Enhanced Expression of Anti-CD19 Chimeric Antigen Receptor in piggyBac Transposon-Engineered T Cells

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Adoptive T cell therapy using chimeric antigen receptor (CAR)-modified T cells is a promising cancer immunotherapy. We previously developed a non-viral method of gene transfer into T cells using a piggyBac transposon system to improve the cost-effectiveness of CAR-T cell therapy. Here, we have further improved our technology by a novel culture strategy to increase the transfection efficiency and to reduce the time of T cell manufacturing. Using a CH2CH3-free CD19-specific CAR transposon vector and combining irradiated activated T cells (ATCs) as feeder cells and virus-specific T cell receptor (TCR) stimulation, we achieved 51.4% ± 14% CAR+ T cells and 2.8-fold expansion after 14 culture days. Expanded CD19.CAR-T cells maintained a significant fraction of CD45RA−CCR7+ T cells and demonstrated potent antitumor activity against CD19+ leukemic cells both in vitro and in vivo. Therefore, piggyBac-based gene transfer may provide an alternative to viral gene transfer for CAR-T cell therapy.

INTRODUCTION

Adoptive immunotherapy using chimeric antigen receptors (CARs) to target tumor-associated antigens represents a novel approach for the treatment of hematological malignancies. In particular, CD19.CAR-T cells promoted substantial clinical responses in the patients with refractory or relapsed acute lymphoblastic leukemia (ALL).1–4

CD19.CAR-T cells remain suboptimal since it requires a step of magnetic cell isolation to enrich the T cell product in CAR+ T cells and more than 21 days of culture to generate sufficient numbers of T cells for clinical adoptive transfer. Furthermore, our CAR included a long IgG1-CH2CH3 spacer that might abrogate the in vivo antitumor activity.5–11

The aim of the present study was to improve the manufacturing of piggyBac-modified CD19.CAR-T cells to achieve high levels of CAR expression and reduce the duration of the in vitro culture. We hypothesized that these goals can be obtained by (1) adding autologous activated T cells (ATCs), which express multiple co-stimulatory molecules, as feeder cells; (2) stimulating T cells via viral antigens rather than mitogenic anti-CD3/CD28 monoclonal antibodies (mAbs); and (3) using a CH2CH3-free CD19.CAR construct to render in vivo persistence and antitumor activity.

RESULTS

Feeder-Irradiated Autologous ATCs Enhance CAR Expression in piggyBac-Modified CD19.CAR-T Cells

We previously reported that feeder cells enhance the expansion rate of piggyBac-modified T cells.3 To determine whether autologous ATCs can improve the expansion and CAR expression of piggyBac-modified CD19.CAR-T cells, irradiated autologous ATCs were added to the culture on days 0 and 7 after transfection. In brief, peripheral blood mononuclear cells (PBMCs) were transfected with CD19.CAR

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encoding PB transposon and stimulated on anti-CD3/CD28 mAb-coated plates supplemented with interleukin (IL)-7 (10 ng/mL) and IL-15 (5 ng/mL), in the presence (CD3/CD28-ATC method) or absence (original method) of autologous ATCs (Figure 1A). While T cell proliferation was comparable in the two culture conditions (Figure 1B), T cells generated with feeder ATCs (CD3/CD28-ATC) exhibited significantly higher levels of CAR expression (10.7% ± 7.8%) compared to those with no feeder ATCs (original) (4.6% ± 3.2%) (p = 0.029) (Figures 1C and 1D). As a consequence, the absolute number of CAR+ T cells at the end of the culture expansion increased from 2.6 ± 2.2 × 10^6 cells (original method) to 7.6 ± 8.0 × 10^6 cells (CD3/CD28-ATC) (p = 0.049) (Figure 1E). These results suggest that irradiated ATCs helps in increasing the frequency of CAR-expressing T cells.

Although higher numbers of CAR+ T cells were obtained using feeder ATCs, their frequencies remain suboptimal for the clinical applicability. These results prompted us to further improve our method of CAR-T cell generation.

**Virus-Specific TCR Stimulation Increases CAR Expression in piggyBac-Modified CD19.CAR-T Cells**

To determine whether physiological T cell receptor (TCR) stimulation enhances CAR expression and expansion of piggyBac-modified CD19.CAR-T cells, we used a mix of four common viral antigens (ACE; AdV5 Hexon, CMV pp65, EBV EBNA-1, and BZLF1) for the stimulation of T cells. In brief, piggyBac-modified CD19.CAR-T cells were stimulated twice by either irradiated ATCs pulsed with ACE viral peptides (ACE CAR) or ATCs and anti-CD3/CD28 mAbs (CD3/CD28-ATC CAR) (Figure 2A). T cell expansion rates were comparable for 14 days of culture (Figure 2B). In contrast, ACE CAR-T cells exhibited significantly higher (33.0% ± 9.7%) CAR expression than the CD3/CD28-ATC CAR-T cells (10.7% ± 7.8%; p = 0.001) (Figures 2C and 2D). Furthermore, the mean fluorescence intensity of the CAR expression was significantly higher in ACE CAR-T cells than those of both original and CD3/CD28-ATC CAR-T cells (Figures S1 and S2). As a consequence, ACE CAR-T cells showed higher absolute numbers of CAR+ T cells (17.2 ± 10^6) at the end of the culture period as compared to CD3/CD28-ATC CAR method (7.6 ± 10^6; p = 0.047) (Figure 2E). CAR+ T cell numbers in the ACE CAR-T group were 6.6-fold higher (p = 0.028) than those generated using the original method (2.6 ± 2.2 × 10^6) (Figures 1E and 2E).

