

LETTER TO THE EDITOR

CD70-targeting CAR-T cells have potential activity against CD19-negative B-cell Lymphoma

To the Editor:

CD19-targeting chimeric antigen receptor T (CAR-T) cell therapy is a revolutionary immunotherapy in treating relapsed or refractory B lineage malignancies. However, relapse with CD19-negative tumor after treatment with anti-CD19 CAR-T cells has been reported in different types of B-cell lymphoid malignancies, with a percentage exceeding 10% in patients with acute lymphoblastic leukemia [1] and up to 38% in patients with non-Hodgkin lymphoma (NHL) [2]. Thus, antigen escape constitutes a significant obstacle for anti-CD19 CAR-T therapy, underscoring the need to develop CAR-T cell therapies directed to alternative antigens. CD70, the membrane-binding ligand of the CD27 (a tumor necrosis factor receptor superfamily), has been reported to be expressed on the malignant cells of diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL) and follicular lymphomas (FL), as well as Hodgkin's lymphoma, Waldenström macroglobulinemia, and multiple myeloma, but rarely on normal B cells or T cells [2–4]. In addition, CD70 is also expressed in various solid tumor types, such as renal cell carcinoma and mesothelioma [3]. Recently, targeting CD70 has emerged as potential novel immunotherapeutic strategy [4–6]. Here, we report on the development of novel, second-generation CAR-T cells against CD70, a target for CAR-T cell therapy of B-cell lymphoma that has not been fully realized.

CAR-T cell-based cancer immunotherapy relies on not only the specificity but also association constant of single-chain variable fragment (scFv). The scFv used in the CAR is synthesized from parent antibody by fusing their light (V_L) and heavy (V_H)-chain variable domains into a single chain using a peptide flexible linker. A previous study demonstrated that the CAR targeting CD79b, in which the scFv was synthesized in a light-heavy (L/H) orientation of variable domain, had superior antigen-specific effector

functions than that of the CAR with heavy-light (H/L) orientation of the variable domain [7]. Using this strategy, we generated two second-generation anti-CD70 CAR constructs, with either a L/H or a H/L scFv configuration derived from a reported anti-CD70 monoclonal antibody, denoted as anti-CD70 (L/H) or anti-CD70 (H/L), respectively (Figure 1A). We also generated a CAR consisting of truncated CD27 (without its intracellular signaling domain) (Figure 1A) coupled with the co-stimulatory domain of 4-1BB and then CD3 ζ signaling domain (denoted as trCD27), which had previously been shown to have CD70-specific tumor recognition [8]. In contrast to solid tumor cell lines, CD70 mRNA was highly expressed in NHL cell lines such as 60 B-cell lymphoma cell lines (Supplementary Figure S1A). Flow cytometry analysis confirmed the high expression of CD70 protein in B-cell lymphoma cell lines Raji, JeKo-1, Mino, and WSU-DLCL2 compared with K562 cells serving as a CD70-negative control (Supplementary Figure S1B). We next characterized three types of anti-CD70 CAR-T cells lentivirally transduced to express anti-CD70 (L/H), anti-CD70 (H/L) or trCD27 CAR constructs, and assessed their effector functions. The anti-CD19 CAR, as the control lentiviral vector, was also generated. The representative transduction efficiency of anti-CD70 (L/H), anti-CD70 (H/L), trCD27, and anti-CD19 CAR was 55.1%, 44.5%, 74.5%, and 74.5%, respectively (Supplementary Figure S2). Proliferation of CAR-T cells following stimulation with anti-CD3/CD28 beads was measured. The anti-CD70 (L/H) CAR-T cells showed a lower rate of *ex vivo* expansion, while anti-CD70 (H/L), trCD27, and anti-CD19 CAR-T cells showed significantly greater proliferative capacity (Figure 1B). It has been well known that some scFvs are associated with ligand-independent tonic signaling, which could contribute to the reduction of efficacy of CAR-T therapy by leading to terminal effector T-cell differentiation and exhaustion [1]. We therefore compared the subsets of anti-CD70 (L/H), anti-CD70 (H/L), trCD27, or anti-CD19 CAR-T cells at 12 days after culture with anti-CD3/CD28 beads. The similar subset distribution of T cells was observed in

