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Efficient Chimeric Antigen Receptor T-Cell Generation Starting with Leukoreduction System Chambers of Thrombocyte Apheresis Sets

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Keywords
Leukoreduction system chamber · T cell · Chimeric antigen receptor T cell · Cell therapy

Abstract
Introduction: Primary human blood cells represent an essential model system to study physiology and disease. However, human blood is a limited resource. During healthy donor plateletpheresis, the leukoreduction system chamber (LRSC) reduces the leukocyte amount within the subsequent platelet concentrate through saturated, fluidized, particle bed filtration technology. Normally, the LRSC is discarded after apheresis is completed. Compared to peripheral blood, LRSC yields 10-fold mononuclear cell concentration. ***Methods:*** To explore if those retained leukocytes are attractive for research purposes, we isolated CD3+ T cells from the usually discarded LRSCs via density gradient centrifugation in order to manufacture CD19-targeted chimeric antigen receptor (CAR) T cells. ***Results:*** Immunophenotypic characterization revealed viable and normal CD4+ and CD8+ T-cell populations within LRSC, with low CD19+ B cell counts. Magnetic-activated cell sorting (MACS) purified CD3+ T cells were transduced with CD19 CAR-encoding lentiviral self-inactivating vectors using concentrated viral supernatants. Robust CD19 CAR cell surface expression on transduced T cells was confirmed by flow cytometry. CD19 CAR T cells were further enriched through anti-CAR MACS, yielding 80% CAR+ T-cell populations. In vitro CAR T cell expansion to clinically relevant numbers was achieved. To prove functionality, CAR T cells were co-incubated with the human CD19+ B cell precursor leukemia cell line Nalm6. Compared to unmodified T cells, CD19 CAR T cells effectively eradicated Nalm6 cells. ***Conclusion:*** Taken together, we can show that lymphocytes isolated from LRSCs of plateletpheresis sets can be efficiently used for the generation of functional CAR T cells for experimental purposes.

Introduction

The scientific examination of primary human cells and the analysis of the data obtained have become an important diagnostic and experimental tool in medical research fields. In order to understand the underlying biological processes of human cell physiology as well as the associated pathologies, it is essential to study primary human material. However, human blood is a limited resource. Human blood cells are usually derived from

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blood drawn from patients or healthy donors through peripheral vein puncture and blood collection. So-called buffy coats represent another source of normal blood cells [1]. This byproduct of about 60 mL remains during the standard production process of erythrocyte concentrate and fresh frozen plasma. The buffy coat is the fraction of uncoagulated blood, which contains most of the white blood cells and platelets. These cells aggregate into a thin layer suspended between the red blood cells and the supernatant plasma during density gradient centrifugation of whole blood donations [2]. Routinely, buffy coats are pooled for thrombocyte concentrate production. Alternatively, they are widely used for research purposes. However, there is limited access due to blood shortage. In our study, we used so-called leukoreduction system chambers (LRSCs), a side product of thrombocyte apheresis collection runs [3], to isolate and validate human primary T cells for research purposes. In contrast to buffy coats, LRSCs yet are not licensed for clinical blood product supply. Similar to buffy coat donors, informed consent is obtained from platelet donors before thrombocyte apheresis. All donors are screened to ensure good health condition and allow compliance with biosafety regulations in the use of otherwise untested samples. Furthermore, blood products are not collected from high-risk populations for blood-borne infections and are tested for viral and bacterial infectious diseases caused by human immunodeficiency virus, hepatitis B virus, hepatitis C virus, *Treponema pallidum*, and West Nile virus [4]. During plateletpheresis, the LRSC reduces the leukocyte amount within the subsequent platelet concentrate by using saturated, fluidized, particle bed filtration technology [5]. As adoptive cell therapies have gained significant importance in the past decades, and chimeric antigen receptor (CAR) T-cell therapy has proven successful in treating certain relapsed and refractory hematological malignancies [6, 7], exploration of new targets, improvement of manufacturing, and novel model systems have become a main area of research. Here, we isolated human T cells from the usually discarded LRSCs with the aim of manufacturing functional human CAR T cells. As the LRSC can be removed under sterile conditions upon apheresis, application of these cell reservoirs could be envisioned for GMP-grade manufacturing of donor-derived T-cell products.