We assessed the secretion of interferon (IFN)-γ in response to the four common viral antigens (ACE; AdV5 Hexon, CMV pp65, EBV EBNA-1, and BZLF1) using the enzyme-linked immunosorbent spot (ELISpot) assay to determine the virus-specific activity of the generated CAR-T cells. Unexpectedly, IFN-γ secretion minimally increased in response to viral antigens by ACE CAR-T cells relative to the background IFN-γ secretion in the negative control (Figure S3).

To determine whether the presence of CAR in T cells attenuated the virus-specific activity, we separately evaluated the virus-specific activity by intracellular cytokine assay in both CAR and NT population of ACE CAR-T cells from donor 1, which demonstrated apparent virus specificity on day 14. Although IFN-γ in response to pp65 antigen was detected in the CAR population, it was minimally detected in the CAR population (Figure S4). To confirm this, we compared...
the virus specificity between CAR-transduced and non-transduced (NT) T cells, both stimulated by autologous ATCs and ACE viral peptides. As compared to ACE CAR-T cells, ACE NT-T cells demonstrated a tendency of increased virus-specific activities against viral antigens (Figure S5). Furthermore, sequential ELISpot assays showed that ACE CAR-T cells seem to maintain the virus specificity by day 7 but lose their specificity by day 14 as compared to ACE NT-T cells (Figure S6). These results suggested that the presence of CAR might be one of the reasons for the attenuated virus-specific activity in ACE CAR-T cells after the second stimulation of ACE peptides.

To characterize the immunophenotypic composition of the T cell products, the cells were harvested and analyzed by flow cytometry by day 14 after transfection. As shown in Figure 3A, ACE CD19.CAR-T cells consisted of 94.2% ± 3.7% CD3+ T cells with a higher percentage of CD8+ (79.9% ± 5.9%) than CD4+ (29.6% ± 17.3%) T cells. CD56+CD3− NK cells account for 1.6% ± 1.0% of the total cells as previously described. Furthermore, ACE CD19.CAR-T cells contained 79.9% ± 5.9% cells which co-expressed CD45RA and CCR7 (Figures 3B and 3C) that resemble naive or stem cell memory-like T cell phenotype. In contrast, ACE NT-T cells exhibited more differentiated phenotype (Figure S7), consistent with their virus-specific activity.

To investigate whether virus-specific TCR stimulation reduces the rate of activation-induced cell death as compared to anti-CD3/CD28 mAb stimulation, 7 days after stimulation, the frequency of apoptotic cells was evaluated by flow cytometry. As shown in Figure S8, ACE
CD19.CAR-T cells showed a trend for a lower frequency of Annexin V+ cells within the CAR+ T cells as compared to CD19.CAR-T cells stimulated with anti-CD3/CD28 mAbs. These results suggest that anti-CD3/CD28 mAb TCR stimulation may induce more apoptosis in T cells leading to an overall lower output of CAR+ T cells.

ACE CD19.CAR-T Cells Exert an Anti-leukemic Effect In Vitro

To evaluate the anti-leukemic effects of ACE CD19.CAR-T cells in vitro, we co-cultured ACE CAR-T cells with SU/SR, tyrosine kinase inhibitors-resistant CD19+ ALL cell line.8,12 As shown in Figures 4A and 4B, ACE CD19.CAR-T cells showed significantly higher elimination of SU/SR cells (94.7% and 87.8%) compared to both CD3/CD28-ATC CAR-T (59.6% and 48.7%) and original CAR-T cells (51% and 34.4%) at the effector:target (E:T) ratios of 1:50 and 1:100, respectively. These results suggest that ACE CD19.CAR-T cells have potent anti-leukemic effect in vitro.

ACE CD19.CAR-T Cells with a New Transposon Vector with No CH2CH3 Hinge Region Show Higher CAR Expression

Recently, several groups have suggested that the long CH2CH3 hinge region may attenuate in vitro lysis of tumor cells or induce killing of monocytes10 and in vivo expansion of CAR-T cells.11 To provide better in vivo persistence and antitumor activity, we constructed a new transposon vector (CH2CH3-free) by removing the spacer region from the original CD19.CAR vector (original) based on the construct developed by Kochenderfer et al.13 (GenBank: HM852952.1). Therefore, this new vector is 645 bp shorter than the original vector as a result of the deletion (Figure 5A). We then generated ACE CAR-T cells using either the original or the CH2CH3-free vector (Figure 5A). Overall, T cell expansion was similar (Figure 5B), but the CH2CH3-free construct led to significantly higher CAR expression (51.4% ± 14%) compared to the original construct (33.0% ± 9.7%) (p = 0.034) (Figures 5C and 5D) and a trend of an increase in the absolute number of CAR+ T cells (27.8 ± 28.0 × 10^6 versus 17.2 ± 17.0 × 10^6) (p = 0.23) (Figure 5E). Similar to the results from the original vector, ACE CAR-T cells with CH2CH3-free transposon vector contained a significant fraction of CD3+CD45RA+CCR7+ T cells (Figure S9).