Abbreviations: IFN, Interferon; NF- κ B, Nuclear factor kappa B; NSG, NOD/SCID/interleukin-2 receptor subunit $\gamma^{-/-}$; Th1, T helper cell type 1; TNF, Tumor Necrosis Factor

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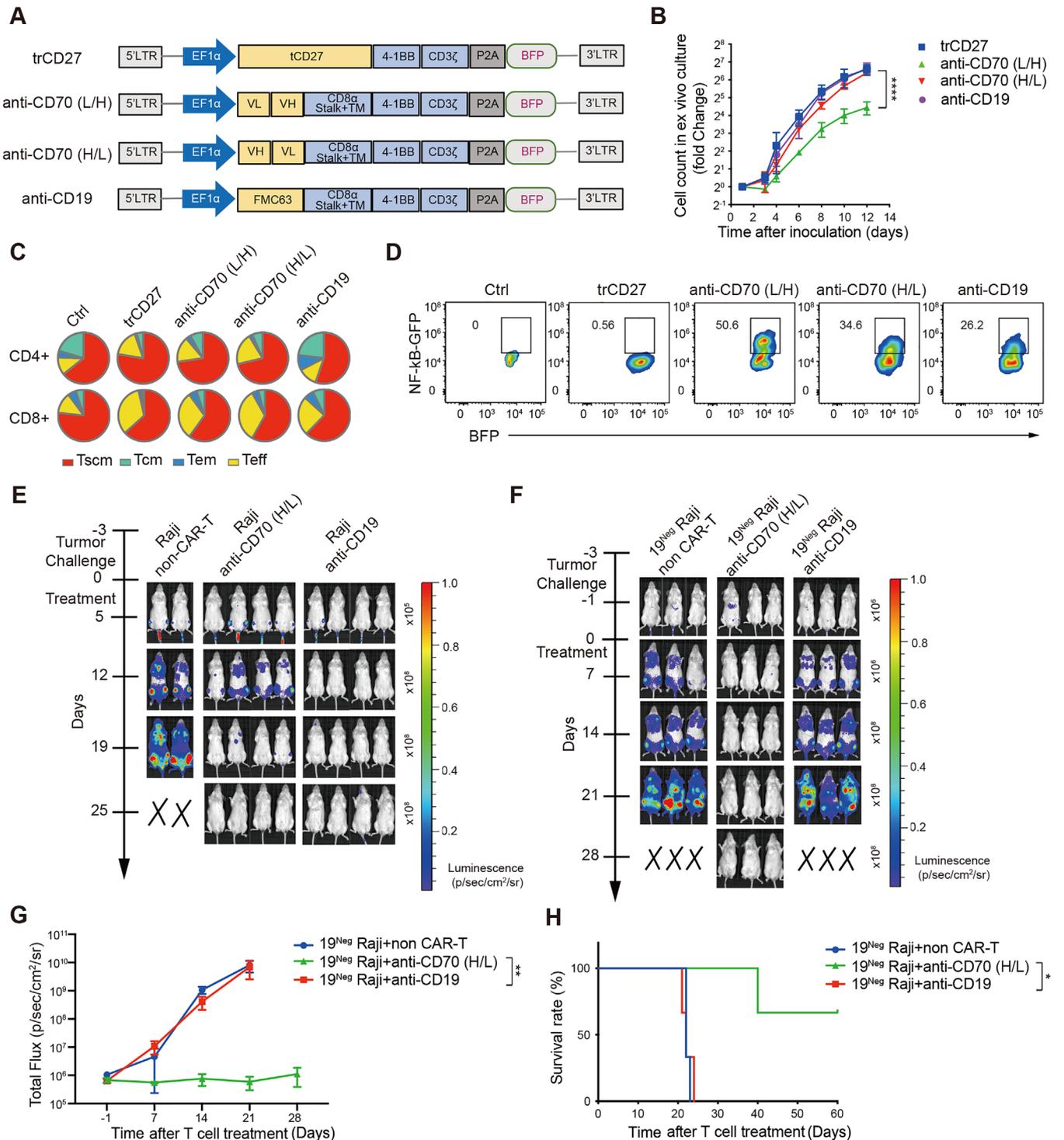