Materials and Methods

Human Cell Lines

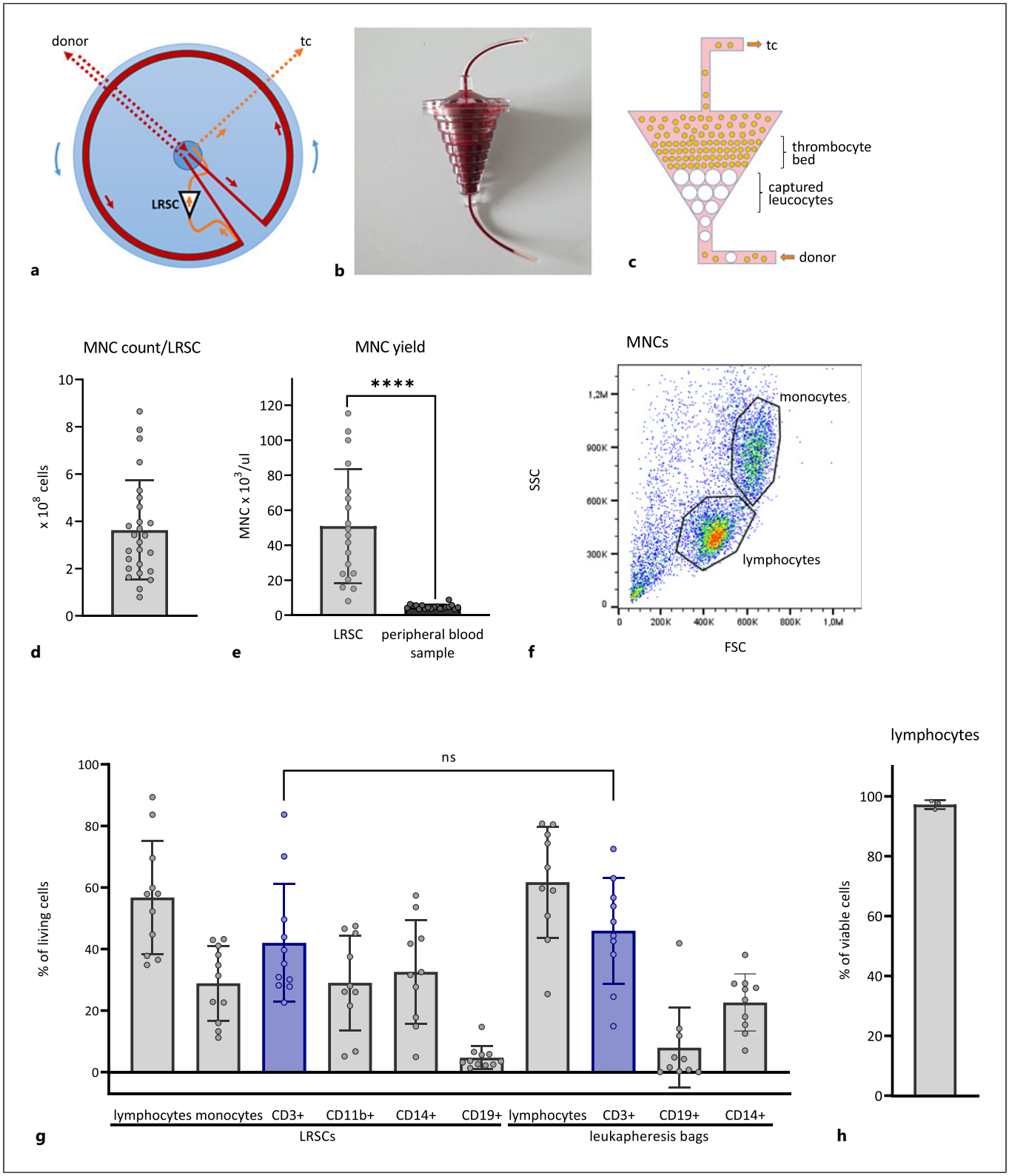
HEK293T cells were cultivated in Dulbecco's modified Eagle medium (Gibco, Carlsbad, USA) supplemented with 10% fetal calf serum (Bio&Sell, Feucht/Nürnberg, Germany), 2% L-glutamine (Bio&Sell, Feucht/Nürnberg, Germany), and 1% penicillin-streptomycin (PAN Biotech, Aidenbach, Germany) in humidified incubators at 37°C and 5% CO_2. Human Nalm6 B cell precursor leukemia cells (ATCC, USA) were cultured in RPMI with 10% fetal calf serum. Cell lines were regularly tested for mycoplasma contamination via polymerase chain reaction. Origin of the cells was validated by short tandem repeat profiling.