Although we compared the frequency of apoptotic cells between two CD19.CAR vectors 7 days after stimulation to investigate whether ACE CAR-T cells with CH2CH3-free construct reduced induced apoptotic cell death, no differences were observed in the frequencies of apoptotic cells between the two ACE CAR-T cell lines with either original vector or CH2CH3-free vector (data not shown).
In addition, we co-cultured CD19+ leukemic cells and analyzed them using flow cytometry to evaluate the in vitro antitumor activity of CH2CH3-free ACE CAR-T cells. Figure S10 shows that ACE CAR-T cells with CH2CH3-free vector demonstrated a comparable antitumor activity (Figure S10A) and IFN-γ secretion (Figure S10B) against SU/SR cells. These results suggested an equivalent in vitro antitumor activity of CH2CH3-free ACE CAR-T cells as compared to those with original vector.

ACE CD19.CAR-T Cells Exhibit an Antitumor Effect In Vivo

To evaluate the antitumor activity of ACE CD19 CAR-T cells in vivo, we intravenously engrafted firefly luciferase (ffLuc)-labeled CD19+ Daudi cells in NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice and subsequently infuse them with either control NT T cells or ACE CH2CH3-free CD19.CAR-T cells 3 days following tumor inoculation (Figure 6A). Mice treated with ACE CD19.CAR-T cells showed significantly lower tumor luminescence on days 7, 11, and 18 after tumor inoculation as compared to the mice in the control T cell group (Figures 6B and 6C). The lower tumor luminescence in mice treated with ACE CD19.CAR-T cells was associated with an improved overall survival rate (p < 0.001) (Figure 6D). These results suggest that ACE CD19.CAR-T cells can control the growth of CD19+ tumor cells in vivo.

DISCUSSION

In the present study, we developed a novel method of piggyBac transposon-based generation of CAR-T cells that has high potential for the clinical translation since it achieves high expression of the CAR in T cells and adequate expansion of T cells that retain memory phenotype.

Non-viral gene transfer methodologies, including piggyBac transposon, are usually characterized by low transfection efficiency, thus requiring magnetic purification of CAR+ cells and long-term ex vivo culture for generating CAR-T cells for clinical use.8 In this study, by adding ATCs as a feeder and using alternative means of TCR stimulation using viral antigens, more than 30% of the expanded T cells expressed the CAR. In addition, CAR expression significantly increased to approximately 50% by just reducing the size of the CAR cassette, eliminating the long IgG1.CH2CH3 spacer. Our experiments also indicate that 27.8 ± 28.0/10^6 CAR+ T cells can be expanded starting from 10/10^6 PBMCs in 14 days of culture. As several studies have shown that 1 – 3 × 10^6/kg CD19+ T cells can induce clinical responses,4,14,15 our proposed strategy seems sufficiently scalable to produce CAR-T cell products for clinical use.

PB-mediated gene transfer requires electroporation, which may cause cell death. Therefore, the maintenance of an appropriate cell density is required for the generation of PB-modified T cells.5 Several approaches can be used to provide feeder cells to PB-modified T cells that include engineered tumor cell lines such as K562 expressing co-stimulatory molecules or total unfractionated PBMCs.5 However, including tumor cell lines in culture might lead to a more complicated approval process for clinical trials to fulfill the regulations in our country. Furthermore, obtaining sufficient number of PBMCs is relatively challenging as compared to the activated ATCs, which can easily be expanded, while PBMCs are a solid option for feeder cells or simulant for T cells. Thus, in addition to that and ATCs reportedly expressing adequate levels of HLA class I, II, and co-stimulatory molecules,16 we selected ATCs as a feeder cell and simulator for CAR-T cells in this study.
The final output of T cells activated and expanded ex vivo is the result of a balance between T cell expansion and T cell death. In the present study, we found that, upon CAR piggyBac gene transfer, T cell stimulation obtained via TCR engagement with viral peptides had less induced activation and cell death, which ultimately led to a significant increase in CAR-T cell counts rather than cross-linking with mAbs. Anti-CD3/CD28 mAb stimulation has been successfully used to generate and expand CAR-T cells for clinical use\textsuperscript{14,15,17,18} using either retroviral or lentiviral vectors to insert the CAR into T cells. A critical step in the procedure of non-viral gene transfer is represented by the electroporation of T cells. Although we do not have a mechanistic explanation, our data suggest that the methodology used to engage the TCR may affect the outcome of electroporated T cells causing more death when antibodies are cross-linking TCR molecules. By contrast, TCR engagement with peptide presented in the context of the major histocompatibility complex (MHC) by ATCs may preserve the physiology of electroporated cells.

