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Point-of-care manufacturing of anti-CD19 CAR-T cells using a closed production platform: Experiences of an academic in Thailand

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Anti-CD19 chimeric antigen receptor (CAR)-T cell therapy has evolved as a standard of care for various forms of relapsed/refractory B cell malignancies in major developed countries. However, access to industry-driven CAR-T cell therapy is limited in developing countries, partly due to the centralized manufacturing system. Here, we demonstrated the feasibility of the point-of-care (POC) manufacturing of anti-CD19 CAR-T cells from heavily pretreated patients and healthy graft donors at an academic medical center in Thailand using a closed semi-automated production platform, CliniMACS Prodigy, and established in-process quality control and release testing to ensure their identity, purity, sterility, safety, and potency. Nine out of the nine products manufactured were used in a pilot study (ISRCTN17901467). However, we did observe that starting T cells with CD4/CD8 ratios of less than one-third had a high chance of manufacturing failure, which could be minimized by serum supplementation. Further analysis of T cell phenotypes in the infused versus circulating CAR-T cells revealed the differentiation from early memory subtypes toward effector cells *in vivo*. The POC manufacturing and quality control settings herein could be applied to other CAR-T cell products and may benefit other academics, especially those in developing countries, making CAR-T cells more accessible.

INTRODUCTION

Cell-based immunotherapy using chimeric antigen receptor (CAR)-engineered T cells represents a major breakthrough in cancer care, which began with the approval of the first two anti-CD19 CAR-T cell products by the US Food and Drug Administration (FDA) in 2017. Anti-CD19 CAR-T cell therapy has rapidly become a standard of care for various relapsed/refractory B cell malignancies in the United States, Europe, and other developed countries, with four products currently approved by both the US FDA and the European Medicines Agency. However, access to industry-driven CAR-T cell therapy is limited in developing countries, including Thailand, mainly due to the extremely high cost of laborious CAR-T manufacturing to create an autologous product in a centralized system and complex logistics.[1,2] In brief, the industrial manufacturing process starts with collection of the patient's peripheral blood mononuclear cells (PBMCs) by leukapheresis, which are then cryopreserved and shipped to the manufacturing facility. Upon the availability of a manufacturing slot, the leukapheresis product is thawed, and the CAR-T transduction process is initiated. Once completed, the expanded cells are transferred into an infusion bag, cryopreserved, and released with quality assurance to the treatment site. The turnaround vein-to-vein time is typically 3−5 weeks,[3] with the long waiting time being another drawback of the centralized CAR-T manufacturing, making it unsuitable for patients with rapidly progressing disease.

Alternatively, decentralized or point-of-care (POC) manufacturing of CAR-T cells at academic medical centers could reduce the acquisition cost and complexity of the manufacturing process (i.e., cryopreservation and cold chain logistics). POC manufacturing enables the delivery of fresh CAR-T cells, which were shown earlier to have higher

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in vitro antitumor activity and lesser microcellular damage than the frozen,[4,5] although whether fresh products would provide better clinical outcomes remains to be determined. Conventional CAR-T manufacturing involves many hands-on, manual operations and open steps in grade A laminar flow cabinets in a dedicated grade B cleanroom.[6,7] A paradigm shift enabling POC manufacturing is the development of bioreactors and closed system manufacturing platforms that mitigate the need for expensive infrastructure and simplify the multistep process.[2,8,9] At our institute, we aim to manufacture anti-CD19 CAR-T cells that ensure levels of quality and safety equivalent to those of approved products. To this end, we used a closed semi-automated platform, CliniMACS Prodigy, that integrates all cell processing steps from T cell separation, CAR transduction, cell expansion, and formulation in a device using a single-use chamber and tubing set under Good Manufacturing Practices (GMP) compliance.[10,11] In-process control (IPC) and quality control (QC) release testing of the finished products were stringently implemented based on product characteristics and relevant guidelines for cell and gene therapy products.[12–14]

Currently, we are conducting a pilot study to evaluate the safety and efficacy of autologous or human leukocyte antigen (HLA)-matched sibling donor-derived anti-CD19 CAR-T cells for the treatment of recurrent/relapsed $CD19^+$ B cell malignancies. To date, nine POC cell products, designated as SiCF-019 cells, have been manufactured and used—seven products were generated from heavily pretreated patients' own cells, and two products were from healthy donors. Here, we demonstrated the phenotypic and functional characteristics of the cell products obtained that qualified them for clinical application, supporting the feasibility of POC manufacturing of CAR-T cells using a GMP-compliant closed system in Thailand. We also shared our real-world good and bad manufacturing experiences, which may benefit other centers that aim to increase the accessibility of CAR-T cell therapy and/or are in the initial setup phase.

RESULTS

Study design and participants

We are conducting a single-arm, single-center pilot study of anti-CD19 CAR-T cells manufactured in the Siriraj Cell Factory for Cancer Immunotherapy, Faculty of Medicine Siriraj Hospital, Thailand. The primary objective of the study was to determine the safety and preliminary efficacy of autologous or HLA-matched sibling donor-derived anti-CD19 CAR-T cells, called SiCF-019, for the treatment of recurrent/relapsed $CD19^+$ B cell malignancies in a Thai adult population. Secondary objectives included the feasibility of POC manufacturing at the minimum target dose of 1×10^6 CAR-T cells per kilogram and the determination of CAR-T cell persistence in patients. At the time of submitting this manuscript, nine patients have been enrolled, two of whom (case nos. 3 and 7) received allogeneic CAR-T cells from their stem cell transplantation donors (see Table S1 for patient clinical information and pretreatment regimens and Table S2 for healthy donor information). A lymphodepleting chemotherapy regimen of intravenous fludarabine (25 mg/m^2) daily for 3 days and cyclophosphamide (60 mg/kg) was planned prior to CAR-T infusion.

Overview of the POC manufacturing of clinical CAR-T cells

The entire manufacturing process was carried out using the CliniMACS Prodigy, which was housed in a Grade C cleanroom, using a single-use, disposable TS520 tubing set, and the preprogrammed T cell transduction (TCT) process, according to the manufacturer's protocol, as schematically illustrated in Figure 1. The semi-automated sequential steps included T cell enrichment and activation on day 0, transduction of T cells with anti-CD19 CAR lentiviral vector (LVV) on day 1, and T cell expansion for up to day 18 or until the desired cell number was reached. After that, the cells were harvested from the device in a final formulation buffer and aliquoted for dosing according to a prescription. The remaining cells were cryopreserved until further infusion. During the TCT process, cells were sampled for IPC testing every 3 days starting from day 5 and for QC release testing at the end of culture. The tests were simply grouped as follows: cell counting, immunophenotyping for immune cell composition (ICC), CAR transduction rate (TCT efficiency), and T cell subset (T-sub) characterization; sterility and other microbiology testing; and other release criteria assays—for example, replication competent lentivirus (RCL), vector copy number (VCN), and cytotoxicity assay.

T cell enrichment and expansion

Seven leukapheresis products were obtained from heavily pretreated patients with non-Hodgkin lymphoma (NHL) or acute lymphoblastic leukemia (ALL), most of which had a low CD4/CD8 ratio (median 0.352, range 0.196–0.657), and two products were from healthy, HLA-matched sibling donors (case IDs H3 and H7) with a normal CD4/CD8 ratio (median 1.163, values of 0.784 and 1.541). Fresh leukapheresis products loaded into the CliniMACS Prodigy were labeled with anti-CD4/CD8 magnetic microbeads and enriched through a magnetic separation column. After column purification, the enriched cells were eluted into a target cell collection bag and sampled for ICC determination. The enrichment process did not significantly affect cell viability (Figure 2A) and yielded high T cell purity (range 78.67%–97.77%). Representative flow cytometric plots of $CD3^+$ cells pre- and post-enrichment are shown in Figure 2B. The T cell composition of the pre-enriched product was 9.92% ± 4.55% in $CD4^+$ T cells and 22.69% ± 12.31% in $CD8^+$ T cells, and the post-column recovery of $CD4^+$ T cells was 31.72% ± 15.81% and that of $CD8^+$ T cells was 55.12% ± 18.52%, with an unchanged CD4/CD8 ratio (Figure 2C; $p = 0.418$). The total number of enriched $CD3^+$ T cells was $1.94 \pm 0.77 \times 10^9$ cells, but only $100–150 \times 10^6$ cells were seeded in the culture chamber for the cultivation process (Figure 3A, left). The remaining cells were frozen for later use.

