Subcutaneous injection of total nucleated cells rapidly isolated following four-hour peripheral whole blood exposure to CD3-directed CAR-T lentiviruses with a synthetic driver results in robust CAR-T proliferation and anti-tumor immunity

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Key Messages

- Here we describe a novel approach for the rapid isolation of total nucleated cells (TNC) reverse eluted off a leukocyte reduction filter from whole blood exposed for four hours to CD3-directed CAR-T Lentiviruses (LV)
- CD3-directed CAR-T LVs induce TCR internalization (CD3 Dimming) in T cells including Naïve and Naïve-derived T cells present within the isolated TNCs, and mediate *in vitro* T cell growth, transgene expression, and function
- Following subcutaneous injection (SC) of TNC, *in vivo* expansion of CAR-T cells first occurs locally at the site of injection and then expands into the systemic circulation and distal tumor sites
- Subcutaneous (SC) injections of CD19 CAR-T LV-loaded TNCs induced significant anti-tumor immunity equivalent to CD19 CAR-T LV-loaded PBMCs isolated from whole blood
- By leveraging this technology, we can develop a rapid, scalable, and cost-effective process for the development of a rapid point-of-care (rPOC) subcutaneous CAR-T therapy

Abstract

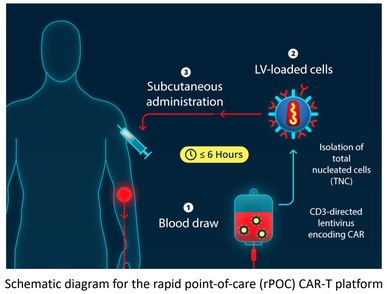
Background: Adoptive cell therapies using Chimeric Antigen Receptors (CARs) show durable clinical benefit for patients with hematologic malignancies, however, challenges remain for enabling this personalized treatment to be delivered in a timely, cost effective, and logistically friendly manner.

Methods: Lentiviral vectors (LV) encoding CD19 and CD22 CARs with a synthetic driver element were packaged with a VSV-G envelope designed with the capability to target and activate $CD3^{pos}$ T cells in whole blood. LV were directly reconstituted in peripheral whole blood for four hours and total nucleated cells (TNC) or peripheral blood mononuclear cells (PBMC) were rapidly isolated utilizing two different closed systems. Immediately following the four-hour exposure event, isolated TNC or PBMC were injected subcutaneously into mice with disseminated Raji luciferase tumor cells. For further characterization, LV-exposed TNC and PBMC were cultured *in vitro* for six days and functionally examined.

Results: Following four-hour exposure to CD3-directed LVs, >90% of T cells, including naïve/naïve-derived ($CCR7^{pos}$ $CD45RO^{neg}$) and central memory ($CCR7^{pos}$ $CD45RO^{pos}$) T cells present within isolated TNC or PBMC exhibited a significant decrease in CD3 surface expression. Subcutaneous injection of gene modified TNC or PBMC resulted in the *in vivo* generation and expansion of large numbers of circulating CAR-T positive cells with complete eradication of disseminated Raji tumors. In parallel cell culture experiments, TNC or PBMC isolated following four-hour LV whole blood exposure exhibited robust expansion without additional T cell receptor (TCR) or CD3 stimulation, while TNC or PBMC not exposed to virus did not show any expansion. Following six days in culture, immunophenotyping by flow cytometry demonstrated that >90% of the cells were $CD8^{pos}$ and $CD4^{pos}$ T cells with CAR-T expression present on central memory ($CCR7^{pos}$ $CD45RO^{pos}$) and effector memory ($CCR7^{neg}$ $CD45RO^{pos}$) T cells. CAR-T antigen specificity to CD19 and CD22 was measured by IFN-gamma release co-culture assays.

Conclusion: We conclude that large numbers of functionally active CAR-T positive cells can be generated both *in vitro* and *in vivo* following a four-hour peripheral whole blood exposure to CD3-directed LVs encoding for CARs with a synthetic driver element. These results provide the basis for an autologous same-day peripheral blood draw to subcutaneous injection rapid point-of-care (rPOC) approach.

