Supporting Information

Scalable and Ultrafast CAR-T Cell Production Using Microfluidics

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1. Materials

Polydimethylsiloxane (PDMS, silicone elastomer kit SYLGARD™ 184 from Dow Corning, USA), which consists of two components: a silicone elastomer base (PDMS base) and a curing agent, was used to fabricate microfluidic devices. Glass coverslips (EUROTUBO® slides, D100002) were used as substrates.

2. Fabrication of microfluidic chips

The master molds with the developed geometry were printed from photopolymer resin (Eryone, China) using a Phrozen Sonic Mini 4K 3D printer. The microfluidic devices were fabricated using the "soft lithography" method. For this, polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, USA) was mixed in the ratio of curing agent to polymer base equal to 1:10. The obtained mixture was poured into the plastic master molds and cured at 80 °C for 2 h. Then, the obtained PDMS replicas were sealed on a glass substrate pre-treated with oxygen plasma in order to activate the surface.

The first obtained microfluidic device consists of a single serpentine-like channel, which acted as the incubation area with effective volume ranges from 8.3 µl to 33 µl with the heights of the channel of 50, 100, 150, and 200 µm.

The second topology consists of a single channel and an enhanced incubation area with effective volume 120 µl and channel height 150 µm, and with pillars in the center of the area for easier and simpler fabrication of the microfluidic device during plasma treatment of the replica and glass.

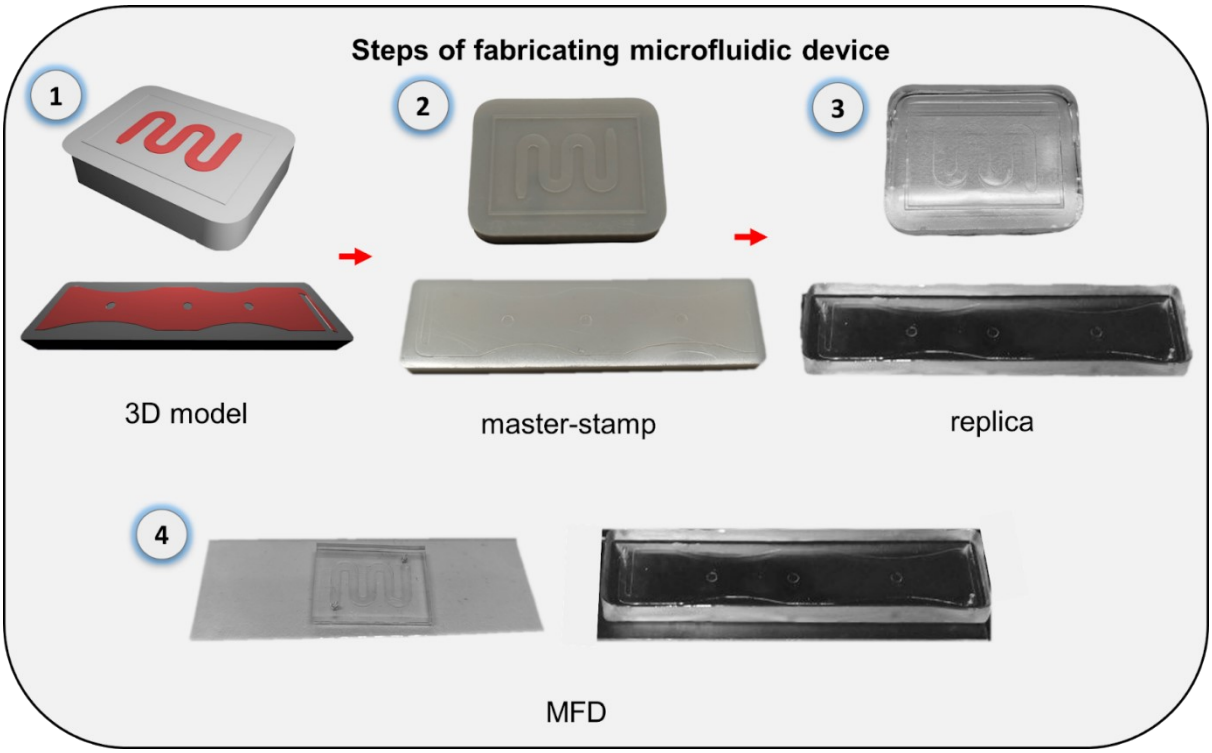

Figure S1. Scheme of the main steps of the microfluidic device fabrication.

