Generation of tertiary lymphoid structures and CD3^{pos} CD8^{pos} CD56^{pos} NKG2D^{pos} CAR TaNK cells following subcutaneous injection of CD3-directed lentiviral vector-loaded PBMC

Anirban Kundu[1], Dongming Zhang[1], Frederic Vigant[1], Junyi Zhang[1], Gregory Schreiber[1], Ewa Jaruga-Killeen[1], Alissa R Kerner[1], Michelle Andraza[1], Wei Zhang[1], John Henkelman[1], Renata Soares[1], Gregory I. Frost[1], & Sid P. Kerkar[1]

[1]EXUMA Biotech Corp., 625 N. Flagler St., Suite 625, West Palm Beach, FL 33401

EXUMA Biotech

Abstract# 560

Key Messages

- Subcutaneous (SC) injection of peripheral blood mononuclear cells (PBMC) loaded for 4hrs with CD3-directed lentivector (LV) generate a novel CAR T and NK-like (TaNK) cell capable of enhanced proliferation and target elimination
- Following SC administration, CAR expression and expansion initiates at the site of injection from tertiary lymphoid structures comprised of T cells, macrophages, and dendritic cells
- CAR^{pos} cells formed subcutaneously effectively traffic to the tumor site within 2 weeks post-injection
- From this novel LV CAR and Driver vector platform, CD19 or CD22 CAR^{pos} cells with a T and NK-like phenotype (CD3^{pos}CD8^{pos}CD56^{pos}NKG2D^{pos}) and unique cytokine profile, mediate target elimination with enhanced systemic proliferation and engraftment compared to traditional CD4/8 41BB CAR-Ts administered intravenously into a lymphoreplete animal model
- Through a subcutaneous initiation from TLS, a rapid and scalable process for the development of a CAR-TaNK therapy with reduced cytokine impact and immunosuppression may be clinically feasible

Abstract

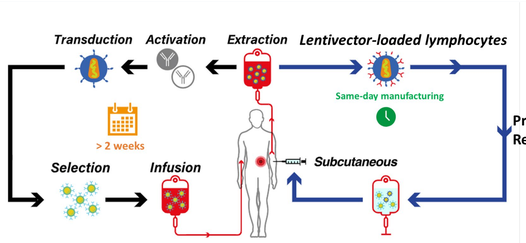
Background: We have previously established the ability of CD3-directed lentiviral vectors encoding for CD19 or CD22 CARs to mediate robust anti-tumor immunity in humanized lymphoreplete mouse models. We now present additional mechanistic data for this novel subcutaneous (SC) CAR-T approach.

Methods: Human PBMCs loaded with a self-inactivating lentiviral vector (LV) encoding CD19 CAR with a synthetic driver element were injected SC into autologous PBMC humanized NSG MHC I/II double knock out (DKO) mice. The LV was packaged with a modified envelope with the ability to target and activate CD3^{pos} T cells. To first track the site of CAR formation in vivo following SC injection, PBMCs were loaded with an LV encoding a CD19 CAR and luciferase, and bioluminescence imaging (BLI) was performed. Additionally, histopathology of the site of injection and distal organs were examined. Evaluation for CAR^{pos} cells was performed through immunohistochemistry and PCR detection. To examine the tropism of the CD3-directed CD19 CAR LV and characterize CAR^{pos} cells, the phenotype of *in vivo* expanded CAR^{pos} cells was evaluated. Further characterization of CAR^{pos} cells was performed with *in vitro* studies.

Results: Following SC injection of LV-loaded PBMC, the first evidence of transgene expression utilizing BLI for luciferase was detected four to five days following SC injection. At the same time point, histological examination of the SC site of injection revealed the formation of tertiary lymphoid structures (TLS) consisting of human CD8^{pos} and CD4^{pos} T cells, CD68^{pos} macrophages, CD68^{pos} dendritic cells, and a few CD20^{pos} B cells. On day 13 post-SC injection, BLI detected the presence of CAR^{pos} cells systemically beyond the site of injection and within subcutaneous Raji tumors implanted on the contralateral side. On day 14 post-SC injection, histologic examination showed sustained TLS within the SC tissue without signs of dermal acute inflammation or ulceration and evidence of CAR^{pos} cells in the spleen. CAR^{pos} cells exhibited robust anti-tumor immunity and expansion in peripheral blood. CAR^{pos} cells consisted of a distinct population of CD8^{pos} T cells with NK-like features (TaNKs) and a CD3^{pos}CD8^{pos}CD56^{pos}NKG2D^{pos} cell phenotype. *In vitro* transduction of CD56 NK cell-depleted PBMC with the CD3-directed LV also led to CAR-TaNK formation.