Unexpectedly, ACE CAR-T cells did not secrete IFN-γ against viral peptides, although they were simulated by viral antigens during the culture. Recently, Ghosh et al.\textsuperscript{19} elucidated the mechanism whereby allogeneic CD19-targeted CAR-T cells provided anti-lymphoma activity without causing an increased incidence of graft-versus-host-disease in the past clinical trials. In their study, alloreactive T cells expressing CD19.CAR with CD28 co-stimulatory domains experienced enhanced stimulation, leading to the progressive loss of their alloreactive effector function. In our study, although ACE CAR-T cells seemed to maintain the virus specificity by day 7, they lost it by day 14. In addition, the virus-specific population seemed to be limited in the CAR/C0 population and ACE NT-T cells maintained the virus specificity on day 14. Together with the naive or stem-cell memory-like phenotype in ACE CAR-T cells, one possible explanation for this is that virus-specific TCR stimulation might have enhanced the expansion of CAR and virus-specific TCR dual-positive T cells and simultaneously downregulated their virus-specific TCR after the second stimulation of ACE peptides. Further studies are warranted to investigate the precise mechanism.

Cytokines’ use to expand CAR-T cells seems also important in determining not only the expansion rate of these cells but also their
immunologic composition. By using the combination of IL-7 and IL-15, 94% of the ex vivo expanded ACE CAR-T cells expressed CD3 and contained both CD4+ and CD8+ T cells, with a slightly higher percentage of CD8+. Moreover, most CAR+ T cells exhibited a CD45RA+CD62L+ phenotype. Although the precise mechanism is still unclear, PB virus-transduced CAR-T cells did not show an augmented memory phenotype. Although the optimal voltage setting (2400 V) provided a 1.71-fold increase of CAR+ T cells exhibited a naive (Tn) or stem-cell memory (Tscm) T cell phenotype that are considered favorable phenotypic characteristics to ensure persistence and antitumor effects upon infusion into patients, as demonstrated by the potent anti-leukemia effects of ACE CAR-T cells in our experiments in vitro and in vivo. Recently Barnett et al. compared the phenotype of CAR-T cells transduced by either lentivirus or PB transposon and revealed that the majority of PB-modified CAR-T cells showed CD45RA−CD62L−, similar to our results. In contrast, lentivirus-transduced CAR-T cells did not show an augmented memory phenotype. Although the precise mechanism is still unclear, PB gene transfer system may induce expansion of CAR-T cells with Tn/Tscm phenotype.

Although our novel method significantly improved the efficiency of CAR-T cell generation, the efficiency was still low as compared to another report of CAR-T cells generated by the non-viral gene transfer system such as Sleeping beauty and piggyBac transposon. Perhaps this could be attributed to the electroporation condition of piggyBac-modified CAR-T cells. Recently, Ramanayake et al. rigorously optimized their electroporation condition and determined that the optimal voltage setting (2400 V) provided a 1.71-fold increase of their CAR-T generation efficiency. Although our electroporation method (4D-Nucleofector device) has a limited option of electroporation conditions as compared to those (Neon Transfection System) previously optimized their electroporation condition and determined that the optimal voltage setting (2400 V) provided a 1.71-fold increase of their CAR-T generation efficiency. Although our electroporation method (4D-Nucleofector device) has a limited option of electroporation conditions as compared to those (Neon Transfection System) recently improved their electroporation efficiency of CAR-T generation 2.5-fold compared to others and ours regarding the numbers of stimulation and the presence of stimulation through the CAR. We stimulated T cells only two times to reduce the culture period and avoid T cell differentiation and exhaustion. Moreover, we used autologous ATCs for the stimulation of CAR-T cells, which may minimally stimulate T cells through the CAR because ATCs included minimal CD19+ cells as compared to PBMCs. In contrast, Ramanayake et al.25 stimulated their piggyBac-modified CAR-T cells three times with PBMCs. In their method, CD19.CAR-T cells could be stimulated through the CAR because ATCs included minimal CD19+ cells as compared to PBMCs. Moreover, Barnett et al.22 compared the efficiency of CAR-T generation was significantly improved by further optimization of the electroporation condition in piggyBac-modified CAR-T cells.

Another reason could be the differences in the culture strategy between others and ours regarding the numbers of stimulation and the presence of stimulation through the CAR. We stimulated T cells only two times to reduce the culture period and avoid T cell differentiation and exhaustion. Moreover, we used autologous ATCs for the stimulation of CAR-T cells, which may minimally stimulate T cells through the CAR because ATCs included minimal CD19+ cells as compared to PBMCs. In contrast, Ramanayake et al.25 stimulated their piggyBac-modified CAR-T cells three times with PBMCs. In their method, CD19.CAR-T cells could be stimulated through the CAR owing to the CD19+ population in PBMCs, and they achieved 765-fold expansion and 72% of the CAR expression in their CD19.CAR-T cells. Similarly, Kebrina et al.23 generated Sleeping beauty-modified CAR-T cells by stimulating with CD19 and co-stimulatory molecules expressing K562 cells. Although most of their CAR+ T cells exhibited a differentiated effector memory phenotype and an increased expression of LAG3, PDCD1LG2, and E2F mRNA related to exhaustion, they achieved high CAR expression (84%) and fold expansion (2,200–2,500) for 4 weeks of culture and presented promising results from their clinical trials. Even though the culture strategies and efficiencies of CAR-T cell generation were different, these findings supported that a non-viral gene transfer system could be an alternative option for generating CAR-T cells for clinical use.

