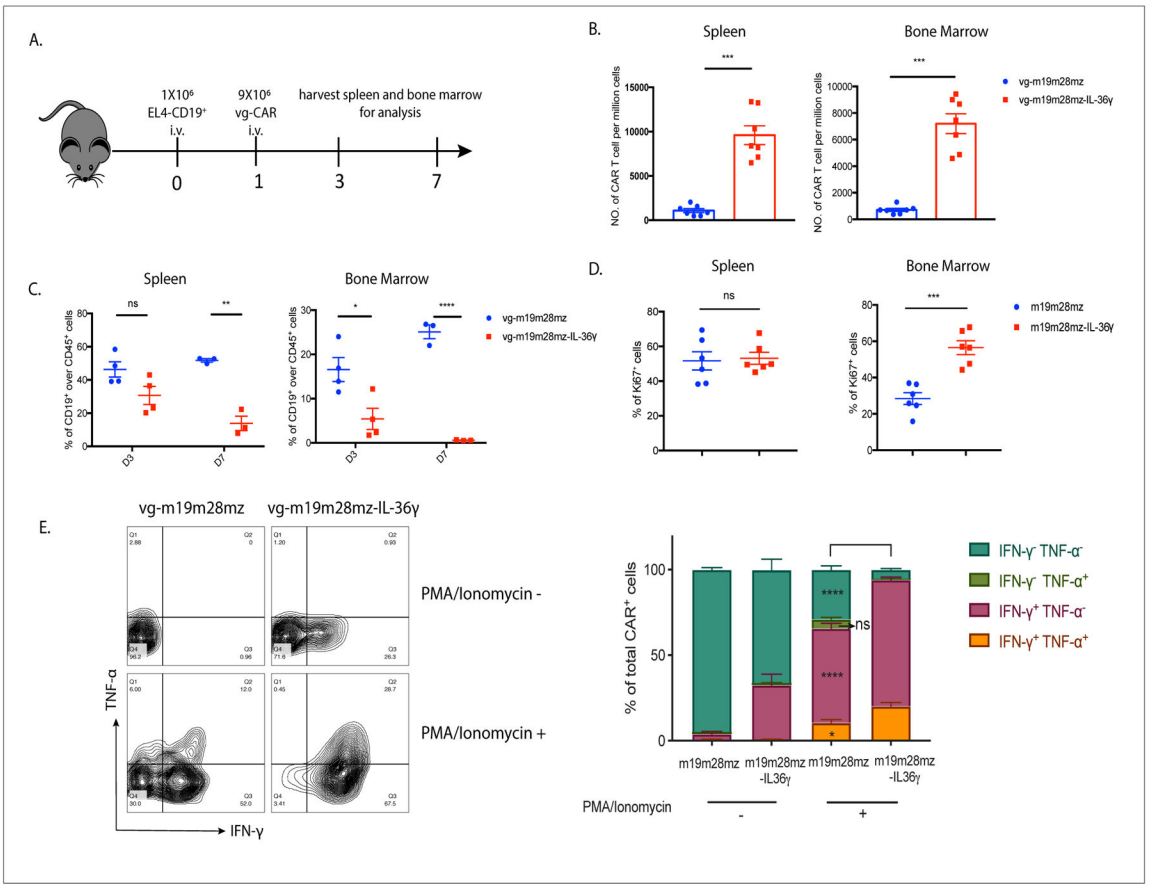

Fig. 3. IL-36γ enhances CAR T cell proliferation and cytokine secretion *ex vivo*.