CAR Construct, Lentiviral Expression Vector, and Viral Particle Production

The second-generation CAR construct (CTL019-alike CAR [8]) used in this study is composed of a CD8 leader sequence as a signal peptide followed by the FMC63 CD19 single-chain variable fragment for CD19 antigen recognition. The connection to the intracellular domains is mediated by a CD8 hinge domain. The 4-1BB intracellular costimulatory domain, which has been revealed to promote CAR T cell persistency and differentiation towards a central memory phenotype, is followed by the CD3ζ signaling domain in a distal position [9]. For CAR T-cell generation, a LeGO-derived [10] lentiviral expression vector encoding the CD19 CAR (CTL019) and the enhanced green fluorescence protein (EGFP) separated by an internal ribosomal entry site (IRES) element was generated. The final construct was verified by sequencing. For viral particle production, HEK293T cells were transfected using a standardized polyethylenimine transfection protocol [11]. The resulting lentiviral supernatant was collected 48 h post-transfection, filtered through a 0.22 µm filter unit and concentrated through centrifugation at 20,000 rpm at 10°C for 2 h. Resuspended lentiviral particles were directly applied to the isolated activated T cells to induce transduction.

Isolation, Cultivation, and Transduction of Primary Human T Cells

LRSCs were obtained from our thrombocyte apheresis collection center in the Division of Transfusion Medicine, Cell Therapeutics and Haemostaseology, LMU Munich. The Trima apheresis devices were equipped with the Trima Accel software version 7 (Terumo BCT, USA). The leukocyte counts of healthy thrombocyte donors were determined by automated blood cell analysis before donation (Sysmex XN-350 Cell Analyzer). Peripheral blood mononuclear cells (MNCs) were carefully isolated from LRSC (ethics certificate LMU #18-821) of utilized Trima Accel thrombopheresis sets (Terumo BCT) via density gradient centrifugation with Ficoll-Paque Plus (Cytiva, California, USA). After several washing steps with 1×PBS and a red blood cell lysis step with ice-cold distilled water, MNCs were obtained. Such LRSC-derived MNCs were counted via trypan blue staining. T lymphocytes were enriched from the MNCs using a magnetic-activated cell sorting (MACS) PAN T cell isolation kit (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The T lymphocytes were then cultured in RPMI 1640 supplemented with HEPES, GlutaMAX (Gibco, Carlsbad, CA, USA), 10% heat-inactivated human serum (Bio&Sell, Feucht/Nürnberg, Germany), 1% L-glutamine (Bio&Sell), and 1% penicillin-streptomycin (PAN Biotech, Aidenbach, Germany). T cells were activated with anti-CD3/CD28 paramagnetic beads (Dynabeads™; Gibco, Carlsbad, CA, USA) at a 1:2 bead:cell ratio. IL-2 (PeproTech, Cranbury, NJ, USA) was added every other day at a final concentration of 100 IU/mL. One day after T-cell isolation and pre-stimulation, concentrated lentiviral particles from 8 transfected 10 cm HEK293T dishes were resuspended in T-cell media and directly applied to 2×10^5 T cells. Transduction efficiency was determined via flow cytometry by assessing the percentage of EGFP+ cells at day 4 post-transduction. Thereafter, successfully transduced T cells were enriched using MACS technology with biotinylated anti-CD19-CAR (FMC63 idiotype) antibodies and paramagnetic anti-Biotin microbeads (Miltenyi, Bergisch Gladbach, Germany).



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Flow Cytometry

CAR detection was performed with an anti-FMC63 idiotype antibody (Miltenyi). CD3 (OKT3), CD4 (OKT4), CD8 (SK1), CCR7 (G043H7), CD95 (DX2), CD45RA (HI100), CD45RO (UCHL1), Tim3 (F38-2E2), LAG3 (7H2C65), CD25 (BC96), CD69 (FN50), CD14 (M5E2), CD11b, CD19 (HIB19), and the respective isotype control antibodies were purchased from BioLegend (San Diego, CA, USA). Measurement was performed on a Beckman Coulter (Brea, CA, USA) CytoFLEX flow cytometer. Viability was assessed via Zombie dye staining (BioLegend).