Rapid Point-of-Care Platform



Schematic diagram for the rapid point-of-care (rPOC) CAR-T platform

1. Anticoagulated peripheral blood is reconstituted with CAR-T LV product for four hours
2. Blood is filtered through a leukocyte reduction filter to rapidly isolate total nucleated cells (TNC)
3. Reverse eluted LV loaded TNC cells from the filter are prepared for subcutaneous injection (vein to SC product in less than six hours)

Results

Figure 1. Isolation of Total Nucleated Cells from Whole Blood

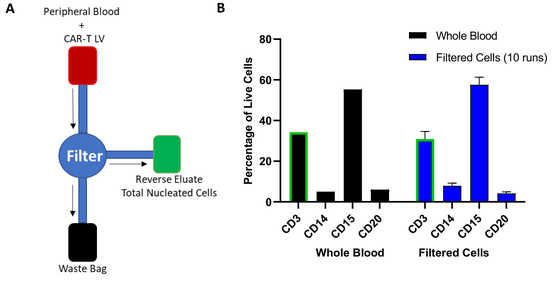


Fig 1: **A**. Leukocyte reduction filter diagram showing direct filtration of cells from whole blood and the reverse elution of total nucleated cells through a bi-directional filter. **B.** Reverse elution of LV-loaded total nucleated cells from whole blood maintains the full composition of leukocytes ($CD3^{pos}$ lymphocytes; CD14 Monocytes; CD15 neutrophils; CD20 B cells) and consistent recovery of $CD3^{pos}$ lymphocytes following 10 independent runs

Figure 2. Change in CD3 and TCR αβ expression after four-hour exposure to CD3-directed LV

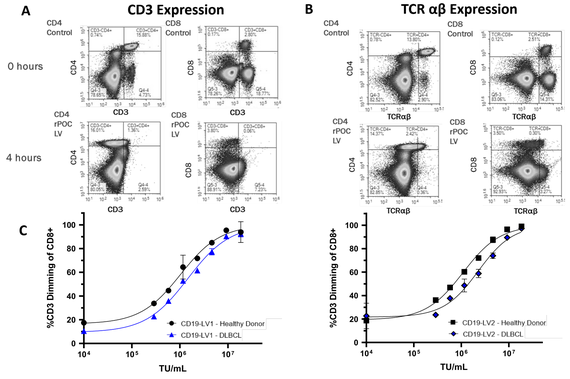


Fig 2: **A**. Flow cytometry demonstrates a decrease in CD3 expression in $CD8^{pos}$ and $CD4^{pos}$ T cells within isolated TNCs following reconstitution of peripheral whole blood with CD3-Directed LV for four hours. **B.** Flow cytometry plot depicting a decrease in TCR αβ expression following reconstitution of peripheral blood with CD3-Directed LV for four hours. **C.** Non-linear curve fit for percentage CD3 decrease (CD3 Dimming) for $CD8^{pos}$ and $CD4^{pos}$ T cells at increasing concentrations (TU/mL) of CD3-directed LV. Data generated from LV reconstitution in peripheral blood from a healthy donor and from a DLBCL patient with two different LV product batches (LV1 and LV2).

Figure 3: CD3-Directed LVs can target Naïve and Naïve-derived T cells

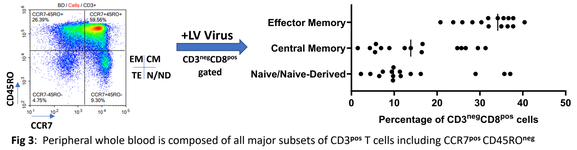


Fig 3: Peripheral whole blood is composed of all major subsets of $CD3^{pos}$ T cells including $CCR7^{pos}$ $CD45RO^{neg}$ Naïve and Naïve Derived (N/ND), $CCR7^{pos}CD45RO^{pos}$ Central Memory (CM), $CCR7^{neg}CD45RO^{pos}$ Effector Memory (EM), and $CCR7^{neg}CD45RO^{neg}$ Terminal Effectors (TE) T cells. Importantly, following CD3-directed LV reconstitution in peripheral blood for four hours, TCR internalization (loss of CD3 expression) is observed equally in all populations of T cells including Naïve/Naïve-derived, Central Memory and Effector Memory T cells

Figure 4: CD3-directed LV can mediate in vitro T cell growth, transgene expression, and function