(**A**) Experiment setup for (B-C) and (E). (**B**) CAR T cell frequency in spleen and bone marrow was determined by flow cytometry at day 3. Data are plotted as mean ± SEM and are pooled from three independent experiments (n=7/group). ***$P < 0.001$ by t test with Welch's correction. (**C**) $CD19^+$ cell frequency in spleen and bone marrow at days 3 and 7 was determined by flow cytometry. Data are plotted as mean ± SEM and are pooled from two independent experiments (n=3-4/group). ns, non-significant, *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ by Student's t test. (**D**) C57BL/6 mice were inoculated with $1x10^6$ EL4-$CD19^+$ tumor cells on day 0, followed by intravenous injection of $2.5x10^6$ CAR T cells derived from $Thy1.1^+$ donors on day 1. Percentage of $Ki67^+$ cells in CAR population ($Thy1.1^+$) was determined by flow cytometry at day 7. Data are plotted as mean ± SEM and are pooled from two independent experiments (n=6/group). ns, nonsignificant, ***$P < 0.001$ by Student's t test. (**E**) CAR T cells were analyzed for the secretion of IFN-γ and TNF-α in the absence or presence of PMA/ionomycin stimulation by intracellular flow cytometry at day 3. Representative plot and quantitative results are shown. Data are plotted as mean ± SEM and are pooled from two independent experiments (n=5-6/group). ns, non-significant, *$P < 0.05$, ****$P < 0.0001$ by two-way ANOVA, Tukey's multiple comparisons test.

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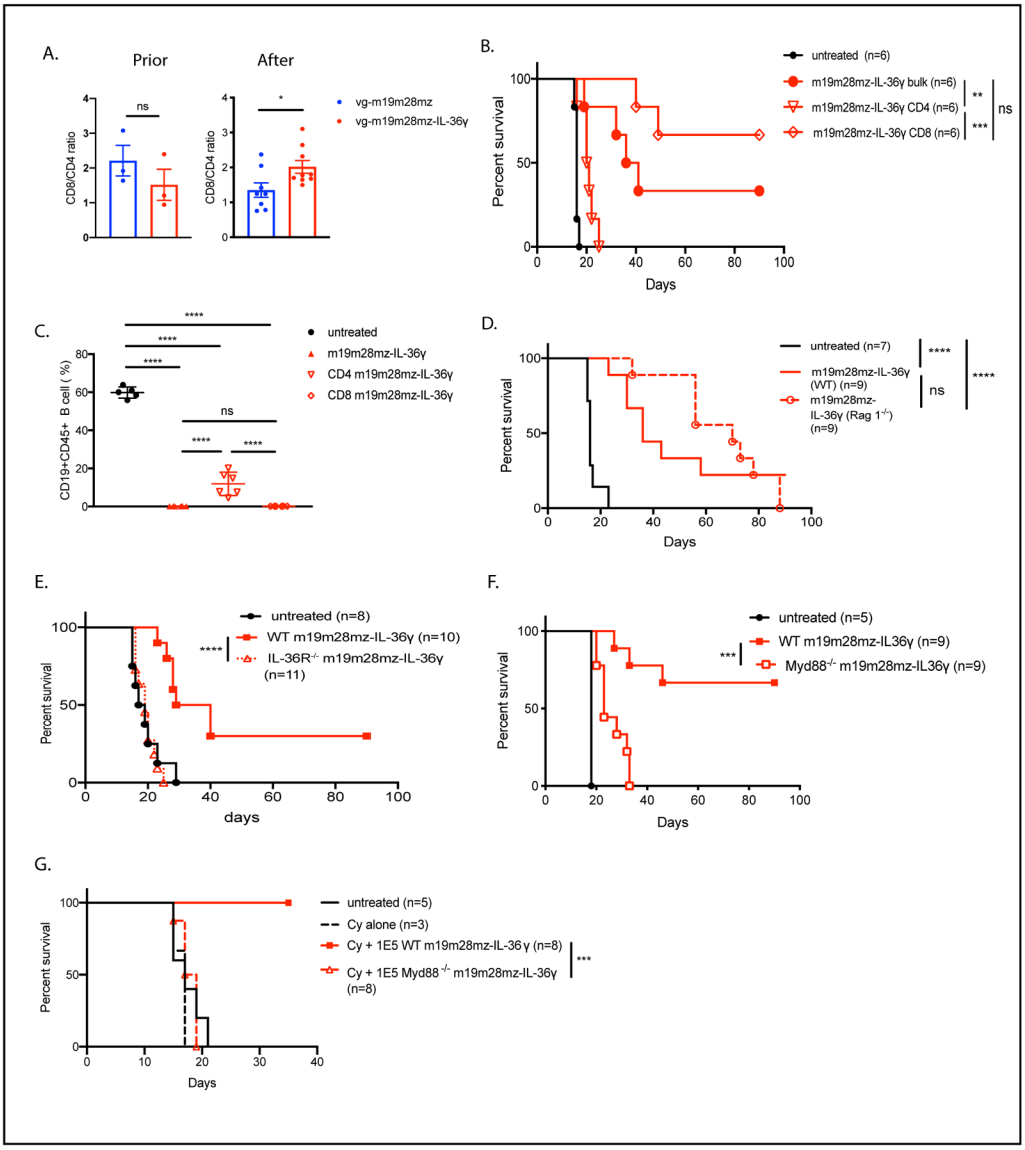