Conclusion: The subcutaneous injection of CD3-directed LV loaded PBMCs leads to the formation of tertiary lymphoid structures at the site of injection and the development of distinct CD3^{pos}CD8^{pos}CD56^{pos}NKG2D^{pos} CAR-TaNK cells. These cells possess enhanced systemic proliferative capacity compared to traditional *ex vivo* manufactured 41BB CAR-T cells in a lymphoreplete mouse model and the ability to eliminate target cells *in vivo* with low numbers of starting cells (10,000 cells).

Production CAR-TaNK Cells for Subcutaneous Administration



Schematic diagram for the rapid manufactured subcutaneous CAR-TaNK platform

1. PBMC from peripheral blood or apheresis are loaded with CAR lentivector for 4 hrs and washed prior to SC injection
2. The novel lentivector envelope binds to CD3, resulting in TCR internalization and cell activation
3. Cell product consists of PBMC with lentivector loaded lymphocytes

Results

Figure 1. Total body CD19-Luciferase CAR expression and histologic examination of the site of subcutaneous injection and distal organs

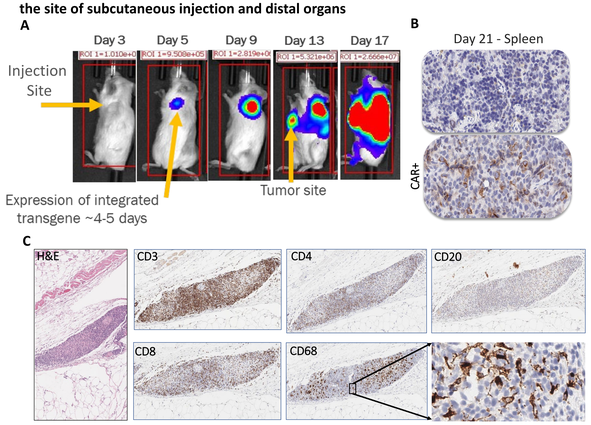


Fig 1: A. B-NDG mice with Raji tumor implanted on the right flank were subcutaneously injected with a CD19-Luciferase CAR LV loaded PBMC on ***Day 0*** on the contralateral left flank. CD19-Luciferase expression was tracked through *in-vivo* bioluminescence imaging (BLI). On ***Day 5***, the first sign of CAR-Luciferase expression was seen at the site of injection. On ***Day 13***, CAR-Luciferase expression was detected beyond the site of injection and in the contralateral-sided tumor. On ***Day 17***, CAR-Luciferase signals were detected systemically. **B.** Mice were sacrificed at various timepoints D3-D21 and organs were harvested. On ***Day 21***, IHC staining for CAR epitope in spleens demonstrates CAR^{pos} cells. Histologic examination of the subcutaneous site of injection on ***Day 7***, ***Day 10***, and ***Day 14*** revealed the formation of tertiary lymphoid structures (TLS). TLS on ***Day 14*** was composed mostly of human CD8^{pos} T cells, CD4^{pos} T cells, CD68^{pos} macrophages, and CD68^{pos} dendritic cells.

Figure 2. Systemic expansion of CAR^{pos} Cells in a PBMC humanized mouse model

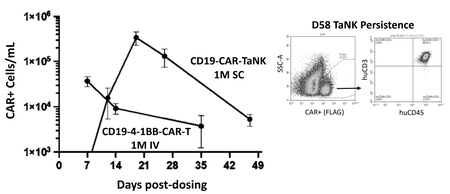


Fig 2: NSG-DKO mice were humanized with donor-matched PBMC IV (10M cells). Traditional CD19-4-1BB-CAR-T cells were produced through the *in-vitro* stimulation of PBMC with anti-CD3/CD28 stim, transduced with a third-generation 41BB-CD19 CAR LV in a GREX for 12 days, washed and cryopreserved, and infused at 1M cells/mouse (>40% CAR-T^{pos}) that had been previously humanized with 10M matched PBMC. For comparison, 1M total PBMC were loaded with 1M TU CAR-TaNK LV for 4hrs, washed, and subcutaneously injected into humanized mice with 10M matched PBMC. CD19-CAR-TaNK cells demonstrated higher peak expansion levels in peripheral blood and persistence compared to traditional 4-1BB-CAR-T cells in this human PBMC lymphoreplete model. Importantly, >99% of all CAR^{pos} cells were CD3^{pos}, indicating successful gene integration by CD3-directed CAR-TaNK LVs.