Enriched T cells were activated on day 0 of the process through CD3/CD28 co-stimulation, followed by transduction with anti-CD19 CAR LVV in culture media supplemented with interleukin-7 (IL-7) and IL-15 at an MOI of 60–90. After 48 h, the LVV was washed out, and the cells were further expanded for an average of 12.7 days (median: 12 days, range: 11–18 days). Cell viability/counting, ICC, and TCT efficiency were determined on day 5 of the process and every 3 days by flow cytometry. Initially

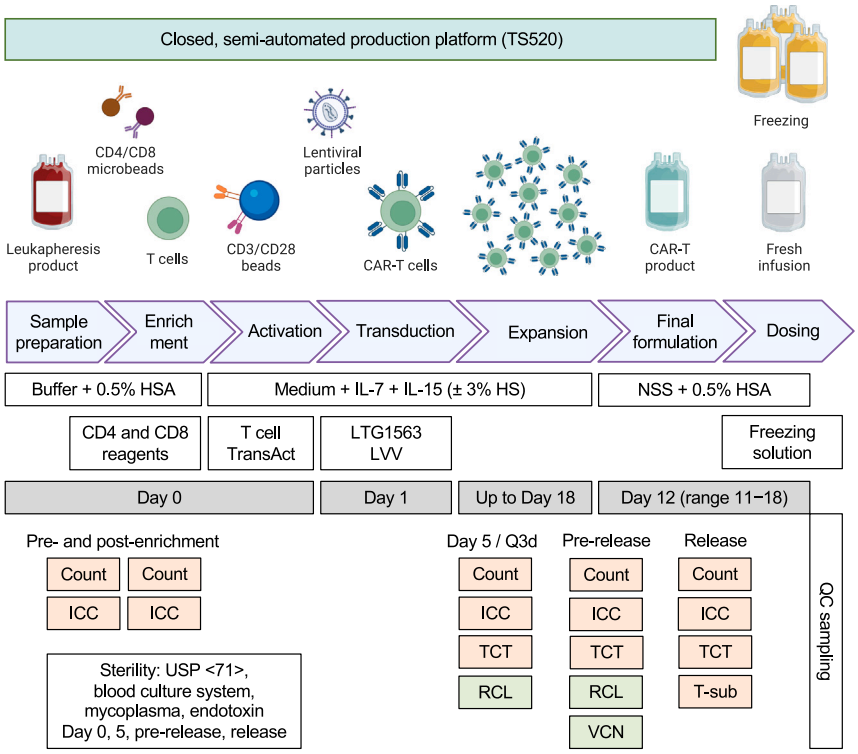


Figure 1. A schematic overview outlining the workflow of CAR-T cell manufacturing using a closed semi-automated production platform

Leukapheresis product was transferred to the CliniMACS Prodigy device with TS520 tubing set and TCT program on day 0, and T cells were enriched and activated through CD3 and CD28 co-stimulation. LVV transduction was performed on day 1, after which T cells were expanded in culture media, typically for 12 days, until the final CAR-T cell product was harvested. The IPC and QC samples were analyzed at the indicated time points. Q3d, every 3 days.

in cases 1–6, 3% human serum (HS) was not added to the media because the TCT process under serum-free conditions had been optimized by the manufacturer. However, we observed an extremely low $CD3^+$ T cell number (29.51 ± 6.28 × 10^6 cells) and a large amount of cell debris on day 5 in cases 2, 5, and 6, where a starting patient's cells (post-enrichment) had very low CD4/CD8 ratios of less than one-third (0.22, 0.29, and 0.20) (Figures 3B and 3C). For case 2, the cells fully recovered and expanded on day 8. For case 5, we tried to rescue the cells by adding an extra 5% HS albumin (HSA) to the media on day 11, when we found out that the cells failed to self-recover, and harvested the cells on day 14 as they were no longer able to proliferate. For case 6, an extra 3% HS was supplemented as early as day 5, and the cells fully recovered on day 11 and were harvested on day 18. To reduce the chance of manufacturing failure, we decided to culture the cells with constant HS supplementation from case 7 onward. The total $CD3^+$ T cell number at the end of culture is summarized in Figure 3A (right). Cell viability in the final products was high (>85%) in all cases (acceptance criteria >70%), including those rescued cases (Figure 3D), and the CD4/CD8 ratio seemed to be more balanced (median: 0.986, range: 0.21–1.90) (Figure 3E).

Transduction efficiency and product purity

The transduction efficiency by means of the percentage of CAR^+ rate in $CD3^+$ T cells was monitored by using CD19 CAR Detection Reagent, which is a flow cytometry-based detection that has previously been shown to have a good correlation with quantitative real-time PCR (real-time qPCR) data,[15] as shown in Figure 4A. The transduction efficiency of our final products was 40.66% ± 16.23%, similar to those analyzed on day 5 of culture (Figure 4B), with a mean VCN of 1.97 ± 0.35 copies per CAR-T cell (all of which met the requirement of authorities at <5 copies per cell).[16] We observed no significant difference in the proportion and ratio of CAR^+ $CD4^+$ T cells and CAR^+ $CD8^+$ T cells among groups with different culture conditions (Figure 4C). The total number of manufactured CAR^+ $CD3^+$ T cells in no HS and 3% HS groups was 1.57 ± 0.32 × 10^9 cells (Figure 4D), far exceeding the dosing need (Figure 4E), and at least three bags of double doses were cryopreserved. For the rescued group, the cell number was just enough for a single dose at 1.5 × 10^6 CAR-T cells per kilogram in case 6. As the minimum target dose of 1 × 10^6 CAR-T cells per kilogram was not reached in case 5, the patient received an adjusted dose of 0.14 × 10^6 CAR-T cells per kilogram. Despite this, patient case 5 achieved complete response (CR) at 1 month and remained in CR at 6 months.

Product impurity, particularly B cell contamination, is a safety concern. The median contaminating B cells in the final products were 0.00% (range: 0.00%–0.04%), monocytes were 0.00% (range: 0.00%–0.01%), neutrophils were 0.01% (range: 0.00%–0.46%), and eosinophils were 0.04% (range: 0.00%–0.29%) (Figure 4F; see also Table S3). A decent amount of natural killer T (NKT) cells were detected (median: 5.84%, range: 4.41%–10.69%), while there was a very minimal number of NK cells (median: 0.12%, range: 0.02%–3.17%).

T cell subset characterization

It is widely accepted that early memory T cell subsets (e.g., naive [T_N], stem cell memory [T_{SCM}] and central memory [T_{CM}]T cells), which are long-lived and possess high self-renewal ability, exhibit long-term persistence and superior antitumor response *in vivo*.[17–19] We assessed the memory T cell subsets in the final products based on the differences in expression of CD62L, CD45RO, and CD95, as depicted in Figure 5A, following the gating strategy shown in Figure 5B. Figure 5C shows that most of the cell products, except for those of