Killing Assays

On day 12 after transduction, the enriched CD19 CAR T cells and untransduced control T cells of the same donor were co-cultured with luciferase-expressing CD19+ human Nalm6 cells [12] at different effector:target (E:T) ratios (1:1, 0.5:1, 0.25:1) in a 96-well flat bottom plate. After 24 and 48 h of co-incubation, luminescence was measured.

Statistical Analysis

Statistically significant differences were determined using GraphPad Prism 8.0 with unpaired or paired two-tailed *t* tests and two-way ANOVA followed by Sidak's multiple comparison test. For all statistical analyses, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$, and ns indicates nonsignificant.

Results

Isolation and Characterization of MNCs from LRSCs

As a lymphocyte source for the CAR T-cell manufacturing, we isolated MNCs out of LRSCs derived from utilized thrombopheresis sets of healthy blood donations. Donors for this study were chosen randomly. The LRSC captures the leukocytes of the separated plasma/thrombocyte layer within the centrifuge part of the apheresis device (Fig. 1a–c). Each LRSC was sufficient for one CAR T-cell production process. Sterile sealed LRSCs were taken out of the apheresis sets and further processed under cell culture conditions. Upon density gradient centrifugation of approximately 7.5 mL of residual LRSC blood, an average amount of $3.6 \times 10^8 \pm 2.1 \times 10^8$ MNCs per LRSC were obtained (Fig. 1d). Compared to donor peripheral blood leukocyte counts before thrombopheresis, LRSCs concentrated MNCs up to 10 times resulting in $50.8 \times 10^3 \pm 32.6 \times 10^3$ cells/μL in LRSC versus $4.8 \times 10^3 \pm 1.3 \times 10^3$ cells/μL in the peripheral blood of thrombocyte donors prior apheresis (Fig. 1e). As demonstrated by forward/side scatter profile analyses, lymphocytes and monocytes represented the vast majority of the obtained blood cells (Fig. 1f). Flow cytometry analyses further revealed 42% ± 19% CD3+ cells within LRSC MNC populations, with CD3+ T lymphocytes being the most prominent cell population followed by CD14+ monocytes. The percentage of the CD3+ population in LRSC-derived MNCs from healthy donors ranged similar to CD3+ cell proportions in CAR T leukapheresis bags derived from patients utilized for manufacturing of clinical CAR T products, both with low B cell counts (Fig. 1g). Flow cytometry also showed >95% cell viability of isolated lymphocytes (Fig. 1h).

Immunophenotypical Subset Analysis and Enrichment of LRSC-Derived CD3+ T Cells

Flow cytometry analyses of distinct LRSC samples showed normal human T-cell subsets within the isolated MNCs comprising naïve, central memory, stem cell memory, effector memory, and terminally differentiated effector memory T cells (Fig. 2a). The T cells displayed typical CD4 and CD8 subpopulations [13] with a CD4/CD8 ratio of 3.2 ± 1.6 (Fig. 2b). Analysis of memory phenotype properties based on CD45RA, CCR7, and CD95 surface expression revealed mostly naïve T cells (Fig. 2c). Additional characterization showed low expression of exhaustion and activation markers of LRSC-derived CD3+ T cells (Fig. 2d). Of note, LRSC CD3+ cells contained 8 +/− 5.1% γδT cells, a promising cell source for allogenic CAR T cells [14] (Fig. 2e). The MNC CD3+ cell fraction could be further enriched by MACS via a negative selection resulting in a pure CD3+ T-cell population (>95%) (Fig. 2f, g).