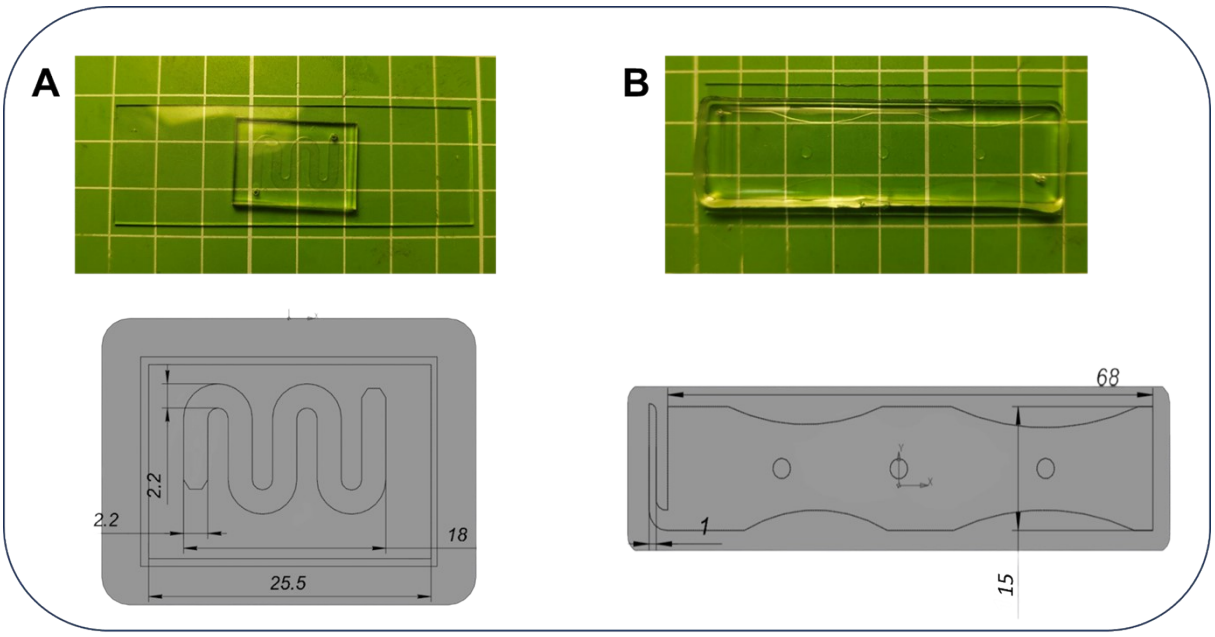

Figure S2. Schemes of microfluidic devices. A. Snake-like geometry with linear parameters. B. Increased incubation area geometry with linear parameters.

3. Variation of the geometrical parameters for enhanced microfluidic transduction

To investigate the geometric properties of the transduction, we varied the heights of the microfluidic device in order to achieve the optimal height to perform the higher efficiency of transduction.

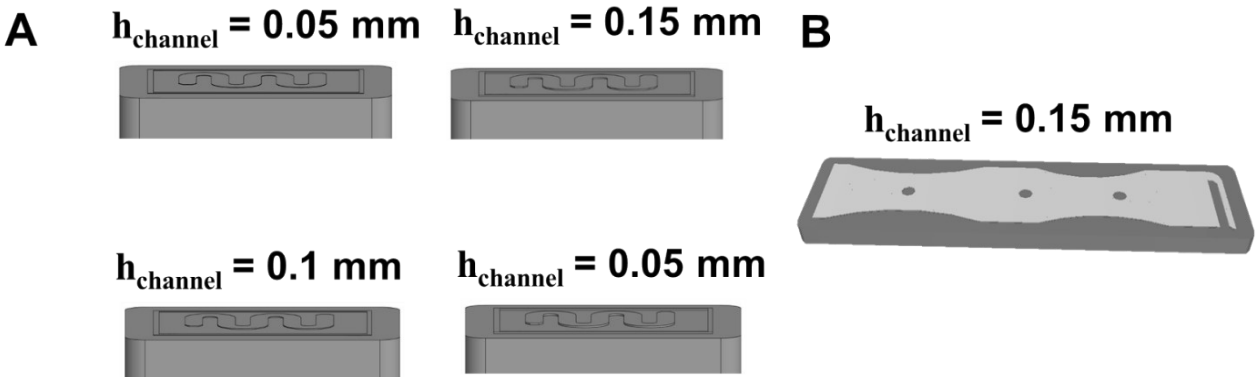

Figure S3. Variations of the heights and geometry.