FIGURE 1 CD70-targeting chimeric antigen receptor-T (CAR-T) cells for lymphoma therapy. **(A)** Schematic representation of lentiviral constructs in which the EF1- α promoter drives expression of different CARs bearing intracellular 4-1BB co-stimulatory and CD3 ζ -activating signaling domains. One anti-CD70 CAR was constructed with truncated CD27 including extracellular and transmembrane portions, shown as trCD27. Another two anti-CD70 CARs were generated with different orientations of a CD70-binding antibody derived single-chain variable fragment (scFv): the light-to-heavy orientation [anti-CD70 (L/H)] and the heavy-to-light orientation [anti-CD70 (H/L)]. A CD19-specific CAR incorporating the FMC63 scFv was also generated, denoted as anti-CD19. Vectors contain a second transgene encoding for the fluorescent reporter BFP via a P2A cleavage peptide to facilitate enumeration of transduction efficiency. VL: variable light chain, VH: variable heavy chain, TM: transmembrane region, LTR: long terminal repeat. **(B)** CAR-T cell proliferation over 12 days in ex vivo CD3/CD28 bead-activated condition. Means and error bars indicating ± 1 SD of 3 healthy donors are shown. **** $P < 0.0001$ for anti-CD70 (L/H) vs. anti-CD70 (H/L) (two-way ANOVA with Tukey's multiple comparison test). **(C)** Phenotype of CAR-T cells as measured by surface expression of CD4 (top) and

anti-CD70 (L/H), anti-CD70 (H/L), and trCD27 CAR-T cells. However, anti-CD70 CAR-T cells showed a higher frequency of stem cell memory T cells when compared to anti-CD19 CAR-T cells (Figure 1C and Supplementary Figure S3). Inclusion of costimulatory 4-1BB in anti-CD19 CAR construct had been reported to cause tonic signaling via continuous TRAF2-dependent activation of the NF- κ B pathway [9]. We verified significantly increased NF- κ B activation in anti-CD70 (L/H) CAR-T cells (Figure 1D).

Due to the dysfunction of anti-CD70 (L/H) CAR-T cells characterized by the aberrant proliferation and abnormal NF- κ B activation, we chose anti-CD70 (H/L) CAR-T cells to define the antitumor activity of the CAR-T cells targeting CD70 antigen. We performed cytotoxicity assays against CD19- and CD70-positive Raji cells and JeKo-1 cells at an effector-to-target ratio of 20:1 for 4 h, and CD107a degranulation and cytokine expression by CAR-T cells were also determined. The T cells transduced with anti-CD70 (H/L) and anti-CD19 CAR constructs demonstrated comparable cytolytic activity and CD107a degranulation against B lymphoma cell lines (Supplementary Figure S4A and S4B). In consistent with these results, the pattern of cytokine production in response to Raji and JeKo-1 cells, especially with upregulation of Th1 cytokines IFN- γ and TNF α , was similar in anti-CD70 (H/L) and anti-CD19 CAR-T cells (Supplementary Figure S4C). We also detected that anti-CD70 (H/L) CAR-T cells, but not BFP-transduced T cells, effectively killed CD19-negative Raji cells (Supplementary Figure S4D). Next, anti-CD70 (H/L) CAR-T cells showed comparable killing efficacy when co-cultured with CD19-positive and CD19-negative Raji cells, respectively (Supplementary Figure S4E).

We finally used anti-CD70 (H/L) CAR-T cells to test therapeutic efficacy against both CD19-positive and CD19-knockout Raji xenografts in NSG mice because our *in vitro* assays demonstrated superior *ex vivo* growth functions of anti-CD70 (H/L) CAR-T cells compared to anti-CD70 (L/H) CAR-T cells (Figure 1B). Anti-CD70 (H/L)