Lentiviral Vector Transduction of LRSC-Derived Human CD3+ T Cells and Functional Validation of the Subsequent Anti-CD19 CAR T Cells

For lentiviral transduction, we used a self-inactivating lentiviral vector expressing a CTL019-alike CAR [8]. CTL019, the first CAR T-cell therapy approved by the FDA in 2017, represents a second generation CAR with 4-1BB as costimulatory domain. Within the LeGO backbone, expression of the CD19 CAR construct is driven by the spleen focus-forming virus promoter [10] (Fig. 3a). The vector backbone also includes an IRES element for co-expression of EGFP, allowing convenient detection of transduced cells by flow cytometry. For transduction, concentrated lentiviral particles were used. Via flow cytometry, we verified stable expression of the CD19-CAR-IRES-EGFP cassette through EGFP and CD19 CAR surface marker detection (Fig. 3b). EGFP expression is correlated to CAR surface expression, with EGFP high expressors emitting high CD19 CAR signals and vice versa (Fig. 3c). In order to achieve uniform CAR T populations, CD19 CAR T cells were purified via

Fig. 1. Peripheral blood mononuclear cells (MNCs) isolation from leukoreduction system chamber (LRSC) and live gate cell type distribution analysis. **a** Schematic top view of the centrifuge part within the apheresis device. Red and orange arrows indicate direction of blood flow within the centrifuge part of the apheresis device. **b** LRSC attained after routine thrombopheresis procedure. **c** Simplified illustration of the principle behind MNC entrapment in LRSCs. **d** LRSC MNC counts of trypan blue stained MNCs upon Ficoll isolation. **e** Comparison between LRSC and peripheral blood-derived MNC yields of thrombocyte donors. Statistically significant differences were detected by ratio-paired two-tailed *t* test. **f** FSC/SSC scatter profile of LRSC-derived MNCs. **g** Cell subtype distribution of LRSC-derived MNCs compared to CD3+ and CD19+ lymphocytes and CD14+ monocytes in CAR T leukapheresis bags. Statistically significant differences were detected by unpaired *t* test. **h** Viability of lymphocytes upon isolation from LRSC. Tc, thrombocyte concentrate; FSC, forward scatter; SSC, side scatter. ****$p < 0.0001$. Each data point represents results derived from cells of a single LRSC.