In conclusion, we developed a novel method of generating PB-modified CD19.CAR-T cells using minimal manipulation and reagents that are good manufacture compliant for clinical application. Together with the simple manufacturing process and cost-effectiveness of DNA transposon technology, piggyBac-based gene transfer provides an alternative to retroviral or lentiviral gene transfer for CAR-T cell therapy. Based on these encouraging data, we currently presented, the efficiency of CAR-T generation could be potentially improved by further optimization of the electroporation condition in piggyBac-modified CAR-T cells.

Figure 6. ACE CD19.CAR-T Cells Potently Reject CD19+ Leukemic Cells in a Mouse Xenograft Model

(A) NSG mice received intravenous injections (i.v.) of 1 x 10⁶ Daudi cells co-expressing eGFP and II7Luc and were subsequently intravenously infused with 1 x 10⁷ CD19.CAR-T cells 3 days after tumor cell injection. Control mice were intravenously infused with 1 x 10⁷ non-transduced T cells. (B) Representative bioluminescent imaging (BLI) of tumor progression following the i.v. administration of tumor cells with either non-transduced or ACE CD19.CAR-T cells are shown. (C) The mean radiance of tumor progression imaged with BLI in mice bearing Daudi cells is shown. *p < 0.01; ***p < 0.001. (D) Kaplan-Meier survival curves present the overall survival. Significance was determined using a log-rank test.
planning to conduct a clinical trial of PB-modified CD19.CAR-T cells using our ACE CAR-T method.

MATERIALS AND METHODS

Plasmids

The piggyBac transposase plasmid (pCMV-piggyBac) and transposon plasmid for CD19.CAR (pIRII-CAR.CD19-28-ζ, 6,438 bp) have been described previously. In brief, pIRII-CAR.CD19-28-ζ encodes a single-chain variable fragment from the CD19 antibody (FMC63) linked to the hinge CH2CH3 domain of human IgG1, the endodomains of a CD28 molecule, and a TCR ζ chain. Both vectors are transcriptionally regulated by the cytomegalovirus (CMV) immediate early gene enhancer/promoter sequence. To provide better in vivo persistence and antitumor activity, we constructed a new transposon vector (short) that does not have the IgG1-CH2CH3 spacer region based on the construct reported by Kochenderfer et al. (GenBank: HM852952.1).

Cell Lines

SU/SR were derived from SU-Ph2 by culture with imatinib as described previously and maintained in RPMI 1640 medium, supplemented with GlutaMAX and 10% fetal bovine serum (HyClone, Laboratories, Logan, USA). No cytokines were fed to the co-culture.

Gene Transfer into T Cells and Expansion of Transgenic T Cells

PBMCs were freshly isolated from peripheral blood of healthy donors and then immediately transfected after the isolation. Healthy volunteers gave written informed consent according to protocols approved by the institutional review board of Shinshu University School of Medicine, and all experiments involving human participants were performed in accordance with the guidelines established by the Declaration of Helsinki. A 4D-Nucleofector device (Program EO-115) and P3 Primary Cell 4D-Nucleofector X Kit (Lonza, Basel, Switzerland) were used to electroporate 1 × 10^7 PBMCs with a pIRII-CD19.CD28-ζ–CAR transposon plasmid with or without the IgG1-CH2CH3 spacer (5 μg) and a pCMV-piggyBac transposase plasmid (5 μg) according to the manufacturer’s instructions. All cells were cultured in animal-derived component-free T cell culture medium (TexMACS Medium; Miltenyi Biotec, Auburn, CA, USA) containing IL-7 (10 ng/mL), IL-15 (5 ng/mL), and IL-16 (5 ng/mL), and Miltenyi Biotec) in 24-well plates at 37°C in a humidified 5% CO2 incubator.

In the original method (Figure 1A), the electroporated cells were transferred to CD3/CD28 mAb (Miltenyi Biotec)-coated plates the day following electroporation without feeder ATCs (original).

We compared two other different culture conditions. In CD3/CD28-ATC condition, electroporated cells were co-cultured with feeder ATCs on day 0 and then transferred to CD3/CD28 mAb-coated plates on day 1. In ACE condition, electroporated cells were stimulated with feeder ATCs pulsed with four viral peptide pools (MACS GMP PepTivator: AdV5 Hexon, CMV pp65, EBV EBNA-1, and BZLF1) on day 0.