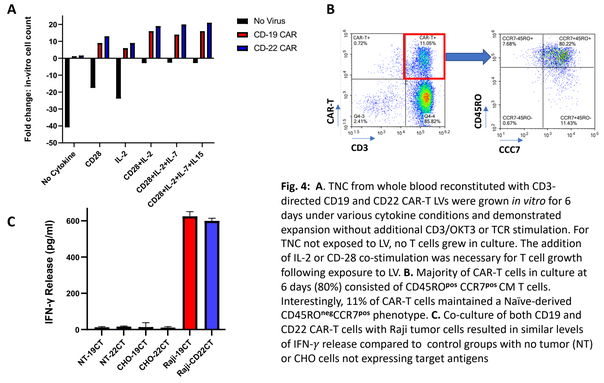


Fig. 4: A. TNC from whole blood reconstituted with CD3-directed CD19 and CD22 CAR-T LVs were grown *in vitro* for 6 days under various cytokine conditions and demonstrated expansion without additional CD3/OKT3 or TCR stimulation. For TNC not exposed to LV, no T cells grew in culture. The addition of IL-2 or CD-28 co-stimulation was necessary for T cell growth following exposure to LV. **B.** Majority of CAR-T cells in culture at 6 days (80%) consisted of $CD45RO^{pos}$ $CCR7^{pos}$ CM T cells. Interestingly, 11% of CAR-T cells maintained a Naïve-derived $CD45RO^{neg}CCR7^{pos}$ phenotype. **C.** Co-culture of both CD19 and CD22 CAR-T cells with Raji tumor cells resulted in similar levels of IFN-γ release compared to control groups with no tumor (NT) or CHO cells not expressing target antigens

Figure 5: Subcutaneous and Systemic Expansion of CAR-T-Luciferase cells

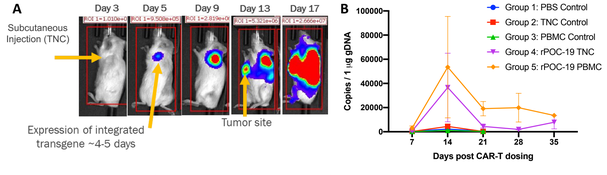


Fig 5: **A.** *In vivo expansion* of CAR-T cells occurs first at the site of subcutaneous injection and then expands systemically into the tumor site on the contralateral side of the mice. An rPOC CD19 LV with luciferase was utilized to track *in vivo* subcutaneous transgene expression and expansion. **B.** Peak CAR-T copies per μg/DNA in peripheral blood were detected at D14 utilizing a PCR-based assay with a primer specific to the rPOC CD19 CAR-T LV. Of note, 5M TNC and PBMC were injected SC with the starting CD3 T cell composition in TNCs being slightly lower than in PBMC.

Figure 6: *In vivo* rPOC TNC vs. PBMC can mediate comparably significant anti-tumor immunity

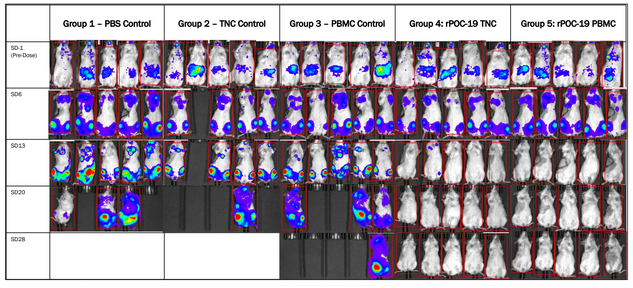


Fig 6: *In vivo* efficacy study for the treatment of systemic disseminated human Burkitt's Lymphoma Models (Raji-luciferase) in B-NDG Mice. Peripheral whole blood was reconstituted with CD3-Directed CD19-CAR-T LVs for four hours and $5x10^6$ TNC (isolated off a leukocyte reduction filter) or $5x10^6$ PBMC (isolated with a ficoll based gradient) were subcutaneously injected into mice. Complete tumor regression was observed in both the TNC and PBMC group by day 20 post injection.

Conclusions

Here we demonstrate:

- A novel, cost-effective, and scalable approach for the *in vivo* generation of CAR-T cells through the rapid isolation of total nucleated cells (TNC) for subcutaneous injection utilizing a bi-directional leukocyte reduction filter from anti-coagulated peripheral whole blood exposed for four hours to CD3-directed CAR-T Lentiviruses (LV)
- CD3-directed CAR-T LVs induce TCR internalization (CD3 dimming) in T cells including Naïve and Naïve-derived T cells present within the isolated TNCs and provide a potent stimulus for initial T cell activation, expansion, and function
- Subcutaneous (SC) injections of CD19 CAR-T LV-loaded TNCs induced significant anti-tumor immunity equivalent to CD19 CAR-T LV-loaded PBMCs isolated form whole blood
- Rapid-point-of-care subcutaneous treatments hold the potential to significantly decrease wait times for therapy and expand patient access to CAR-T therapies

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