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CD19 target-engineered T-cells accumulate at tumor lesions in human B-cell lymphoma xenograft mouse models

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ABSTRACT

Adoptive T-cell therapy with CD19-specific chimeric antigen receptors (CARs) is promising for treatment of advanced B-cell malignancies. Tumor targeting of CAR-modified T-cells is likely to contribute therapeutic potency; therefore we examined the relationship between the ability of CD19-specific CAR (CD19-CAR)-transduced T-cells to accumulate at $CD19^+$ tumor lesions, and their ability to provide anti-tumor effects in xenograft mouse models. Normal human peripheral blood lymphocytes, activated with immobilized RetroNectin and anti-CD3 antibodies, were transduced with retroviral vectors that encode CD19-CAR. Expanded CD19-CAR T-cells with a high transgene expression level of about 75% produced IL-2 and IFN-γ in response to CD19, and lysed both Raji and Daudi $CD19^+$ human B-cell lymphoma cell lines. Furthermore, these cells efficiently accumulated at Raji tumor lesions where they suppressed tumor progression and prolonged survival in tumor-bearing $Rag2^{-/-}\gamma c^{-/-}$ immunodeficient mice compared to control cohorts. These results show that the ability of CD19-CAR T-cells to home in on tumor lesions is pivotal for their anti-tumor effects in our xenograft models, and therefore may enhance the efficacy of adoptive T-cell therapy for refractory B-cell lymphoma.

1. Introduction

Chimeric antigen receptor (CAR)-based T-cell therapy is a promising approach to targeted cancer immunotherapy. CARs are hybrid proteins consisting of an extracellular single chain fragment of variable region (scFv) fused to intracellular lymphocyte signaling domains CD28 or 4-1BB (CD137), coupled with CD3ζ, to mediate T-cell activation. This particular configuration of CARs is widely used in current preclinical and clinical studies [1]. Independent of the human leukocyte antigen system, CAR-transduced T-cells can directly recognize and kill tumor cells that express specific cell surface antigens.

CD19 antigen is an attractive target for CAR-based T-cell therapy since it is a B-cell lineage-specific surface molecule, which is expressed on normal and most malignant B-cells but not on hematopoietic stem cells [2]. Preclinical studies have demonstrated that CD19-specific CAR (CD19-CAR) T-cells eliminated $CD19^+$ B-cell malignancies [3–5]. Recent phase I clinical trials using CD19-CAR T-cells have been shown to be safe and exhibit impressive anti-tumor effects for advanced $CD19^+$ chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), and B-cell lymphoma patients [6–11]. However, the optimal CAR-based T-cell therapy for maximizing therapeutic potency is yet to be realized.

Both persistence and tumor targeting of adoptive transferred CAR-modified T-cells are likely to contribute to therapeutic potency. Preclinical and early clinical trial results have shown that persistence of CD19-CAR T-cells *in vivo* was correlated with anti-tumor effects for B-cell malignancies [4,5,12]. Thus, CAR designs

Abbreviations: CAR, chimeric antigen receptor; CD19-CAR T-cells, CD19-specific CAR-transduced T-cells.

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which involve a signaling feature, that activates and prolongs T-cell survival *in vivo*, have been extensively pursued [13–16]. In contrast, much less is known regarding the correlation between the anti-tumor effects of CD19-CAR T-cells and their tumor targeting ability.

In the present study, we generated large numbers of primary T-cells that expressed CD19-CAR with a CD28 signaling domain. These expanded T-cells exhibited CD19-dependent redirected effector functions *in vitro*. We also observed that they accumulated at the site of tumors and exhibited anti-tumor effects at these sites in human B-cell lymphoma xenograft immunodeficient mice. Our results show that efficient accumulation at tumors of CD19-CAR T-cells can enhance CAR-based T-cell therapy for B-cell lymphoma.

2. Materials and methods

2.1. Plasmids

The gamma retroviral vector SFG-1928z that encodes CD19-specific scFv fused to CD28- and CD3ζ intracellular signal domains has been described [3] (Fig. 1A). A luciferase expression plasmid pEF-Luc was generated by ligating the luciferase gene from pGL3 (Promega, Madison, WI) into a pEF-PGKneo vector containing the EF1α promoter and Neomycin resistance gene cassette [17].

2.2. Peripheral blood lymphocytes and cell lines

Peripheral blood lymphocytes (PBLs) from three healthy donors were obtained with the approval of the Jichi Medical University Institutional Review Board, and written consent of each donor.