Fig. 4. Anti-tumor efficacy requires the CD8$^+$ CAR T cell subset, and is mediated by autocrine IL-36 signaling via the Myd88 pathway.

(**A**) The ratios of CD8/CD4 CAR T cells prior to injection (left) and 3 days after injection in the spleen (right) were determined by flow cytometry. Data in the left panel are plotted as mean ± SEM. Each dot represents one independent experiment run (n=3/group). Data in the right panel are plotted as mean ± SEM. Each dot represents one animal. Data are pooled from three independent experiments (n=8-9/group). ns, non-significant, $*P < 0.05$ by Student's t test. (**B**) Survival of C57BL/6 mice that were inoculated with 1x10^6 EL4-CD19$^+$ tumor cells and treated with 2.5x10^6 bulk, CD4-isolated, or CD8-isolated m19m28mz-IL-36γ CAR T cells the next day. Data are pooled from two independent experiments (n=6/group). $**P < 0.01$, $***P < 0.001$ by log-rank test. (**C**) In experiment (B), peripheral B cell level on day 7 after CAR T cell transfer was determined by flow cytometry. Data are pooled from two independent experiments (n=6/group). ns, non-significant, $****P < 0.0001$ by one-way ANOVA test. (**D**) Survival of WT or Rag1$^{-/-}$ EL4-CD19$^+$-bearing mice treated with

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$2.5x10^6$ m19m28mz-IL-36γ CAR T cells. Data are pooled from two independent experiments (n=7-9/group). ns, non-significant, ****$P < 0.0001$ by log-rank test. (**E-F**) Survival of EL4-$CD19^+$-bearing mice treated with $2.5x10^6$ m19m28mz-IL-36γ CAR T cells derived from WT and IL-$36R^{-/-}$ mice (E) or WT and $Myd88^{-/-}$ mice (F). Data are pooled from two independent experiments (n=8-11/group in (E), n=5-9/group in (F)). ***$P < 0.001$, ****$P < 0.0001$ by log-rank test. (**G**) C57BL/6 mice were pre-conditioned with 250 mg/kg cyclophosphamide i.p. 3 days prior to EL4-$CD19^+$ tumor inoculation, followed by intravenous injection of $1x10^5$ m19m28mz-IL-36γ CAR T cells derived from WT or $Myd88^{-/-}$ donor the next day. Survival from one experiment run was shown (n=3-8/group). ***$P < 0.001$ by log-rank test.