4. Dependence of transduction efficiency on height

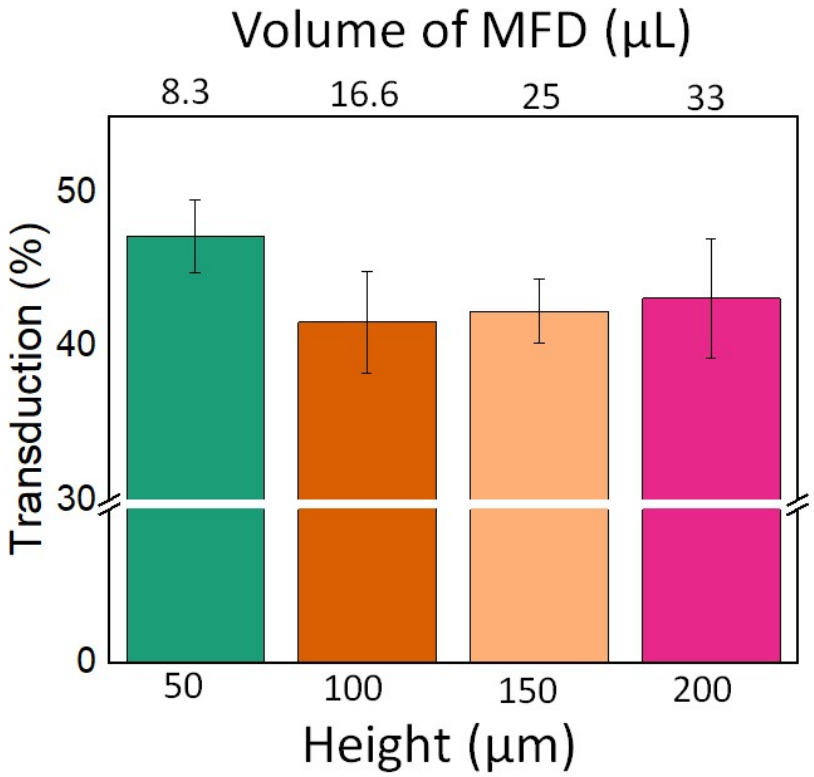

Figure S4. Efficiency of transduction according to the different heights in the MFD.