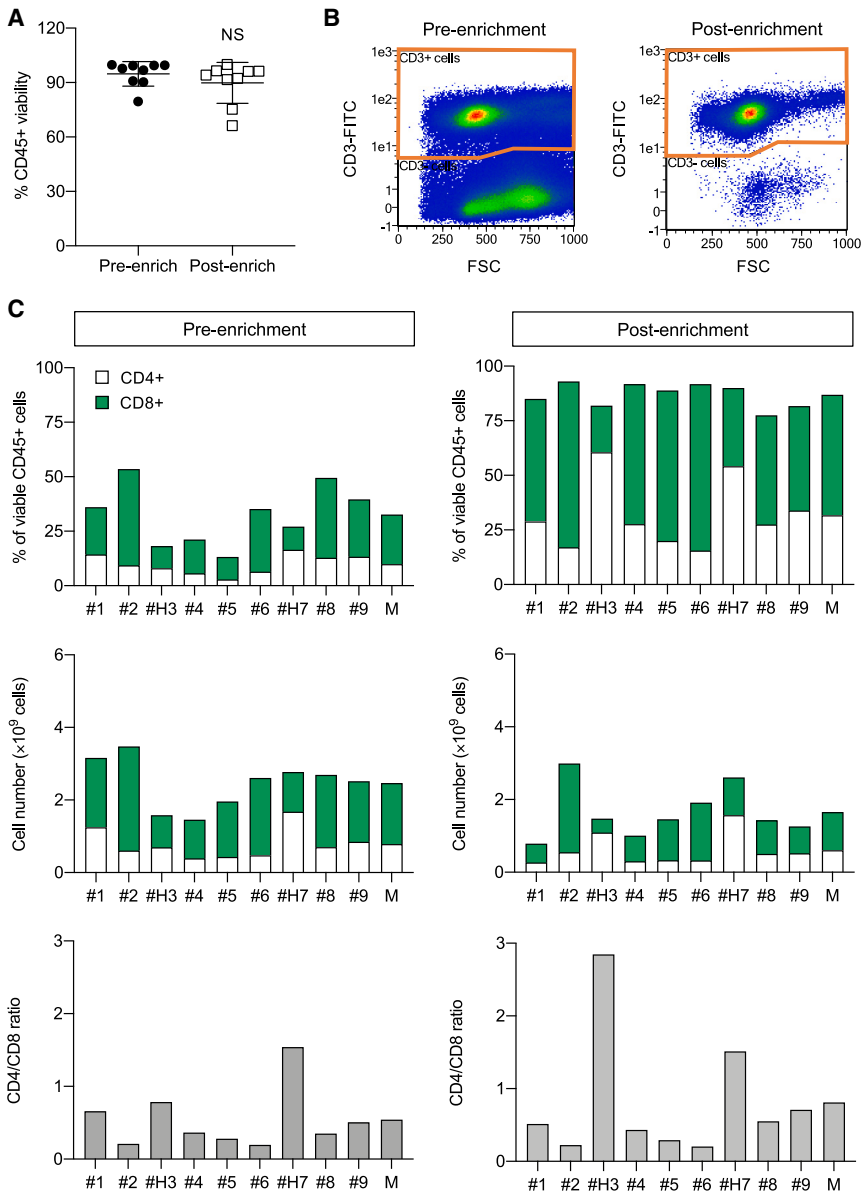


Figure 2. T cell composition of leukapheresis products and recovery post-enrichment

Leukapheresis product was collected from an enrolled patient or a healthy donor (case IDs H3 and H7) and processed within 24 h without cryopreservation. (A) Viability of $CD45^+$ leukocytes in the leukapheresis products (pre-enrichment) and the enriched $CD4^+/CD8^+$ T cells after magnetic separation (post-enrichment). (B) Representative flow cytometric plots showing the purity of $CD3^+$ T cells in viable $CD45^+$ cells pre- and post-enrichment. (C) Percentages and numbers of $CD4^+$ and $CD8^+$ T cells among viable $CD45^+$ cells (upper and center) and the calculated CD4/CD8 ratio (lower) pre- and post-enrichment. M, mean value; NS, not significant.

case 1 and the rescued cases, consisted mostly (~60%–80%) of T_{SCM} and T_{CM}, with little T_N, for both $CD4^+$ and $CD8^+$ T cells, indicating that the current manufacturing protocols yielded favorable T cell subsets.

Release testing and product stability

The results of standard release criteria assays for clinical applications other than phenotypic analyses, including the absence of mycoplasma, endotoxin, RCL, VCN, and product sterility, are summarized in Figure 5D (see also Tables S4–S7 for IPC outcomes). We intend to transport the product to the Stem Cell Transplantation Unit within 1 h upon release; however, the product stability by means of $CD3^+$ T cell viability was tested in its primary package, a 150-mL infusion bag, at 4°C by flow cytometry for up to 24 h. Figure 5E shows that the SiCF-019 cell products were stable at 4°C for at least 24 h.

Functional characterization of the manufactured CAR-T cells

Cytotoxicity of the manufactured cells against target tumor cells was evaluated *in vitro* to validate their effector cell function before cell infusion. Both $CD19^+$ and $CD19^-$ tumor cells were included to determine the CD19 antigen specificity. Briefly, tumor cells were labeled with PKH67 green fluorescence and cocultured with SiCF-019 cells at various effector-to-target (E:T) ratios for 4 h. The cell death rates of $PKH67^+$ tumor cells were then evaluated by the annexin V/7-AAD assay using the gating strategy shown in Figure 6A. The results show that although SiCF-019 cells induced both $CD19^+$ and $CD19^-$ tumor cell death in a dose-dependent manner, they had a more profound effect toward $CD19^+$ cells. For example, the death rates at the E:T ratio of 5:1 were 61.65% ± 13.90% in $CD19^+$ Z-138 cells and 44.88% ± 20.38% in $CD19^+$ REH cells versus 22.31% ± 10.12% in $CD19^-$ K562 cells ($p < 0.001$ and $p < 0.05$, respectively), suggesting the antigen-specific killing by SiCF-019 cells. Notably, SiCF-019 cells produced under different culture conditions showed similar cytotoxic potential.

We also assessed the production of major T helper 1 cytokines such as tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ) in the supernatants of SiCF-019 cells upon exposure to Z-138 cells, the most responsive cells, compared to their untransduced counterparts. Figure 6B shows that SiCF-019 cells exposed to Z-138 cells produced a significant amount of TNF-α but had a substantially greater IFN-γ level than untransduced T cells even without stimulation with target tumor cells.