On day 7, 2 × 10^6 cells were transferred into G-Rex10 culture flasks (Wilson Wolf Manufacturing, New Brighton, MN, USA) with 30 mL of IL-7 (10 ng/mL)/IL-15 (5 ng/mL)-containing TexMACS with 2 × 10^6 of ACE-pulsed irradiated ATCs for the ACE CAR-T and with 2 × 10^6 of unpulsed irradiated ATCs for CD3/CD28-ATC condition, or without stimulation for original condition. On day 14, CAR-T cells cultured under all conditions were collected and analyzed.

Preparing Feeder ATCs

For preparing feeder ATCs, PBMCs were stimulated on CD3/CD28mAb-coated plates for 3 days then transferred to regular plates and maintained for 6–8 days supplemented with IL-7 (10 ng/mL)/IL-15 (5 ng/mL). The cells were then harvested and cryopreserved on days 9–11 after initial stimulation. ATCs were thawed 2–3 days before use and re-stimulated on CD3/CD28Abs-coated plates for 2–3 days then irradiated at 30 Gy just before being added to the culture, as previously reported.

Flow Cytometry

With the use of the BD FACSCalibur with BD Cell-Quest Pro software (BD Biosciences, Franklin Lakes, NJ, USA), the surface markers of the expanded CAR-T cells were analyzed using allopurinol (APC-)conjugated CD3 mAbs, phycoerythrin (PE)-conjugated CD4 mAbs, APC-conjugated CD8 mAbs, APC-conjugated CD45RA mAbs, PE-conjugated CD56 mAbs, PE-conjugated CD62L mAbs, and PE-conjugated CCR7 mAbs (all mAbs were purchased from Miltenyi Biotec). T cell expression of CAR was examined using a specific anti-idiotype single-chain variable fragment (scFV) mAbs, which was kindly provided by Dr. Lawrence Cooper (MD Anderson Cancer Center). The data were analyzed using FlowJo software (TreeStar; http://www.flowjo.com/).

ELISpot Assay

We performed the ELISpot assay to examine viral antigen-specific IFN-γ production by either ACE CAR-T or ACE NT-T cells using the human IFN-γ ELISpot PLUS kit (Mabtech, Nacka Strand, Sweden) according to the manufacturer’s instructions. In brief, we cultured ACE CAR-T or NT-T cells (5 × 10^4 cells/well) in the presence of pp65, hexon, EBNA1, BZLF1, or medium alone (control). After 20 hr of incubation, the spots were counted by an automated ELISpot reader (Autoimmun Diagnostika, Strassberg, Germany).

Intracellular Cytokine Assay

We performed intracellular cytokine staining to determine whether the CAR+ population could respond to viral antigens. In brief, ACE CAR-T cells were stimulated with pp65, hexon, EBNA1, BZLF1, or medium alone (control). After 4 hr of incubation, cells were harvested and stained with the anti-idiotype scFV mAbs described earlier. Then, the cells were fixed and permeabilized according to the manufacturer’s instruction. Finally, the cells were stained with IFN-γ and analyzed by flow cytometry.
Annex V Apoptosis Assay

Electroporated cells were stimulated with either viral peptides (ACE) or CD3/CD28 mAbs (CD3/CD28-ATC) in the presence of autologous ATCs. Seven days after stimulation, the cells were harvested, stained with Annexin V (IMMUNCYTO Cytotoxicity Detection Kit; MBL International Corporation, Woburn, MA, USA), APC-conjugated CD3, and Alexa Fluor 488-conjugated specific anti-idiotypic scFv mAbs, and analyzed via flow cytometry.

Co-culture Experiments

To examine the anti-leukemic activity of CAR-T cells, ACE CD19.CAR-T cells, CD3/CD28-ATC CD19.CAR-T cells, and the original CD19.CAR-T cells were added to 5 × 10⁶ CD19⁺ leukemic cells (SU/SR) at E:T ratios of 1:5, 1:50, and 1:100 into the wells of a 48-well culture plate containing RPMI 1640 medium plus 10% fetal bovine serum without cytokines and cultured for 5 days. At the indicated points, the number of viable cells was determined using trypan blue exclusion test, and the percentage of T cells and CD19⁺ cells were determined by flow cytometry using APC-conjugated CD3 mAbs and PE-conjugated CD19 mAbs. IFN-γ levels in culture media were measured by ELISA assay (R&D Systems, Minneapolis, MN).

In Vivo Experiments

NSG mice were originally generated by Jackson Laboratory (Bar Harbor, ME, USA) and purchased from Charles River Laboratories Japan (Yokohama, Japan). All mouse experiments were performed in accordance with the Animal Husbandry and Institutional Animal Care and Use Committee guidelines of the Nagoya University Graduate School of Medicine.

NSG mice received intravenous injections of 1 × 10⁶ luciferase-transduced Daudi cells. Three days later, they were intravenously infused with 1 × 10⁶ CD19.CAR-T cells. The cell counts of the infused cells were based on the total cell number. Untreated animals served as controls.

Biodistribution of Daudi cells was assessed twice weekly using the In Vivo Imaging System (Xenogen Corporation, Alameda, CA, USA) as described previously. Mice were euthanized at predefined endpoints or when euthanasia criteria were met in accordance with the Center for Comparative Medicine at the Nagoya University Graduate School of Medicine.