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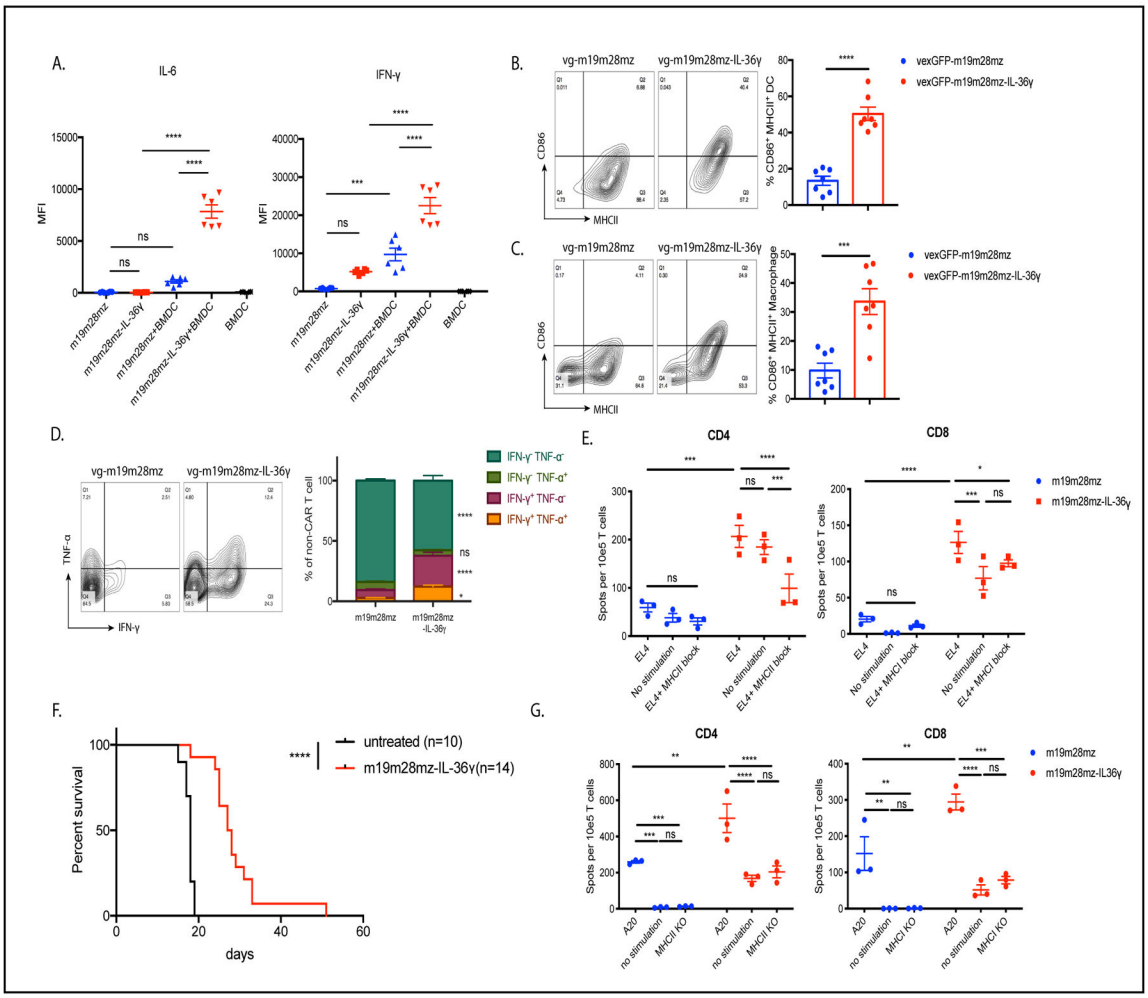

Fig. 5. IL-36γ activates myeloid cells and exposure to IL-36γ–secreting CAR T cells promotes tumor recognition by endogenous T cells.