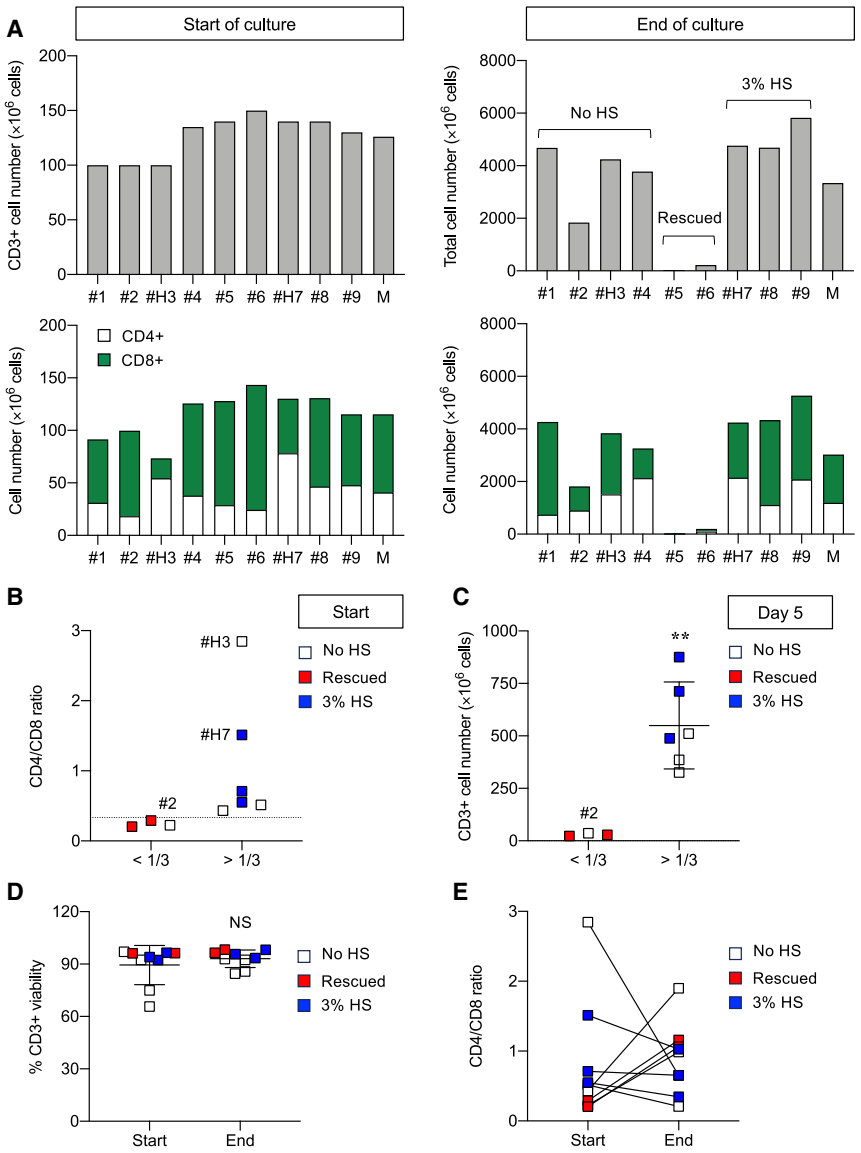


Figure 3. T cell composition at the start and end of culture

(A) Number of total CD3$^+$ T cells loaded into the culture chamber at the start and at the end of culture on day 12 or as indicated (i.e., day 11 for case IDs H7 and 8, day 14 for case 5, and day 18 for case 6 [upper]). Numbers of CD4$^+$ and CD8$^+$ T cells are also shown (lower). Cultured cells were classified according to the culture conditions into three groups: no HS (nos. 1–4), rescued (nos. 5 and 6), and 3% HS (nos. 7–9). M, mean value. (B and C) Cells were grouped according to CD4/CD8 ratios at the start of culture (cutoff at one-third, dashed line) (B), and total CD3$^+$ T cell numbers were compared between groups with relatively lower (<1/3) and higher (>1/3) ratios on day 5 of culture (C). $^{**}p < 0.01$; two-sided Student's t test. The culture conditions are also labeled. (D) Viability of CD3$^+$ T cells at the start and end of culture. NS, not significant ($p > 0.05$); two-sided Student's t test. (E) CD4/CD8 ratio at the start and end of culture.

Exhausted T cells during culture were associated with poor expansion

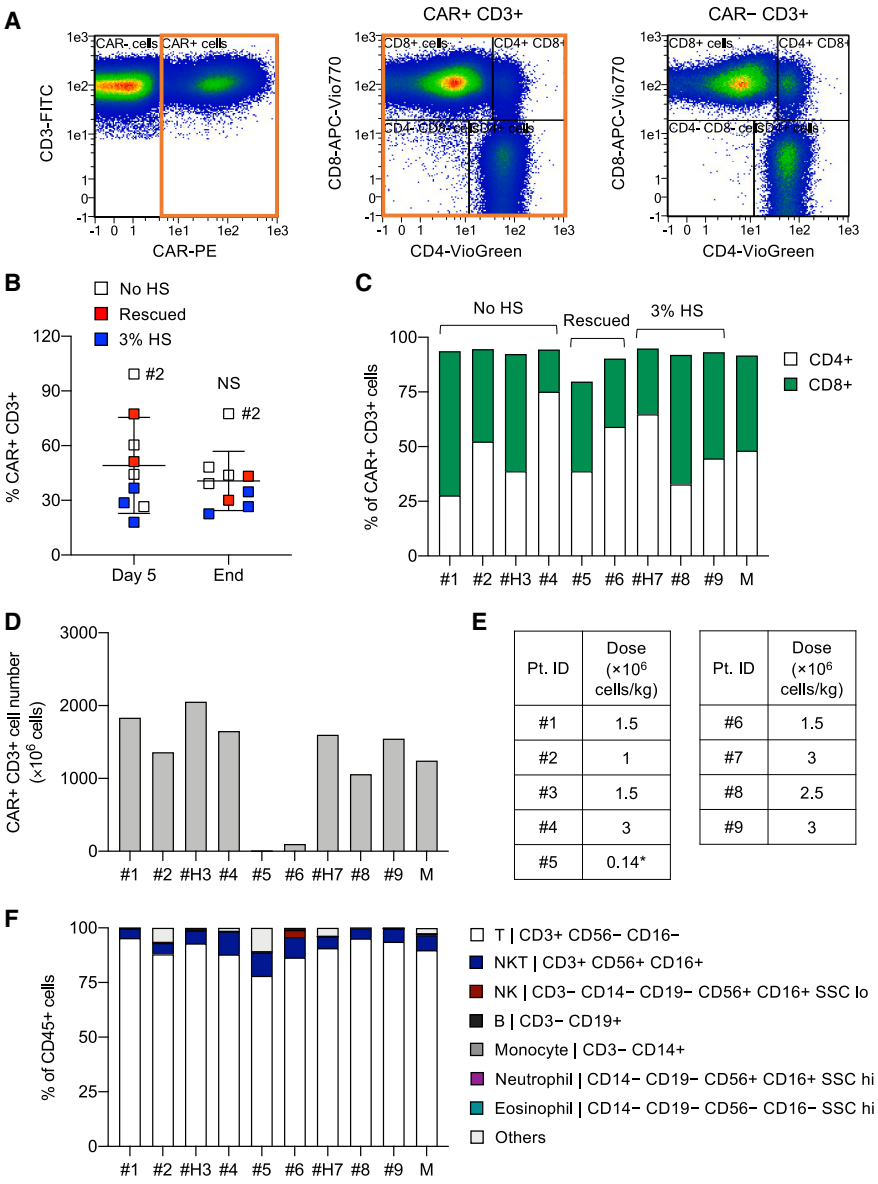
To investigate whether T cell exhaustion is associated with poor *in vitro* expansion in rescued cases 5 and 6, we analyzed T cell exhaustion markers TIM3 (CD366) and PD1 (CD279) in parallel with the numbers of total CD3$^+$ T cells and CAR-T cells at various times. In both cases, the CD3$^+$ T cell number dropped tremendously after transduction, as evaluated on days 5, 7, or 8 of culture (Figure 7A), concomitant with a remarkable increase in TIM3 and PD1 (Figure 7B). An addition of HSA (case 5) and HS (case 6) gradually reversed T cell exhaustion. However, we were not able to rescue T cell culture in case 5, likely because HSA was added too late. We believe the best practice is to rescue the culture right after T cell exhaustion and poor expansion are detected, as in case 6.

CAR-T cell persistence and differentiation in patients

While a full assessment of patient responses is currently in progress and beyond the scope of this paper, we observed that all patients—except for case 7, who died from severe cytokine release syndrome (CRS)—achieved CR within 1 month and that lymphoma responses were sustained, with the majority of patients having CR maintained for at least 6 months (see Table S8). Here, the persistence of the manufactured CAR-T cells in patients was analyzed by means of viable circulating CAR-T cells in peripheral blood by flow cytometry using the gating strategy shown in Figure 8A. CAR-T cells peaked in most patients in the first week (day 7) and, in all cases, within the first month after infusion (Figure 8B). Analysis of T cell subsets was also performed at the peak of CAR-T persistence using the gating strategy shown in Figure 8C. Figure 8D shows that the majority of circulating CD4$^+$ CAR-T cells were T_{CM}, while the majority of circulating CD8$^+$ CAR-T cells were either T_{CM} or T_{EM}, indicating the *in vivo* differentiation of T cells from the T_{SCM} subpopulation to more mature subtypes.