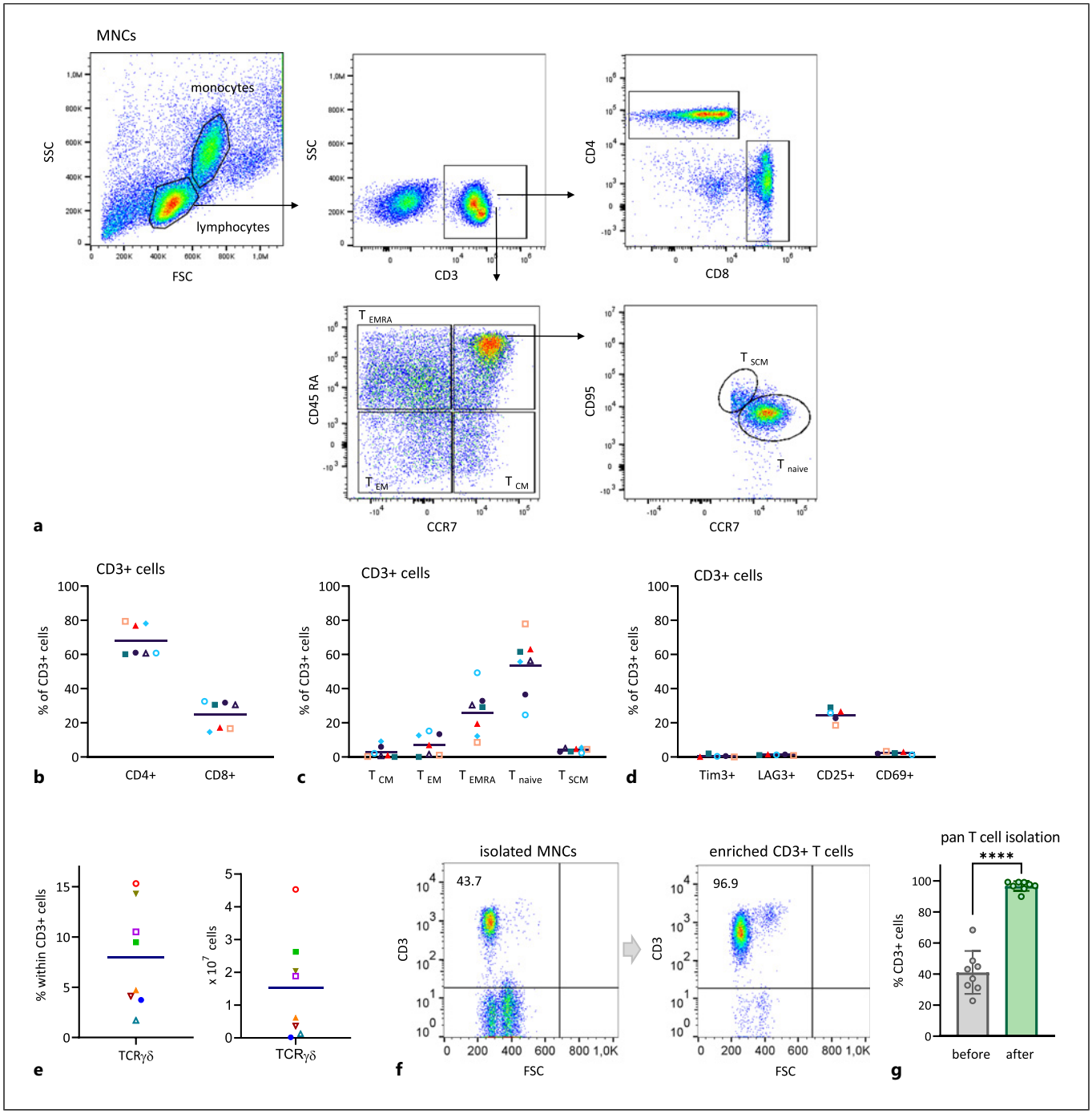

Fig. 2. Characterization and enrichment of LRSC-derived CD3+ T cells. **a** Representative gating strategy for CD3+ T lymphocyte memory subsets: T_{EMRA} $CD45RA^+CCR7^-$, T_{EM} $CD45RA^-CCR7^-$, T_{CM} $CD45RA^-CCR7^+$ and $CD45RA^+CCR7^+$, T_{SCM} $CD45RA^+CCR7^+CD95^+$, and $T_{naïve}$ $CD45RA^+CCR7^+CD95^-$. **b**, **c** CD4+/CD8+ T lymphocyte proportion and memory subsets within the CD3+ population. Each symbol represents one donor. **d** Exhaustion and activation marker expression levels within the CD3+ population. Each symbol represents one donor. **e** Percentage of γδT cells in LRSC-derived CD3+ cells (left) and absolute γδT cell numbers per LRSC (right). **f** Exemplary enrichment of CD3+ cells via the PAN T cell isolation kit. **g** MACS CD3+ cell selection resulted in >95% CD3+ T lymphocyte populations. T_{EMRA}, terminally differentiated effector memory; T_{EM}, effector memory; T_{CM}, central memory; T_{SCM}, stem cell memory; TCRγδ, γδT cells. Statistically significant differences were detected by paired two-tailed *t* test, $^{****}p < 0.0001$. Each data point represents results derived from cells of a single LRSC.

MACS with FMC63 paramagnetic beads. This enrichment resulted in 80% CD19 CAR expressing CD3+ T cells (Fig. 3d, e). Starting with 2×10^5 cells, in vitro expansion to clinically relevant CD19 CAR T cell numbers could be achieved in media containing IL-2 and anti-CD3/CD28 beads (Fig. 3f). Transduction efficiency remained unaltered