CAR-T cells and anti-CD19 CAR-T cells were injected into NSG mice 3 days after intravenous injection of CD19-positive Raji cells. Tumor burden was assessed by bioluminescence imaging, which showed complete elimination of lymphoma by day 12 for anti-CD19 CAR-T cells and day 25 for anti-CD70 (H/L) CAR-T cells (Figure 1E). To evaluate the ability of anti-CD70 (H/L) CAR-T cells to prevent antigen escape, NSG mice were engrafted with CD19-knockout Raji cells. Tumor-bearing mice were administered with one intravenous infusion of anti-CD19 or anti-CD70 (H/L) CAR-T cells. The bioluminescence imaging results revealed that tumors were significantly suppressed in mice treated with anti-CD70 (H/L) CAR-T cells, yielding near-complete tumor clearance (Figures 1F and 1G), which highlights the potential effect of anti-CD70 (H/L) CAR-T cells against CD19-negative B-cell malignancies. Conversely, there was no significant difference of tumor burden between mice treated with anti-CD19 CAR-T cells and T cells without CAR transduction. Moreover, anti-CD70 (H/L) CAR-T cell-treated mice also demonstrated significantly prolonged survival compared with the other two groups (Figure 1H).

Herein, we report on the development of a novel CAR product targeting CD70 using an anti-CD70 scFv and demonstrated potential antitumor effects of anti-CD70 CAR-T cells in CD19-positive and -negative B-cell lymphoma models. However, CD70 expression levels in tumor cells were different amongst the B-cell tumor types. For example, CD70 positivity has been reported in 33% of FL cases, 25% of MCL, and 71% of DLBCL [3, 4]. One potential way to combat the problem of antigen escape following CAR-T cell therapy is to target alternative or more than one antigen receptor, and CARs targeting both CD19 and CD20 in B-cell lymphoma are in clinical development [10]. Together, our findings suggest that anti-CD70 CAR-T cells represent a new therapeutic option for the treatment of patients who have CD19-negative recurrence of lymphoma. Future strategies combining dual targeting of CD19 and CD70 are warranted.

CD8 (bottom) on CAR-positive cells via flow cytometry. The summary data for indicated CAR-T cell populations are shown. Tscm: CD45RA⁺/CD62L⁺, Tem: CD45RA⁻/CD62L⁺, T_{em}: CD45RA⁻/CD62L⁻, T_{eff}: CD45RA⁺/CD62L⁻. The experiments were repeated 3 times with T cells from 2 healthy donors. (D) Flow cytometry plots demonstrate antigen-independent (tonic) signaling of indicated CARs measured in Jurkat NF- κ B reporter (GFP) cells. Viable Jurkat cells were plated and monitored for GFP expression within BFP-positive (CAR-transduced) population 3 days after lentiviral transduction in the absence of target antigen. Data are representative of two experiments. (E) Tumor progression in NSG mice bearing Raji xenografts treated with anti-CD70 (H/L) or anti-CD19 CAR-T cells. Weekly bioluminescence imaging was performed after tumor injection (CAR-T cells were injected on day 0). X indicates a mouse death. (F) NSG mice were injected with CRISPR-mediated CD19-knockout (19^{NeG}) Raji cells on day -3. Bioluminescent images of the tumor burden in mice treated with anti-CD70 (H/L) or anti-CD19 CAR-T cells are shown. X indicates a mouse death. (G) Quantification of the total flux analyzed by *in vivo* bioluminescence imaging of luciferase activity from (E). ** $P < 0.01$ for anti-CD70 (H/L) vs. anti-CD19 CAR-T cells. Data shown as bar graph with mean \pm SD (6 mice per group). (H) Kaplan-Meier survival analysis of tumor-bearing mice from (E). P values were determined with a log-rank Mantel-Cox test. * $P < 0.05$ for anti-CD70 (H/L) vs. anti-CD19 CAR-T cells. EF-1 α : elongation factor-1 α ; BFP: blue fluorescent protein; SD: standard deviation; ANOVA: analysis of variance; NF- κ B: nuclear factor kappa B; GFP: green fluorescent protein

DECLARATIONS ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The animal experiments were approved by the Institutional Animal Care and Use Committee, Zhejiang University. The studies involving human participants were reviewed and approved by an independent Ethics Committee of The Second Affiliated Hospital, College of Medicine, Zhejiang University. The participants provided their written informed consent to participate in this study.

CONSENT FOR PUBLICATION

Not applicable.

DATA AVAILABILITY STATEMENT

The raw data and analyses supporting the conclusions of this article are openly provided upon request, to any qualified researcher.

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CONFLICT OF INTERESTS

All authors declare no competing interests.

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