DISCUSSION

Anti-CD19 CAR-T cell therapy has achieved remarkable clinical success in B cell malignancies. Aside from that, recent studies have suggested the feasibility and efficacy of anti-CD19 CAR-T cell therapy in autoimmune diseases.[20,21] All approved products and a majority of ongoing CAR-T cell clinical trials targeting CD19 and many other antigens and diseases are autologous, resulting in costly manufacturing, particularly if the production platform is centralized and transportation between manufacturing facilities



Pt. ID	Dose (×10^6 cells/kg)
#1	1.5
#2	1
#3	1.5
#4	3
#5	0.14*

Pt. ID	Dose (×10^6 cells/kg)
#6	1.5
#7	3
#8	2.5
#9	3

Figure 4. T cell transduction efficiency and CAR-T cell yield

(A) Flow cytometric gating strategy for characterization of CAR$^+$ and CAR$^-$ T cells among viable CD45$^+$ cells. (B) Percentage of CAR$^+$ rate among viable CD3$^+$ T cells on day 5 and at the end of culture. NS, not significant ($p > 0.05$); two-sided Student's t test. The culture conditions are also labeled. (C) Percentages of CAR$^+$ CD4$^+$ and CAR$^+$ CD8$^+$ T cells among viable CAR$^+$ CD3$^+$ T cells. (D) Total number of manufactured CAR$^+$ CD3$^+$ T (CAR-T) cells in the final cell product, SiCF-019 cells. (E) The dosing of CAR-T cells in the enrolled patients. Pt., patient. An asterisk indicates a value below the minimum target dose of 1×10^6 cells per kilogram. (F) Cellular composition reported as percentages of the defined population in viable CD45$^+$ cells in the final cell product (see Table S3 for all values).

and treatment sites with a complex cold chain is needed. Access to centralized, industrial CAR-T cells is not considered economically feasible for most local Thai patients. To date, commercial anti-CD19 CAR-T cells, such as tisagenlecleucel (Kymriah, Novartis) and axicabtagene ciloleucel (Yescarta, Kyte-Gilead), have not been reviewed or approved by the Thai FDA. Here, we demonstrated the feasibility of POC manufacturing of anti-CD19 CAR-T cells with quality and safety assurance in a non-profit, non-commercial setting at an academic medical center in Thailand using the CliniMACS Prodigy system, which can broaden the availability of CAR-T cells and expand treatment access to local patients. Abstracting from intellectual property rights, POC cell products are considerably less costly, and the estimated production cost could be further reduced if we could bring down the price of LVV, for example, by obtaining them in large quantities or via contract development and manufacturing organization services.

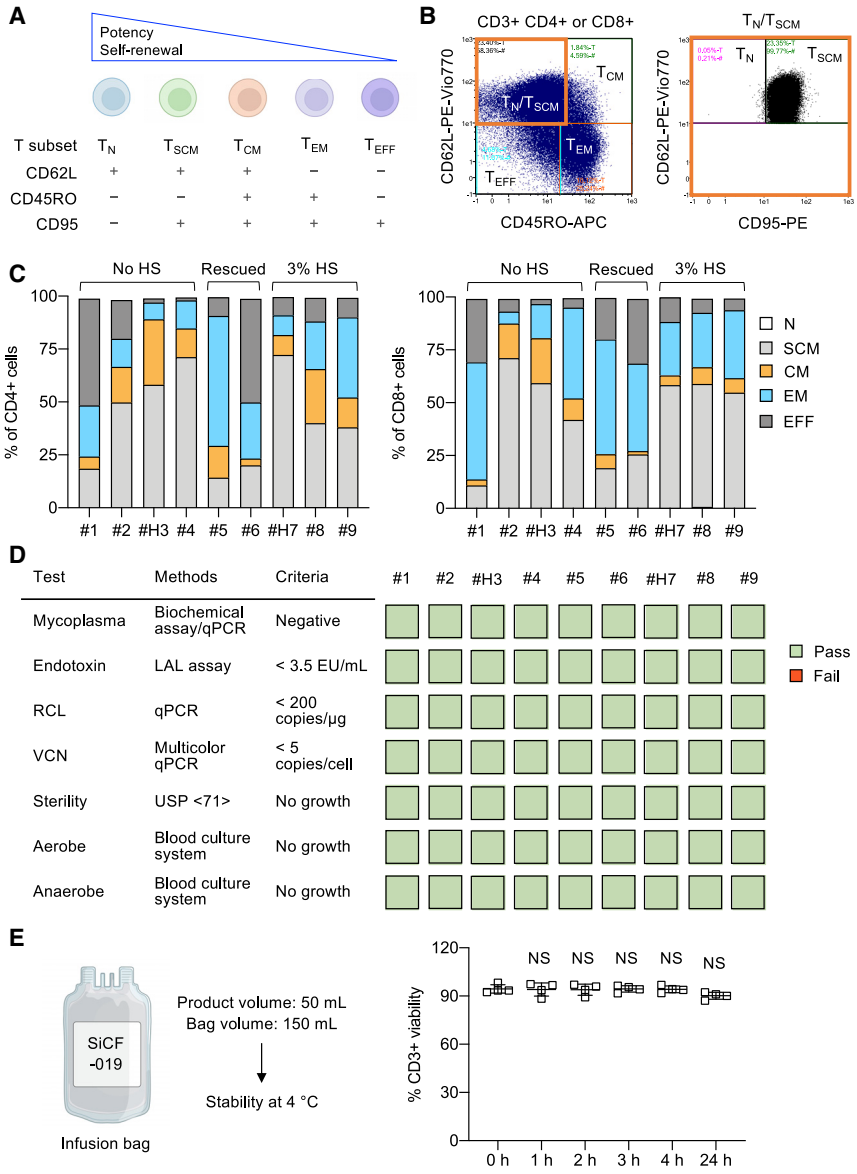


Figure 5. Immunophenotypic characterization and QC release testing of the final cell product

(A) A schematic diagram of different T cell subsets and their combinatorial expression of multiple markers. (B) Flow cytometric gating strategy for characterization of T cell subsets in the final cell product. (C) Percentages of T_N, T_{SCM}, T_{CM}, T_{EM}, and T_{EFF} cells among viable $CD4^+$ and $CD8^+$ T cells. (D) Results of QC release testing with respect to safety and sterility. (E) Stability of the final cell product in its primary package at 4°C based on viability of $CD3^+$ T cells. NS, not significant ($p > 0.05$) versus control at 0 h; two-sided Student's t test. T_{EM} and T_{EFF}, effector memory and effector T cells, respectively.

In the current pilot study, only patients who had previously received multiple lines of therapies were eligible; hence, their T cells had a relatively low fitness, as evaluated by the CD4/CD8 ratio (median 0.352). Despite this, we were able to manufacture CAR-T cell products that far exceeded the dosing need from most patients' materials (median: 13 doses, range: 7–20 doses), except for the rescued cases, in whom the CD4/CD8 ratio was less than one-third (median: 0.21). We observed that these T cells with relatively lower CD4/CD8 ratios (cases 2, 5, and 6) were more susceptible to LVV transduction—their CAR^+ rate on day 5 was 72.15% ± 15.35% versus 35.75% ± 13.73% in those with higher ratios ($p = 0.016$) (Figure S1). As a result, tonic signaling of CAR, which has been shown to be associated with T cell exhaustion,[22,23] might be overactivated. Indeed, we detected a severe T cell exhaustion (high $TIM3^+$ $PD1^+$) 5–8 days post-transduction in rescued cases 5 and 6, which might be attributable to their poor cell expansion (Figure 7). Our findings suggested that starting T cells with CD4/CD8 ratios of less than one-third increases the chance of manufacturing failure on CliniMACS Prodigy under serum-free conditions, which is in line with the multivariate analysis from a cohort study in Japan reporting CD4/CD8 ratios of less than one-third at apheresis as one of the risk factors for failure of tisagenlecleucel production among diffuse large B cell lymphoma patients.[24] We postulated that the addition of serum as early as possible post-transduction could help restore T cell exhaustion and poor expansion. To reduce the chance of manufacturing failure, we decided to culture the cells with constant serum supplementation from case 7 onward. We did not observe the significant differences in transduction efficiency on day 5 of culture ($p = 0.07$), the number of total CAR-T cells at the end of culture ($p = 0.21$), and *in vitro* antitumor response ($p > 0.05$) between the culture conditions with and without HS at the end of culture (Figures 4 and S2). HS obtained from male donors with an AB blood type has been used as a growth supplement for the expansion of therapeutic cells. However, as we occasionally experience a shortage of healthy male AB donors, who account for only approximately 3% of all Thai blood donors, we alternatively collected and validated the use of HS from a matching blood type for CAR-T cell manufacturing herein. Notably, we did not find any impact on the manufacturing from the use of granulocyte-colony-stimulating factor (G-CSF)-mobilized leukapheresis product from healthy donors as a starting material.