5. Gating strategy

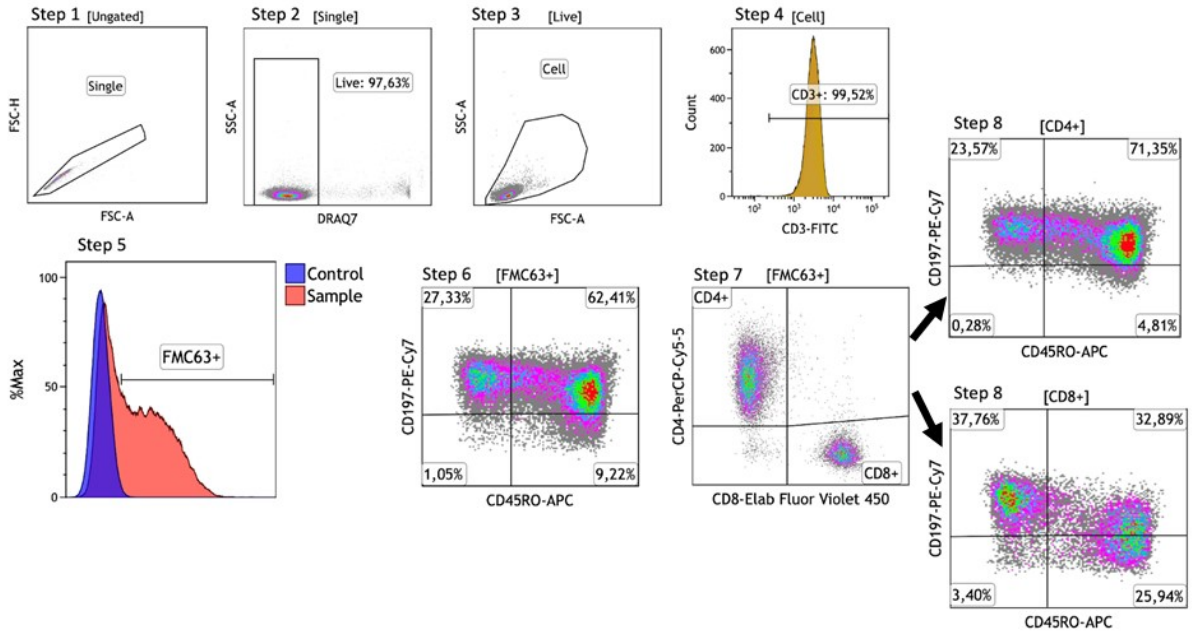

Figure S5. Gating strategy for CAR-T assessment.

Gating strategy included several steps:

1. *Singlet Selection*: using Forward Scatter Height (FSC-H) and Forward Scatter Area (FSC-A) parameters, we identified and selected single cells, while excluding doublets or clumps
2. *Alive Cell Identification*: from gated singlets, we have selected and calculated the percentage of alive cells using the dye DRAQ7
3. *Lymphocyte Identification*: we used Side Scatter Area (SSC-A) and FSC-A to further refine the population to lymphocytes, ensuring that only relevant cell types are analyzed.
4. *CD3+ Cell Identification*: we gated CD3+ cells in the histogram to focus on T-cells specifically
5. *CAR-T Cell Analysis*: from the gated CD3+ population, we calculated the percentage of FMC63+ cells, which represent CAR-T cells.
6. *CD4+ and CD8+ T-cell Evaluation:* we evaluated the percentages of CD4+ and CD8+ T-cell within the total CD3+ population. This differentiation is essential for understanding the functional capacity of the CAR-T cell product.

7- 8. *Subpopulation Assessment*: We analyzed memory subpopulations based on CCR7 (CD197) and CD45RO markers. This step provides insights into the differentiation status of T-cells, identifying naïve, central memory, effector memory, and terminally differentiated subsets.

6. CAR-T cells characterization

T-cell donors and CAR-T cell manufacturing

CAR-T cells were manufactured using T-cells obtained from two healthy donors following the ultrafast protocol. The cryopreserved CAR-T cells were thawed and cultured in a standard medium for 72 hours to assess their functionality post-thaw. After this incubation period, the transduction efficiency was re-evaluated using flow cytometry, yielding transduction levels of 28% and 6.9% for the two donors.

Cytotoxicity assay

For the cytotoxicity assay, 50×10³ CAR-T cells were co-cultured with 200×10³ NALM-6 tumor cells (a B-cell acute lymphoblastic leukemia cell line) in a total volume of medium suitable for a 72-hour incubation period. Following the initial 72-hour co-culture, a second round of cytotoxicity testing was performed by adding another 200×10³ NALM-6 cells and culturing the mixture for an additional 72 hours.

Two control groups were included to validate the results:

1. NALM-6 only: Cultures containing only NALM-6 tumor cells without the addition of CAR-T cells.
2. NALM-6 + T-cells: Cultures containing NALM-6 tumor cells mixed with non-transduced T-cells.

Flow cytometry and cell counting

After the second round of cytotoxicity testing, total cell counts were determined, and flow cytometry was performed to evaluate the proportion of tumor cells and CAR-T cells in the cultures. The proliferation of CAR-T cells and the residual tumor burden were assessed based on these measurements.

After two rounds of cytotoxicity, the number of CAR-T cells increased from 50×10^3 to $460\times10^3 \pm 488$. While the proportion of tumor cells in the total cellular composition was less than 1% **(Figure S7)**, which corresponds to $20\times10^3 \pm 5.66$. In the control groups, where only NALM-6 or NALM-6 + T-cells were cultured, all cells were tumor lineage at the time of evaluation, and the cell counts were 3354×10^3 and 4125×10^3, respectively. Thus, the *in vitro* experiment demonstrates that CAR-T produced using ultrafast technology characterized by high proliferative activity and high antitumor cytotoxicity.