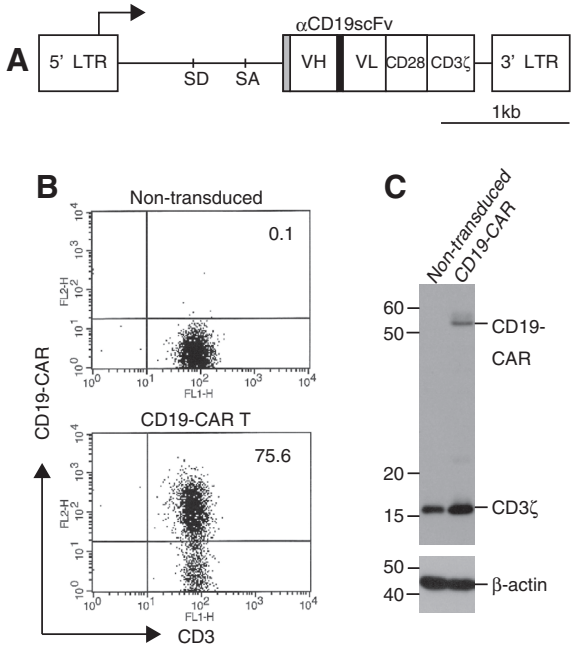

Fig. 1. Generation of CD19-CAR modified T-cells using RetroNectin/anti-CD3 stimulation. (A) Schematic structure of SFG-1928z retroviral vector. VH and VL: variable heavy- and light chain; SD: splice donor; SA: splice acceptor; LTR: long terminal repeat; Gray box: CD8α signal peptide; Black box: $(GGGGS)_3$ linker. PBLs were stimulated with RetroNectin/anti-CD3 and transduced twice with SFG-1928z retroviral vectors. They were propagated on $CD19^+$ feeder cells. (B) Surface expression of CD19-CAR on transduced or non-transduced T-cells was examined by flow cytometry. All numbers show the percentages of $CD3^+$ $CD19\text{-}CAR^+$ populations. Representative data was shown in three independent experiments. (C) CD19-CAR expression in cell lysates from transduced T-cells or the control non-transduced T-cells was detected by Western blotting with anti-CD3ζ; β-actin is a loading control.

PBLs were cultured with X-VIVO 15 (Takara Bio, Shiga, Japan), supplemented with 5% human AB serum (NOVA Biologics, Oceanside, CA) and 1 nanomolar recombinant human interleukin-2 (IL-2) (Life technologies, Carlsbad, CA). The retrovirus packaging cells PG13 [18] and mouse fibroblast NIH3T3 cells that expresses human CD19 (3T3/CD19) [19] were cultured with DMEM, supplemented with 10% FBS. Human Burkitt lymphoma cell lines Raji and Daudi (Health Science Research Resources Bank, Osaka, Japan) were maintained in RPMI1640 supplemented with 10% FBS. Raji cells expressing luciferase (Raji/Luc) were established by transfection with the pEF-Luc plasmid, followed by G418 selection.

2.3. Retroviral transduction and T-cell expansion

For retroviral transduction, PBLs were stimulated with 20 μg/ml of immobilized RetroNectin (Takara Bio) and 10 μg/ml anti-CD3ε antibodies (R&D Systems, Minneapolis, MN) for 3 days and transduced with retroviral particles using a preloading method [20]. Briefly, PG13 viral producer cells were established by stable transduction of SFG-1928z retroviral vectors (Fig. 1A). RetroNectin-coated plates were loaded with retroviral particles obtained from the PG13 cells, and centrifuged (1000*g*, 2 h, 32 °C). Stimulated PBMCs were added to the preloaded plates, and incubated overnight. The procedure was repeated on the next day. For selective *ex vivo* expansion, transduced T-cells were co-cultured with γ-irradiated (50 Gy) 3T3/CD19 cells at 1:1 ratio. On days 5 and 10, the 3T3/CD19 cells were added to the T-cell cultures.

2.4. Flow cytometry

We analyzed cell surface expression of CD19-CAR on transduced T-cells by flow cytometry using a BD LSR with CellQuest software (BD Biosciences, San Jose, CA). Antibodies used for CD19-CAR detection were biotin goat anti-mouse F (ab′), PE Streptavidin (Jackson Immunoresearch, West Grove, PA), and FITC anti-human CD3 (Biolegend, San Jose, CA). Isotype-matched antibodies were used as controls.

2.5. Western blotting

CD19-CAR cellular protein expression was examined by Western blotting. Cell lysate prepared from CD19-CAR T-cells was separated on 12% polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were incubated with a mouse monoclonal anti-human CD3ζ antibody (BD Biosciences) or a rabbit anti-human β-actin antibody (Cell Signaling, Danvers, MA) as a control, followed by anti-mouse immunoglobulin conjugated with horseradish peroxidase. Proteins recognized by antibodies were visualized with an enhanced chemiluminescent detection system (GE Healthcare, Buckinghamshire, UK).

2.6. ELISA

Supernatants from the duplicate wells of co-cultures of 3T3/CD19 cells with CD19-CAR T-cells at 1:1 ratio were harvested after 48 h incubation. Human IL-2 and IFN-γ levels in supernatants were measured by ELISA kits (Biolegend).

2.7. Cell lytic activity

Cell lytic activity of CD19-CAR T-cells was examined by standard 4-h chromium release assays [21].