We harvested the cells on day 11–12 according to the manufacturer's standard protocol, except for the rescued cases, which

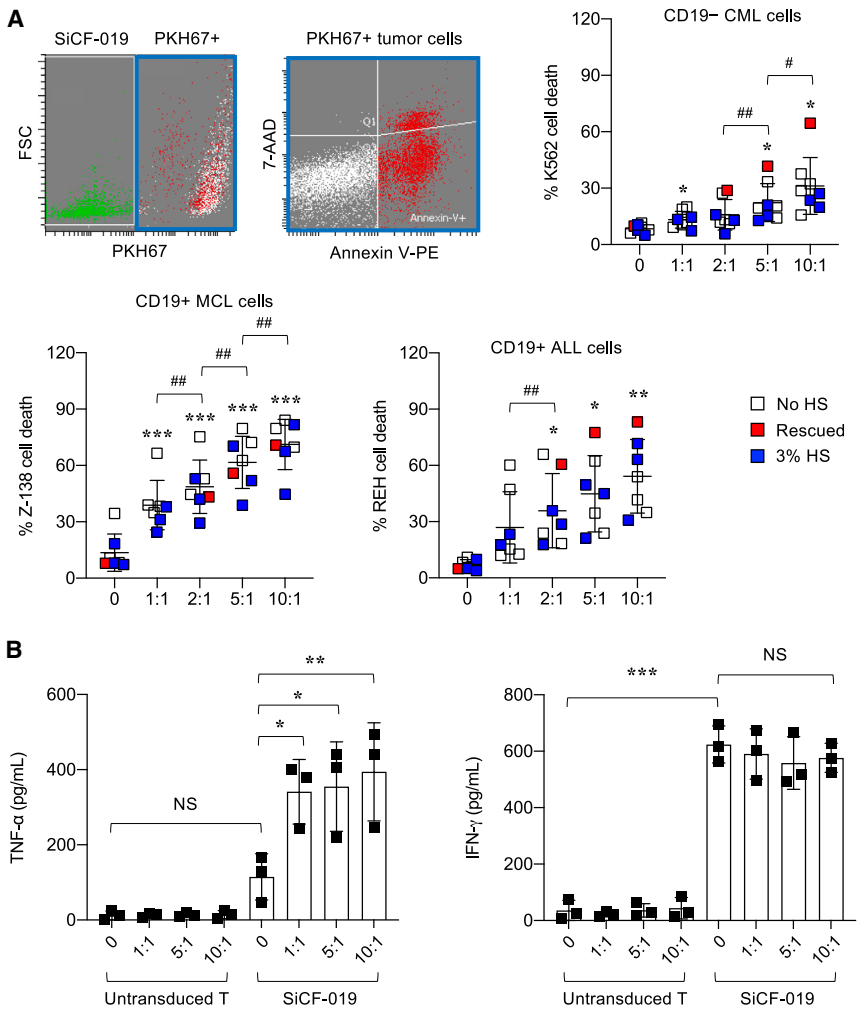


Figure 6. Functionality of the final cell product

(A) Cytotoxicity of the manufactured SiCF-019 (effector) cells against $CD19^+$ and $CD19^-$ tumor (target) cells. (Top left) Flow cytometric gating strategy for specifically detecting the cell death of PKH67-labeled tumor cells after incubation with SiCF-019 cells by annexin V/7-AAD assay. Percentages of total cell death of K562, Z-138, and REH cells, comprising annexin V^+ and/or 7-AAD^+ cells, at various E:T ratios at 4 h are plotted. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus basal control (without SiCF-019); $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus indicated groups; one-way ANOVA with Tukey's multiple comparison test. The culture conditions are also labeled. (B) Quantitative measurement of TNF-α and IFN-γ by ELISA in cell-free supernatant collected from the coexposure of untransduced T cells or SiCF-019 cells with tumor cells at various E:T ratios at 4 h. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus indicated groups; one-way ANOVA with Tukey's multiple comparison test. NS, not significant.

were extended for up to 18 days. Our data show that we obtained abundant CAR-T cells far exceeding the dosing need, even for a dosing at 3×10^6 CAR-T cells per kilogram (the maximum dose we infused into this set of patients), indicating that it is possible to shorten the manufacturing time to 7−8 days, which is highly desirable. Reducing the turnaround time would not only lower the cost but also expedite patient treatment, emphasizing the main goals of POC manufacturing. Our final POC cell products, SiCF-019 cells, meet all the release specifications for clinical application with respect to identity/characterization, purity, sterility, safety, and potency. Further analysis of T cell subsets revealed substantial proportions of T_{SCM} and T_{CM}, which are early memory T cells possessing long-term persistence and antitumor activity *in vivo*, in the majority of cell products, which is consistent with previous studies using a similar platform and reagents.[14,19,25] Since 2018, CliniMACS Prodigy has been commonly used for POC manufacturing of CAR-T cells in academic medical centers in the United States,[2,26–29] Spain,[19,30–32] Russia,[27] and India[14] for clinical studies and hospital exemption, while other manufacturing platforms include the Lonza Cocoon, Wilson Wolf G-Rex bioreactor, and Gibco CTS Rotea.

We quantified live, circulating CAR-T cell persistence in the peripheral blood of patients by flow cytometry using antigen-based detection of anti-CD19 CAR and reported that CAR-T cells were detectable in all patients—except for case 7, who died from severe CRS—for at least 6 months after cell infusion (Figure 8). The immunophenotypic analysis of these CAR-T cells revealed that the majority of CAR^+ cells in their highly active state (within the first month) were $CD8^+$ T cells (CD4/CD8 ratio <0.5), indicating that *in vivo* expansion was driven by $CD8^+$ CAR-T cells, consistent with a previous report.[26] Additionally, we demonstrated for the first time that circulating CAR-T cells at its peak persistence were mostly T_{CM} and T_{EM}, thus confirming that CAR-T cells differentiated into more mature subtypes, which generally exert greater effector function *in vivo*.[33,34]

In summary, the data presented here demonstrate the feasibility of POC manufacturing of functional, second-generation anti-CD19 CAR-T cells with quality and safety assurance using a GMP-compliant closed system in Thailand. We also shared good and bad experiences from our cell factory regarding CAR-T cell manufacturing from patients' materials with extremely low T cell fitness, which carries a high risk of manufacturing failure. These settings of POC manufacturing platform and QC testing could be applied to other CAR-T cell products targeting various antigens and diseases that are currently undergoing research and development in our institute and may benefit the setup of cell factories in other