Statistical Analysis

The results from three culture experiments were expressed as the means ± SD. An unpaired t test was used to identify any significant differences among the three independent groups. Statistical significance was defined as p < 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes ten figures and can be found with this article online at https://doi.org/10.1016/j.omtm.2017.12.003.

AUTHOR CONTRIBUTIONS

Conceptualization, Y.N.; Methodology, D.M., N.N., M.T., Y.N.; Investigation, D.M., N.N., M.T., N.K., Y.O., Y.N.; Resources, M.H.W., G.D., C.M.R.; Writing – Original Draft, D.M., S.S.; Writing – Review & Editing, S.S., N.N., M.H.W., G.D., C.M.R., Y.T., Y.N.; Funding Acquisition, Y.T., Y.N.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

1. Davila, M.L., Riviere, I., Wang, X., Bartido, S., Park, J., Curran, K., Chung, S.S., Stefanski, J., Borquez-Ojeda, O, Olesniewa, M., et al. (2014). Efficacy and toxicity management of H-28c CAR T cell therapy in B cell acute lymphoblastic leukemia.Sci. Transl. Med. 6, 224ra25.
2. Maude, S.L., Frey, N., Shaw, P.A., Aplenc, R., Barrett, D.M., Bunin, N.J., Chew, A., Gonzalez, V.E., Zheng, Z., Lacey, S.F., et al. (2014). Chimeric antigen receptor T cells for sustained remissions in leukemia. N. Engl. J. Med. 371, 1507–1517.
3. Lee, D.W., Kochenderfer, J.N., Stetler-Stevenson, M., Cui, Y.K., Delbrook, C., Feldman, S.A., Fry, T.I., Orentas, R., Sabatino, M., Shah, N.N., et al. (2015). T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukemia in children and young adults: a phase 1 dose-escalation trial. Lancet 385, 517–528.
4. Turtle, C.J., Hanafi, L.A., Berger, G., Cooley, T.A., Cherian, S., Hudecek, M., Sommermeyer, D., Melville, K., Pender, B., Budiarto, T.M., et al. (2016). CD19.CAR-T cells of defined CD4⁺/CD8⁺ composition in adult B cell ALL patients. J. Clin. Invest. 126, 2123–2138.
5. Nakazawa, Y., Huye, L.E., Dotti, G., Foster, A.E., Vera, J.F., Manuri, P.R., June, C.H., Rooney, C.M., and Wilson, M.H. (2009). Optimization of the PiggyBac transposon system for the sustained genetic modification of human T lymphocytes. J. Immunother. 32, 826–836.
6. Nakazawa, Y., Huye, L.E., Salaman, V.S., Leen, A.M., Ahmad, N., Rollins, L., Dotti, G., Gottschalk, S.M., Wilson, M.H.M., and Rooney, C.M. (2011). PiggyBac-mediated cancer immunotherapy using EBV-specific cytotoxic T-cells expressing HER2-specific chimeric antigen receptor. Mol. Ther. 19, 2133–2143.
7. Nakazawa, Y., Saha, S., Galvan, D.L., Huye, L., Rollins, L., Rooney, C.M., and Wilson, M.H. (2013). Evaluation of long-term transgene expression in piggyBac-modified human T lymphocytes. J. Immunother. 36, 3–10.
8. Saito, S., Nakazawa, Y., Sueki, A., Matsuda, K., Tanaka, M., Yanagisawa, R., Masuda, Y., Sato, Y., Osabe, S., Imakai, T., et al. (2014). Anti-leukemic potency of piggyBac-mediated CD19-specific T cells against refractory Philadelphia chromosome-positive acute lymphoblastic leukemia. Cytotherapy 16, 1257–1269.
9. Hudecek, M., Lupo-Stanghellini, M.T., Kosaih, P.L., Sommermeyer, D., Jensen, M.C., Rader, C., and Riddell, S.R. (2013). Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T cells. Clin. Cancer Res. 19, 3153–3164.
10. Watanabe, N., Bagaim, P., Sukumaran, S., Ansari, S., Hesslop, H.E., Rooney, C.M., Brunner, M.K., Leen, A.M., and Vera, J.F. (2016). Fine-tuning the CAR spacer improves T-cell potency. Oncoimmunology 5, e1253656.

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11. Almåsbak, H., Walseng, E., Kristian, A., Myhre, M.R., Suso, E.M., Munthe, L.A., Andersen, J.T., Wang, M.Y., Kvalheim, G., Gaudernack, G., and Kyte, J.A. (2015). Inclusion of an IgG1-Fc spacer abrogates efficacy of CD19 CAR T cells in a xenograft mouse model. Gene Ther. 22, 391–403.

12. Hirase, C., Maeda, Y., Takai, S., and Kanamura, A. (2009). Hypersensitivity of Ph-positive lymphoid cell lines to rapamycin: possible clinical application of mTOR inhibitor. Leuk. Res. 33, 450–459.