(**A**) IL-6 and IFN-γ in the supernatant of CAR T cells co-cultured with BMDCs, or CAR T cells and BMDCs cultured alone for 24 hours were determined by Luminex. Data are plotted as mean ± SEM and pooled from two independent experiments (n=6/group). ns, non-significant, ***$P < 0.001$, ****$P < 0.0001$ by one-way ANOVA test. (**B-C**) The expression of CD86 and MHCII on $CD11b^-$ $CD11c^+$ DCs and $F4/80^+$ $CD11b^+$ macrophages in the spleen of tumor-bearing mice treated with $9x10^6$ vexGFP-m19m28mz or vexGFP-m19m28mz-IL-36γ CAR T cells was analyzed by flow cytometry. Representative flow plot (left, B and C) and quantification of % $CD86^+$ $MHCII^+$ cells over $CD11b^-$ $CD11c^+$ DCs (right, B) and $F4/80^+$ $CD11b^+$ macrophages (right, C) are shown. Data are plotted as mean ± SEM and pooled from three independent experiments (n=7/group). ***$P < 0.001$, ****$P < 0.0001$ by Student's t test. (**D**) Splenocytes from tumor-bearing mice treated with $9x10^6$ vexGFP-m19m28mz or vexGFP-m19m28mz-IL-36γ CAR T cells were harvested at day 3 and stimulated with PMA and ionomycin for 4 hours. Production of IFN-γ and TNF-α on endogenous T cells (gated as $CD3^+$ $vexGFP^-$ population) were determined by intracellular flow cytometry. Representative flow plot of each condition (left) and quantification (right) are shown. Quantitative data are graphed as mean ± SEM and pooled from two independent

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experiments (n=5-6/group). ns, non-significant, *$P < 0.05$, ****$P < 0.0001$ by two-way ANOVA, Tukey's multiple comparisons test. (**E**) C57BL/6 mice were inoculated with $1x10^6$ EL4-CD19$^+$ tumor cells on day 0, then on day 1 treated with $2.5x10^6$ m19m28mz or m19m28mz-IL-36γ CAR T cells derived from Thy1.1$^+$ donors. On day 8, host CD4$^+$ and CD8$^+$ T cells (Thy1.2$^+$) from spleen were harvested, separately sorted, and cultured with EL4-CD19$^+$ cells in the presence or absence of MHC I or MHC II antibodies. IFN-γ release was determined by ELISpot assay. Data are plotted as mean ± SEM and are representative of two independent experiments (n=3/group). ns, nonsignificant, *$P<0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by two-way ANOVA, Tukey's and Sidak's multiple comparisons test. (**F**) Long-term surviving mice initially inoculated with $1x10^6$ EL4-CD19$^+$ tumor and treated with $2.5x10^6$ m19m28mz-IL-36γ CAR T cells were re-challenged with $1x10^5$ EL4-CD19$^-$ cells on day 90+. Data are pooled from two independent experiments (n=10-14/group). ****$P < 0.0001$ by log-rank test. (**G**) WT BALB/c mice were inoculated with $1x10^6$ A20 tumor cells on day 0, then on day 7 treated with $3x10^6$ CAR T cells derived from CD45.1$^+$ donors. Host CD4$^+$ and CD8$^+$ T cells (CD45.2$^+$) from spleen were sorted and cultured with A20 cells, A20-MHC I KO, or A20-MHC II KO cells. IFN-γ release was determined by ELISpot assay. Data are plotted as mean ± SEM (n=3/group). ns, nonsignificant, *$P<0.05$, **$P < 0.01$, ***$P < 0.001$ by two-way ANOVA, Tukey's and Sidak's multiple comparisons test.