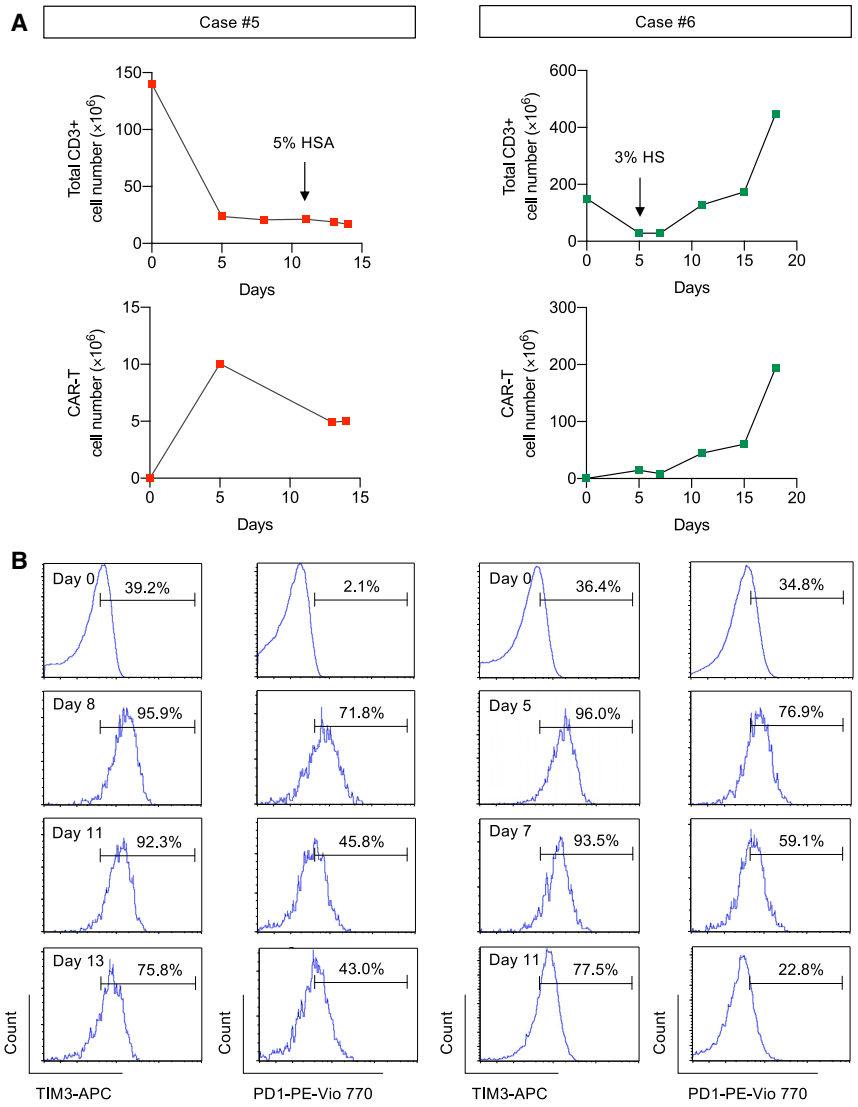


Figure 7. Expression of T cell exhaustion markers in culture with poor expansion

(A) Expansion kinetics of total CD3⁺ T cells and CAR-T cells on various days of culture in rescued cases 5 and 6, which required additional serum supplementation (i.e., 5% HSA or 3% HS). (B) Flow cytometric analysis of the exhaustion markers TIM3 and PD-1 in cultured CD3⁺ T cells at various time points.

academic medical centers, making CAR-T cells more accessible, especially in developing countries.

MATERIALS AND METHODS

Subjects and ethics statement

This study was approved by the Siriraj institutional review board (COA no. Si 966/2020; SIRB Protocol no. 472/2562(EC1)) and conducted in accordance with the 1975 Declaration of Helsinki. All participating patients and healthy donors gave written informed consent. The trial is registered on the ISRCTN registry (ISRCTN17901467, https://www.isrctn.com/ISRCTN17901467).

Collection of PBMCs

PBMCs were obtained from enrolled patients and healthy donors (case IDs H3 and H7) by leukapheresis using a Spectra Optia apheresis system (Terumo Blood and Cell Technologies, Tokyo, Japan) at the Siriraj Blood Donation Center, Siriraj Hospital. Leukapheresis products were transported to the cell factory within 2 h, stored at 4°C, and processed within 24 h without cryopreservation.

Anti-CD19 CAR LVV

LTG1563 carrying a second-generation anti-CD19 CAR construct with FMC63 scFv-based targeting domain, CD8 hinge region, TNFRSF19 transmembrane domain, 4-1BB costimulatory domain, and CD3ζ chain intracellular signaling domain was obtained from Miltenyi Biotec (catalog no. 170-076-018; Bergisch Gladbach, Germany). The LVV was manufactured and tested under a quality management system in compliance with GMP guidelines and was previously used in numerous clinical studies.[26–28] For the present feasibility study, the LVV was an in-kind contribution from Miltenyi Biotec.

POC manufacturing of CAR-T cells using semi-automated platform

All reagents used were GMP products purchased from Miltenyi Biotec, unless otherwise specified. Full details of the key resources can be found in Table S9. Briefly, the TS520 tubing set was installed in the CliniMACS Prodigy device and primed with CliniMACS PBS/EDTA buffer containing 0.5% HSA (Grifols, Barcelona, Spain) and TexMACS culture media supplemented with 350 IU/mL IL-7 and 450 IU/mL IL-15 with or without 3% HS obtained from healthy male donors with AB or matching blood types (Siriraj Blood Donation Center). Once the leukapheresis product was connected and loaded, erythrocyte reduction was performed by density gradient centrifugation in the CentriCult chamber. After that, the remaining cells were labeled with CliniMACS CD4 and CD8 reagents and enriched by magnetic separation. A total of 1–1.5 × 10^8 enriched T cells were seeded into the chamber to initiate culture and activated via CD3 and CD28 using T cell TransAct prior to LVV transduction. After expansion, the cells were harvested in a formulation buffer, which was a normal saline solution for injection (Thai

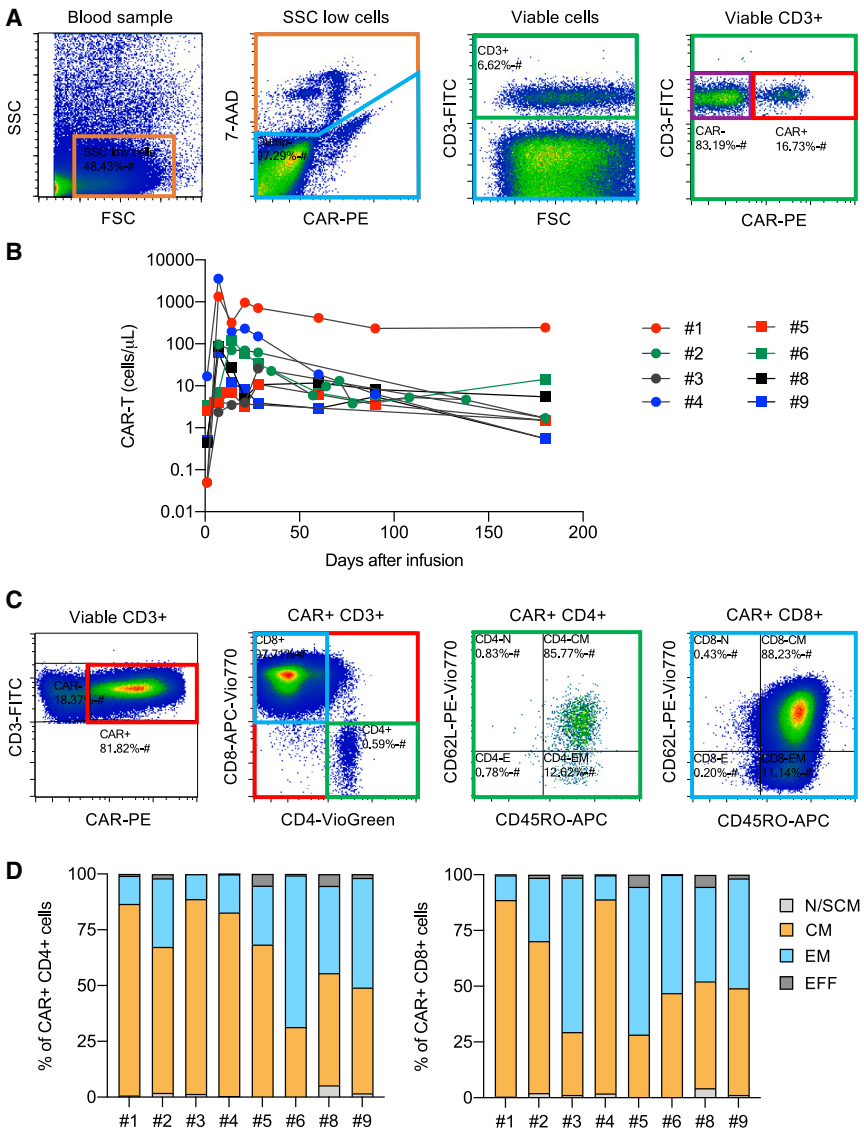


Figure 8. Detection of circulating CAR-T cells in the peripheral blood of patients and their differentiation status

(A) Flow cytometric gating strategy for detection of viable circulating CAR-T cells in blood samples. (B) Absolute number of viable CAR-T cells in patients at various time points for up to 6 months after cell infusion. (C) Flow cytometric gating strategy for characterization of T cell subsets in blood samples. (D) Percentages of $T_{N/SCM}$, T_{CM}, T_{EFF}, and T_{EM} cells among viable CAR^+ $CD4^+$ and CAR^+ $CD8^+$ cells at the peak of CAR-T persistence.