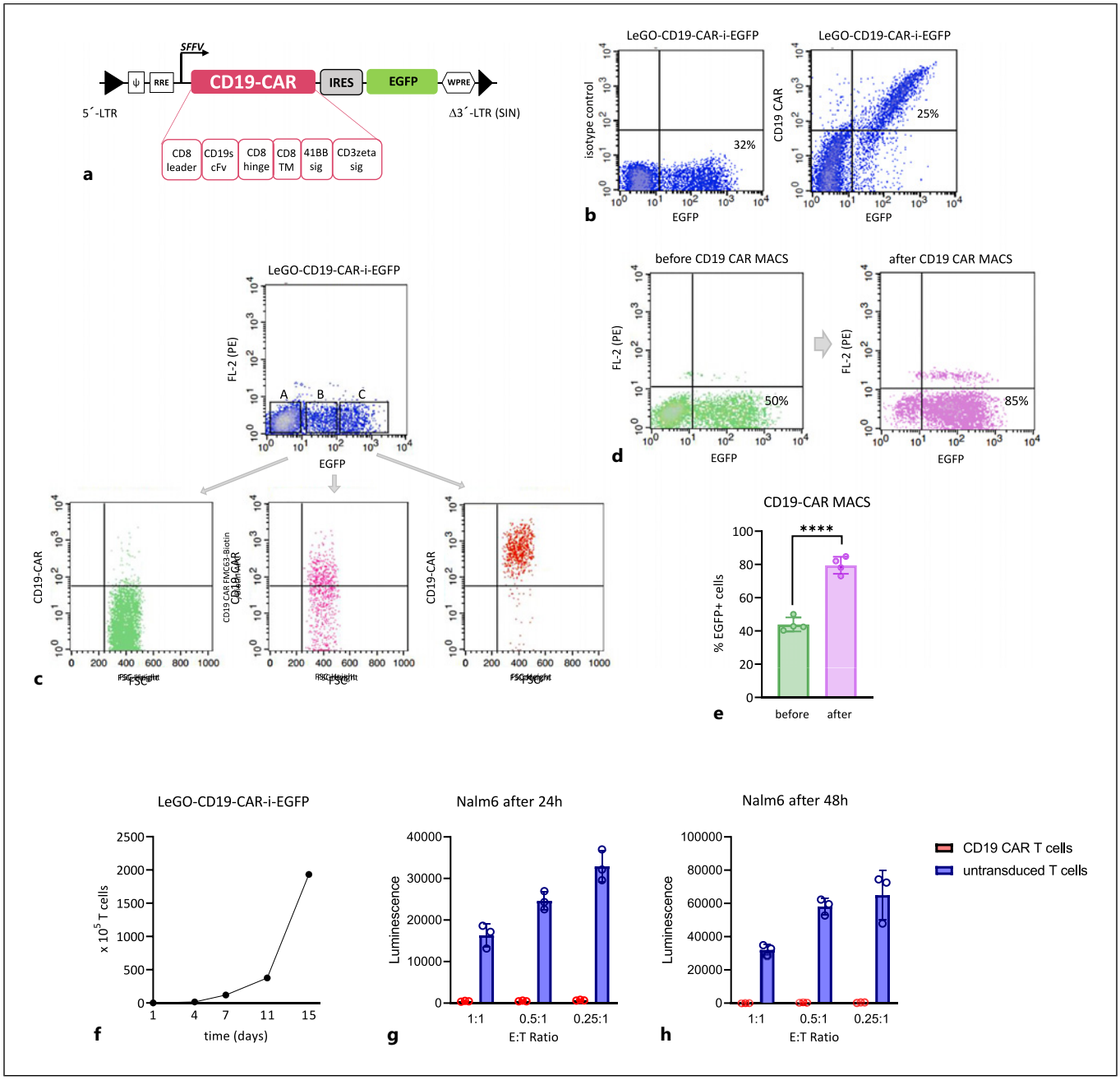

Fig. 3. Lentiviral transduction, CAR expression, expansion, and killing activity of LRSC-manufactured CAR T cells. **a** Scheme of the self-inactivating lentiviral vector encoding the CD19 CAR and EGFP separated by an IRES sequence. **b**, **c** Primary human T cells isolated from LRSCs were transduced with LeGO-CD19-CAR-i-EGFP lentiviral vectors. Transduced T cells were stained with CD19-CAR FMC63-Biotin/Biotin-PE and assessed by flow cytometry. **d** MACS separation allowed for enrichment of CD19-CAR-i-EGFP-expressing T cells. **e** Percentages of EGFP-expressing T cells before and after MACS. Data represent mean ± SD from 4 different donors. Statistically significant differences were detected by paired two-tailed *t* test. **f** Dynabead-aided expansion of transduced T cells in IL-2 rich media over the span of 15 days resulting in up to 2×10^8 cells. The data show representative results obtained from three independent biological replicates. **g**, **h** Cell-associated luminescence level assessment of 24 and 48 h co-incubated transduced and enriched CAR T cells and luciferase-expressing Nalm6 target cells at different effector:target (E:T) ratios. The data show representative results obtained from three independent biological replicates. SFFV, spleen focus-forming virus promoter; IRES, internal ribosomal entry site; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; LTR, long terminal repeat; ScFv, single-chain fragment variable; TM, transmembrane; sig, signaling.

over expansion time (data not shown). Finally, to prove the CAR T cells' functionality in cytotoxicity studies, we demonstrated effective killing of human CD19+ Nalm6 cells at low effector:target cell ratios upon co-incubation with LRSC-derived CD19 CAR T cells. Untransduced T cells served as control cells (Fig. 3g, h).