13. Kochenderfer, J.N., Feldman, S.A., Zhao, Y., Xu, H., Black, M.A., Morgan, R.A., Wilson, W.H., and Rosenberg, S.A. (2009). Construction and preclinical evaluation of an anti-CD19 chimeric antigen receptor. J. Immunother. 32, 689–702.

14. Wang, X., and Rivière, I. (2016). Clinical manufacturing of CAR T cells: foundation of a promising therapy. Mol. Ther. Oncolytics 3, 16015.

15. Kochenderfer, J.N., Dudley, M.E., Kassim, S.H., Somerville, R.P., Carpenter, R.O., Sterler-Stevenson, M., Yang, J.C., Phan, G.Q., Hughes, M.S., Sherry, R.M., et al. (2015). Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor. J. Clin. Oncol. 33, 540–549.

16. Ngo, M.C., Ando, J., Leen, A.M., Ennamuri, S., Lapteva, N., Vera, J.F., Min-Venditti, A., Mims, M.P., Heslop, H.E., Bollard, C.M., et al. (2014). Complementation of antigen-presenting cells to generate T lymphocytes with broad target specificity. J. Immunother. 37, 193–203.

17. Ahmed, N., Brawley, V.S., Hegde, M., Robertson, C., Ghazi, A., Gerken, C., Liu, E., Dakhova, O., Ashtoort, A., Corder, A., et al. (2015). Human epidermal growth factor receptor 2 (HER2)-specific chimeric antigen receptor-modified T cells for the immunotherapy of HER2-positive sarcoma. J. Clin. Oncol. 33, 1688–1696.

18. Brezniak, R.J., Davila, M.L., Riviere, I., Park, J., Wang, X., Cowell, L.G., Bartido, S., Stefanski, J., Taylor, C., Olszewska, M., et al. (2013). CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. Sci. Transl. Med. 5, 177ra38.

19. Ghosh, A., Smith, M., James, S.E., Davila, M.L., Velardi, E., Argyropoulos, K.V., Gunset, G., Perna, F., Kreines, F.M., Levy, E.R., et al. (2017). Donor CD19 CAR T cells exert potent graft-versus-lymphoma activity with diminished graft-versus-host activity. Nat. Med. 23, 242–249.

20. Xu, Y., Zhang, M., Ramos, C.A., Durett, A., Liu, E., Dakhova, O., Liu, H., Creighton, C.J., Gee, A.P., Heslop, H.E., et al. (2014). Closely related T-memory stem cells correlate with in vivo expansion of CAR-CD19-T cells and are preserved by IL-7 and IL-15. Blood 123, 3750–3759.

21. Sommermeyer, D., Hudecek, M., Kosash, P.L., Gogishvili, T., Maloney, D.G., Turtle, C.J., and Riddell, S.R. (2016). Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4+ subsets confer superior antitumor reactivity in vivo. Leukemia 30, 492–500.

22. Barnett, B.E., Hermansson, D.L., Smith, J.B., Wang, X., Tan, Y., Martin, C.E., et al. (2016). piggyBacTM-produced CAR-T cells exhibit stem-cell memory phenotype. Blood 128, 2167.

23. Kebrina, P., Singh, H., Huls, M.H., Figliola, M.J., Bassett, R., Olvares, S., Jena, B., Dawson, M.J., Kumaresan, P.R., Su, S., et al. (2016). Phase I trials using Sleeping Beauty to generate CD19-specific CAR T cells. J. Clin. Invest. 126, 3363–3376.

24. Matti, S.N., Huls, H., Singh, H., Dawson, M., Figliola, M., Olvares, S., Rao, P., Zhao, Y.J., Multani, A., Yang, G., et al. (2013). Sleeping beauty system to redirect T-cell specificity for human applications. J. Immunother. 36, 112–123.

25. Ramanayake, S., Bilmon, I., Bishop, D., Dubosq, M.C., Blyth, E., Clancy, L., Gottlieb, D., and Micklethwaite, K. (2015). Low-cost generation of Good Manufacturing Practice-grade CD19-specific chimeric antigen receptor-expressing T cells using piggyBac gene transfer and patient-derived materials. Cytotherapy 17, 1251–1267.

26. Jena, B., Matti, S., Huls, H., Singh, H., Lee, D.A., Champlin, R.E., and Cooper, L.J. (2013). Chimeric antigen receptor (CAR)-specific monoclonal antibody to detect CD19-specific T cells in clinical trials. PLoS ONE 8, e57838.

27. Vera, J., Savoldo, B., Vigouroux, S., Biagi, E., Pole, M., Rosag, C., Wu, J., Heslop, H.E., Rooney, C.M., Brenner, M.K., and Dotti, G. (2016). T lymphocytes redirected against the kappa light chain of human immunoglobulin efficiently kill mature B lymphocyte-derived malignant cells. Blood 108, 3890–3897.

28. Nishio, N., Diaconu, I., Liu, H., Cerdillo, V., Caruana, I., Hoyos, V., Bouchier-Hayes, L., Savoldo, B., and Dotti, G. (2014). Armed oncolytic virus enhances immune functions of chimeric antigen receptor-modified T cells in solid tumors. Cancer Res. 74, 5195–5205.