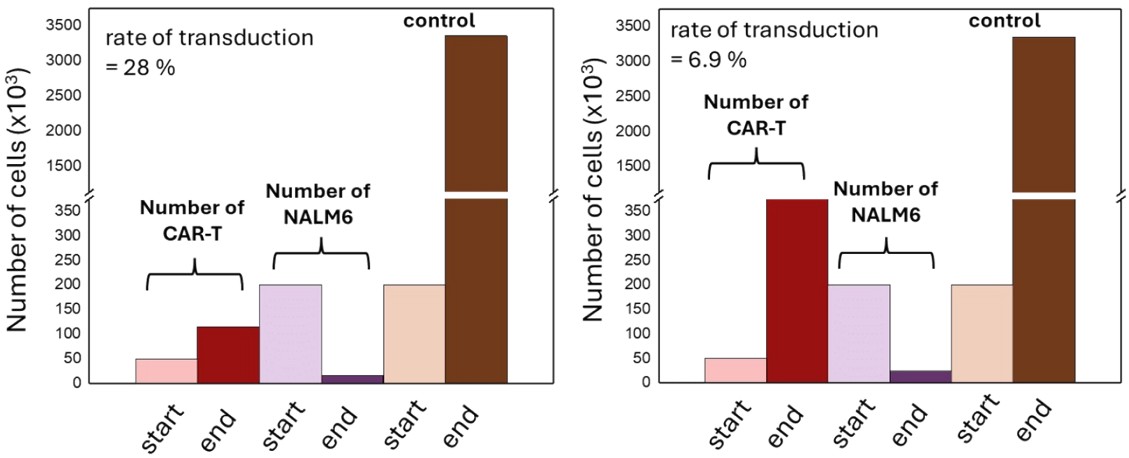

Figure S6. Cell counts at the beginning and end of a series of experiments with two donors.

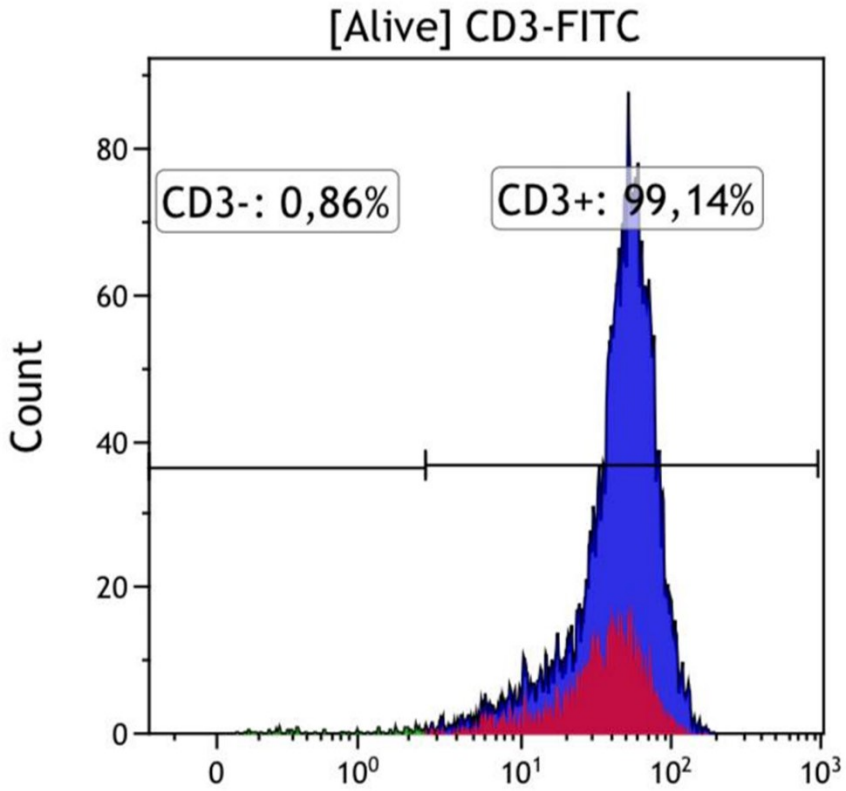

Figure S7. Number of CD3+ T-cells at the end of the experiment with NALM6.