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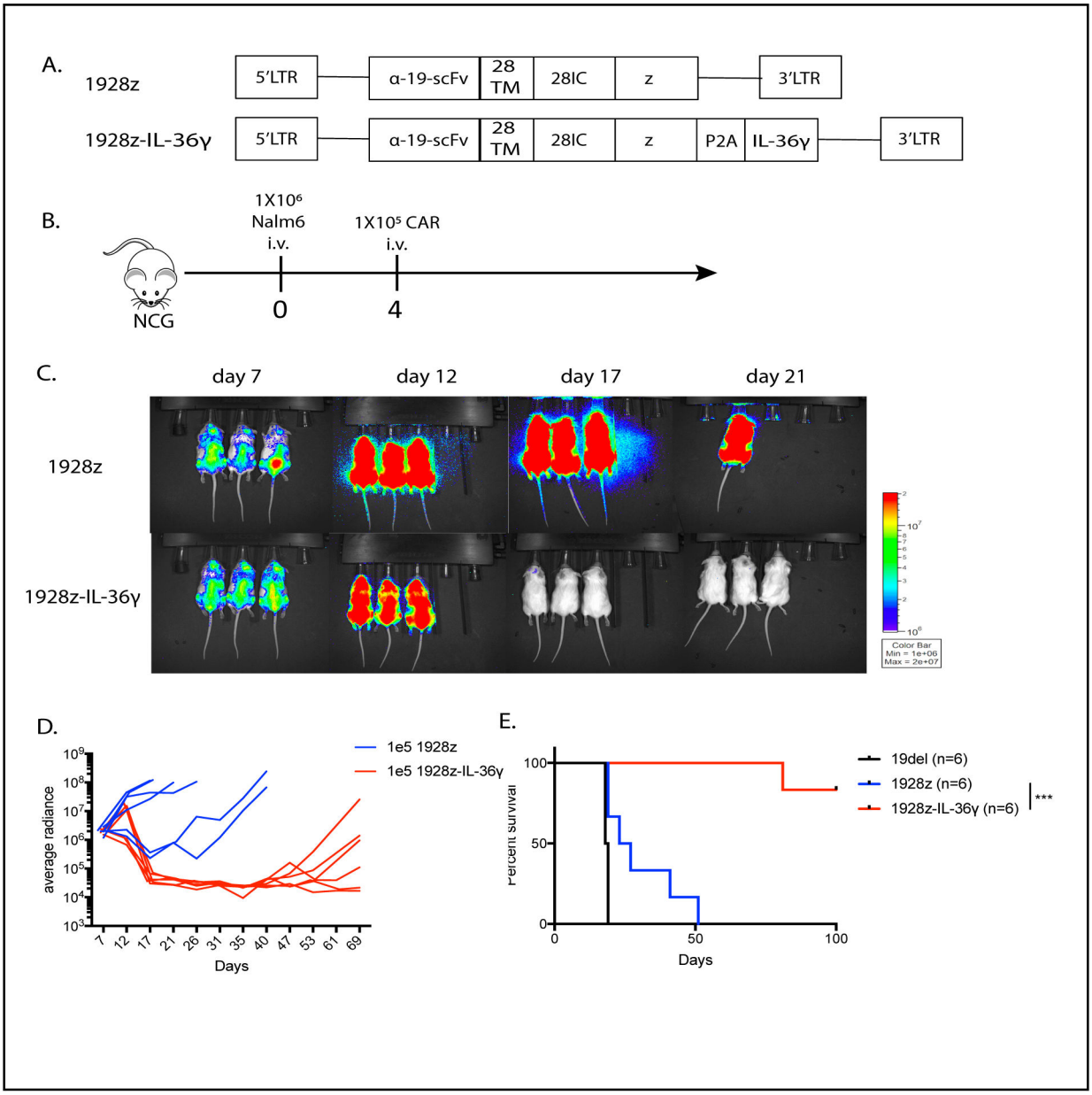

Fig. 6. IL-36γ–secreting CAR T cell enhances tumor eradication in a xenograft model.

(**A**) Schematic of vectors encoding a human CD19–targeting CAR with or without IL-36γ. **(B)** Experiment setup in (C-E). (**C-D**) Bioluminescence image (C) and quantification of average radiance (D) at indicated time points are shown. Each line in (D) represents an individual animal and data are pooled from two independent experiments (n=6/group). (**E**) Survival curve pooled from two independent experiments (n=6/group). ***$P < 0.001$ by log-rank test.