Discussion

Human primary blood cells are highly requested for experimental studies in various research areas. There are a few established methods to obtain immune cells from buffy coats or direct blood samples. Here, we present a further source of viable and functional leukocyte populations, highly suitable for experimental CAR T-cell research approaches. LRSCs represent an easily accessible and sterile source of donor-derived MNCs, which are normally discarded after the plateletpheresis [15], and compared to buffy coats are often provided for free. MNCs from LRSCs provide high amounts of lymphocytes that can be further enriched via MACS purification to obtain pure CD3+ T-cell populations.

Other groups have already characterized viability and composition of LRSC-derived cells [3, 16, 17] and showed the principal feasibility for the generation of gene-modified cells [18–20]. In our study, we could show that isolated LRSC CD3+ T cells represent a very useful starting population for the generation of human CAR T cells for research purposes. The initial viability of CD3+ T cells ranged >95%. Lentiviral transduction and MACS purification resulted in 80% CAR-expressing T cells with robust killing efficiency measured via bioluminescence measurements. The convenient procedure allowed the rapid generation of clinically relevant CAR T cell numbers. CAR T companies usually provide around 3×10^8 CAR T cells for infusion into patients as single dosage [21]. Additionally, the here obtained cells can be used for a variety of experimental approaches including refinement of CAR T cell freezing/thawing protocols, CAR T vector optimization, improvement of the CAR T-cell expansion process, and in vivo CAR T xenograft mouse experiments. Moreover, thrombocyte donors normally are HLA class I and II characterized [22]. Therefore, LRSCs of thrombocyte donors are also an attractive source to study CAR and TCR-engineered T cells in the context of HLA expression and function.

In clinical routine, the manufacture of CAR T cells relies on apheresis products with leukapheresis bags representing the only established starting material. In a single case, B-cell contamination has been suggested to bear side effects for CD19 CAR T production [23, 24]. Compared to clinical grade leukapheresis bags, CD19+ B-cell populations were found not increased in LRSC-derived MNCs. Upon sterile sealing of the LRSC from the apheresis set, application under GMP conditions might also become feasible soon. Of note, viability of LRSC-derived cells was recently shown to be superior to standard apheresis collection methods [3]. It can be envisioned, that such LRSCs, enlarged for greater volumes, can also serve for collection of lymphocytes for clinical CAR T-cell production in the future; however, compatibility for further blood product processing, e.g., sterile docking to other bags, has yet to be developed. As allogeneic, "off-the-shelf" CAR T cells gain more attention, LRSC could also represent an economic surrogate source for the manufacture and study of such CAR T cells.

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Statement of Ethics

Human peripheral blood mononuclear cells were derived from LRSCs according to ethics certificate LMU #18-821.

Conflict of Interest Statement

The authors confirm that none of the material has been published or is under consideration elsewhere. S.K. has received honoraria from TCR2 Inc., Novartis, BMS, and GSK SK and is inventor of several patents in the field of immuno-oncology. S.K. received license fees from TCR2 Inc. and Carina Biotech. S.K. received research support from TCR2 Inc., Tabby Therapeutics, Plectonic GmbH, and Arcus Bioscience for work unrelated to the manuscript. A.G. received research support from Tabby Therapeutics. The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Author Contributions

The experiments were conceived and designed by Christian Wichmann, Linping Chen-Wichmann, Sebastian Kobold, and Christian Kellner. Stefani Xhaxho, Sophie Kreissig, Roland

Windisch, Adrian Gottschlich, and Irmgard Kohler performed the experiments. Essential advice and materials were provided by Andreas Humpe. Christian Wichmann and Stefani Xhaxho wrote the manuscript. All authors have approved the final version of the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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