Otsuka Pharmaceutical, Samut Sakhon, Thailand) containing 0.5% HSA, typically on day 12 of culture.

Immunophenotyping

Immunophenotypic characterization of the manufactured SiCF-019 cells and the frequency and quantity of each immune cell population were determined by flow cytometry using MACSQuant Analyzer 10 (Miltenyi Biotec). Antibodies used in the panel for ICC included CD45-VioBlue, CD4-VioGreen, CD3-FITC, CD16-PE, CD56-PE, CD19-PE-Vio 770, CD14-APC, CD8-APC-Vio 770, and 7-AAD (Miltenyi Biotec). The TCT efficiency was determined in $CD45^+$ $7\text{-}AAD^-$ $CD3^+$ with a $CD4^+$ or $CD8^+$ subpopulation by antigen-based detection using CD19 CAR Detection Reagent (biotin conjugation; Miltenyi Biotec) and a biotin-PE secondary antibody. For the T-sub panel, CD45RO-APC, CD62L-PE-Vio 770, CD95-PE, and CD197-VioBlue antibodies were co-stained with CD3-FITC, CD4-VioGreen, CD8-APC-Vio 770, and 7-AAD. Briefly, the samples were incubated with cocktails of fluorescent-conjugated primary antibodies, 7-AAD, and biotin-PE, if any, in an analysis buffer containing 0.5% BSA for 10 min at 4°C in the dark. CD19 CAR Detection Reagent was added prior to antibody cocktails, if applicable, incubated for 10 min at room temperature in the dark, and washed twice in an analysis buffer.

VCN analysis

Genomic DNA was isolated from the manufactured SiCF-019 cells using a PureLink Genomic DNA Mini Kit (Invitrogen, Waltham, MA) and probed for the lentiviral gag gene (FAM) and human reference gene *PTBP2* (JOE) with a Taq-based qPCR assay using MACS COPYcheck Kit (Miltenyi Biotec) according to the manufacturer's protocol. CFX384 Multicolor Real-Time PCR (Bio-Rad, Hercules, CA) was used to measure the Ct values for FAM and JOE dyes, and the copy numbers of gag and *PTBP2* were calculated from a standard curve. VCN per transduced CAR-T cell was calculated by the following formula: [gag copy number/*PTBP2* copy number] ÷ [transduction rate/100] × 2.

Detection of RCL

The RCL assay is based on the detection of the vesicular stomatitis virus glycoprotein (VSV-G) lentiviral envelope gene, which must not be packed into the LVV and transduced cells.[35] Genomic DNA was prepared and qPCR analysis was carried out on CFX384 Real-Time PCR (Bio-Rad) using SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA). Initial enzyme activation was performed at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and primer annealing/extension at 60°C for 1 min. The sequences of forward and reverse primers were 5′-AGGGAACTGTGGGATGACTG-3′ and 5′-GAACA CCTGAGCCTTTGAGC-3′, respectively. The copy number of VSV-G was calculated from a standard curve of

VSV-G (catalog no. 8454, Addgene, Watertown, MA) dilution. Non-transduced cells on day 0 of the TCT process (starting T cells) were used as a negative control.

Microbiology testing

Leukapheresis product and IPC and final cell products were tested for sterility at the Department of Microbiology, Faculty of Medicine Siriraj Hospital by the following: (1) the compendial US Pharmacopeia (USP) <71> direct inoculation method with tryptic soy broth at 20°C–25°C and fluid thioglyocollate (thiol) broth at 35°C for 14 days, as well as (2) a continuous-monitoring automated blood culture system using a BacT/ALERT FA Plus aerobic (bacteria and fungi) bottle (catalog no. 410851, bioMérieux, Durham, NC) and a BD BACTEC anaerobic bottle (catalog no. 442021, BD, Franklin Lakes, NJ) for up to 14 days. Products were released when IPC samples passed the generally employed 6- or 7-day incubation periods of the blood culture system and cautiously used while awaiting the release of final sterility reports. Bacterial endotoxin detection was also performed at the Department of Microbiology by a turbidimetric Limulus amebocyte lysate assay using a PYROGENT-5000 Test Kit (Lonza, Basel, Switzerland).

Mycoplasma testing for IPC and product release was determined in-house by a MycoAlert PLUS Mycoplasma Detection Kit (Lonza), which is a rapid, selective biochemical assay based on the activity of mycoplasmal enzymes, due to time constraints, and subsequently validated by molecular testing by qPCR at the Research Division, Faculty of Medicine Siriraj Hospital.

Functional analyses

Cytotoxicity assay of the manufactured SiCF-019 cells against various target tumor cells, including $CD19^+$ human ALL-derived REH cells (American Type Culture Collection [ATCC], Manassas, VA), $CD19^+$ human mantle cell lymphoma-derived Z-138 cells (ATCC), and $CD19^-$ human chronic myeloid leukemia-derived K562 cells (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan), was performed. Briefly, target tumor cells were fluorescently prelabeled with PKH67 dye (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions, plated in a round-bottom 96-well plate, and cocultured with SiCF-019 cells at various E:T ratios of 1:1, 2:1, 5:1, and 10:1. Notably, total effector cells were counted without considering the transduction rate. The mixture was then subjected to the annexin V/7-AAD assay (BD Biosciences, San Jose, CA) at 4 h using the BD FACS Canto flow cytometer (BD Biosciences), where target cell death was defined by annexin V^+ and/or 7-AAD^+ cells in the $PKH67^+$ population. Cytokine release assay was used to validate the effector function of SiCF-019 cells. Upon coculture of SiCF-109 cells with $CD19^+$ Z-138 cells at various E:T ratios for 4 h, the supernatant was collected, and the levels of human TNF-α and IFN-γ were measured using an ELISA MAX Deluxe Set Human Kit (BioLegend, San Diego, CA) according to the manufacturer's instructions. Absorbance was read at 450 and 570 nm using VICTOR Nivo multimode plate reader (PerkinElmer, Waltham, MA).

Persistence of the manufactured CAR-T cells in patients

Peripheral blood was collected from patients at various times—for example, 1, 7, 14, and 21 days, and 1, 2, 3, and 6 months ± 7 days after CAR-T cell infusion. The persistence of CAR-T cells in patients was analyzed by flow cytometry using antigen-based detection of anti-CD19 CAR, as mentioned above, as this method allows the detection of CAR-T cell survival and immunophenotyping when co-stained with 7-AAD and other specific antibodies. Upon the completion of the staining process, red blood cells were lysed using 1X BD FACS Lysing Solution (BD Biosciences) and analyzed by MACSQuant Analyzer 10 (Miltenyi Biotec).

Statistical analyses

Statistical analyses were conducted using GraphPad Prism software 8.0. An unpaired, two-sided Student's t test was used for two-group comparisons. One-way ANOVA with Tukey's multiple comparison test was used for multiple comparisons. The data represent mean ± SD from three or more independent experiments. $p < 0.05$ is considered statistically significant.

DATA AND CODE AVAILABILITY

The authors confirm that the data supporting the findings of this study are present within the article and its supplemental information. All other data are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

S.L. and S.I. conceived and designed the study. S.L., P.K., M.J., S.C., Y.U., and T.W. performed the experiments. S.K., W.O., C.K., N.D., and S.I. enrolled the subjects and collected the data. S.L., P.K., M.J., S.K., W.O., and C.K. performed the data analysis. S.L. interpreted the data. S.I., J.K., and P.P. supervised the procedures. S.I. provided the resources and supervised the study. S.L. and S.K. coordinated the project. S.L. wrote the manuscript. S.I. edited the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

All authors declare no competing interests. The research was conducted in the absence of any commercial, proprietary, or financial relationships that could be construed as a potential conflict of interest. All authors and their immediate family members have not been affiliated or associated with Miltenyi Biotec or any other related companies.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omton.2024.200889.

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