7. Phenomenon of pseudotransduction

The **Figure S8** shows the result of the evaluation of the transduction level using flow cytometry on day 1 and day 9+ after transduction. However, the high level of transduction is attributable to pseudotransduction.

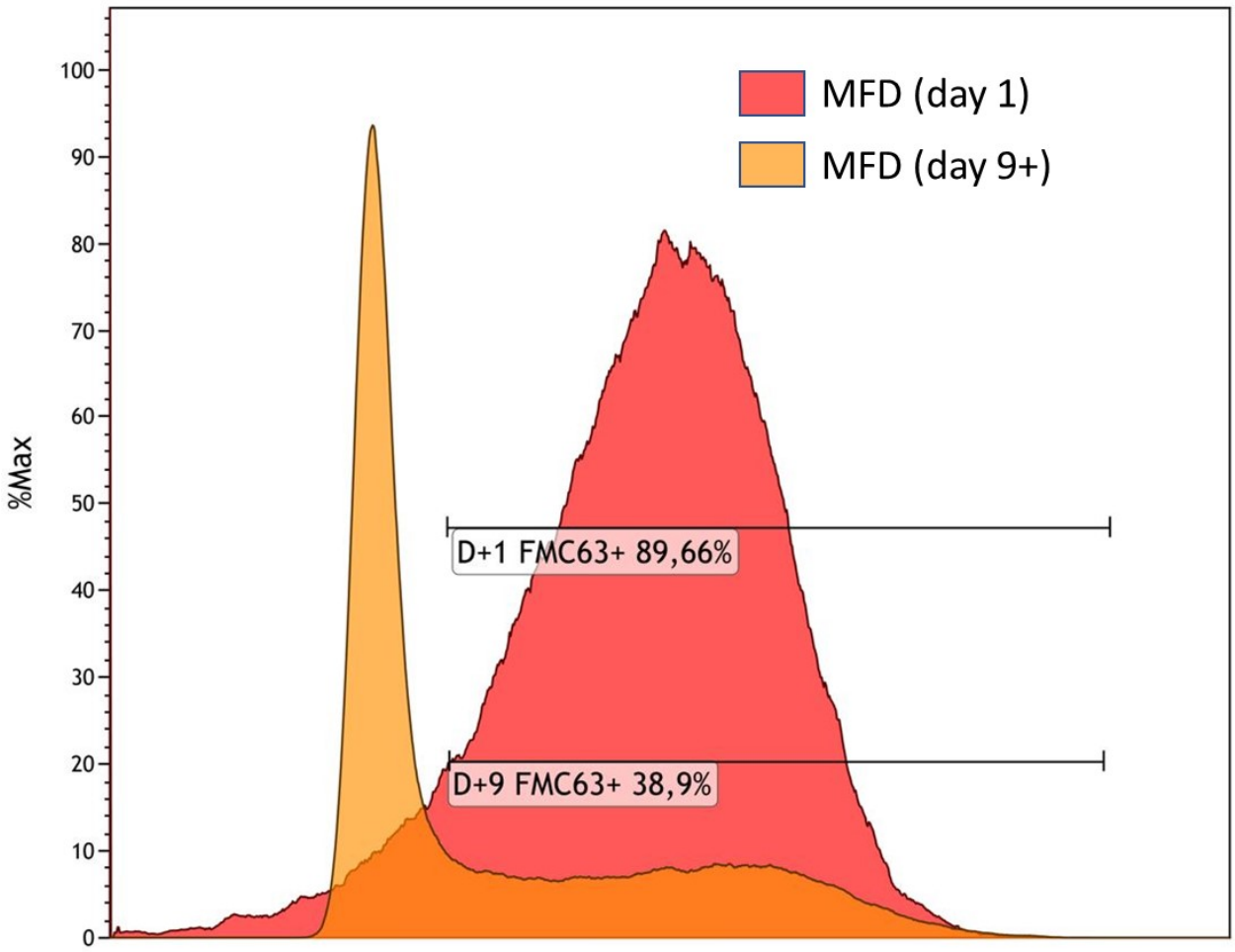

Figure S8. Transduction efficiency of T-cells incubated in the MFD on Day 1 and Day 9+.