Figure 3: In Vivo and In Vitro generation of CAR^{pos} cells with T and NK like (TaNK) phenotype

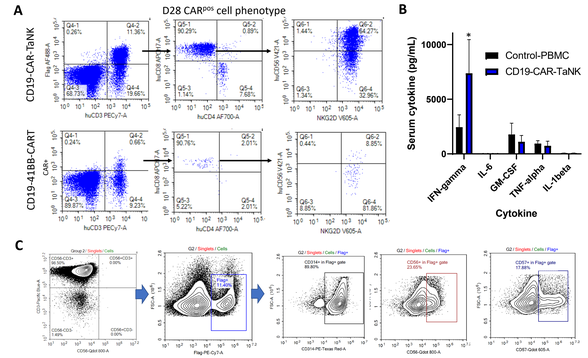


Fig 3: **A.** From the *in vivo* experiment described in Fig. 2, peripheral blood 28-days post-dosing was examined by flow cytometry for CAR expression and markers of T and NK cells. A significantly higher number of CAR^{pos} (FLAG) cells were seen in mice treated with SC CD-19-CAR-TaNKs compared to IV CD19-4-1BB-CAR-T cells. The majority of CAR^{pos} cells from the SC TaNK treatment were CD3+CD8+CD56+NKG2D+. **B.** Serum from mice 21 days post-dosing with SC CD-19-CAR-TaNKs in humanized mice was examined for human cytokines. In a panel of 42 cytokines (not all data shown), a significant increase was only seen in IFN-gamma following TaNK treatment. * P < 0.05 compared to control. **C.** TaNK LV were loaded onto CD56 depleted PBMC and kept *in vitro* for further characterization. CAR-TaNKs generated from CD56 negative PBMC were able to gain NK marker expression (NKG2D, CD56, CD57) following exposure to TaNK LVs.

Figure 4: Novel driver responsible for CAR-TaNKs enables sustained cell expansion without exhaustion following serial tumor antigen challenge

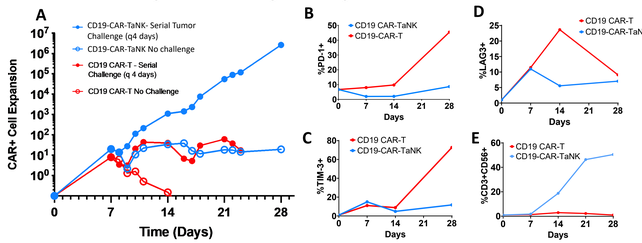


Fig 4: Serial tumor challenge assay. **A.** PBMC were *in vitro* transduced with CD19-CAR- LV vectors differing only by the presence or absence of driver transgene. Cells were first cultured with 10ng/mL IL-7 and 10ng/mL IL-2 for 7 days. After 7 days, cultures were washed and maintained without cytokine support and were serially challenged with Raji tumor cells every 4 days or fed media alone. CAR^{pos} cells with the synthetic driver transgene demonstrated antigen-dependent CAR-TaNK cell expansion with serial Raji tumor challenge with reduced cytokine withdrawal-induced cell death following antigen withdrawal. **B-D.** Surprisingly, following serial Raji tumor challenge, CD19 CAR-TaNK cells show reduced exhaustion markers: %PD-1+, %TIM-3+, and %LAG3+, compared to CD-19 CAR-T cells. **E.** Driver facilitates NK marker conversion in CD-19-CAR-TaNKs cells during continued antigen challenge.

Figure 5: CAR-TaNK's maintain strong activity against heavy lymphoma tumor burden *in vivo*

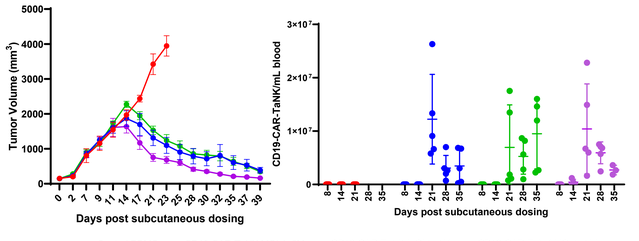


Fig 5: NSG mice with Raji tumor implanted on one flank were subcutaneously injected with different LV MOI of CD19-CAR-TaNK LV loaded PBMC on the contralateral flank. **A**. Robust anti-tumor immunity was observed at the 1M and 5M dose levels with MOI of 1. **B.** Peak expansion was observed at Day-21 with > 1e7 CAR^{pos} cells/mL of peripheral blood in all groups. Of note, CAR^{pos} cells do not exhibit continued expansion and regression in vivo despite a large established tumor burden.

Conclusions

These studies support a novel approach to adoptive cellular therapy that warrants clinical investigation:

- The generation of a unique anti-tumor T and NK-like CD19 CAR TaNK cell capable of enhanced proliferation with robust expansion, and decreased exhaustion markers compared to current standard CAR-T vector designs and cell products in lymphoreplete humanized mouse models.
- Subcutaneous administration of PBMC loaded with a CD3-directed lentivector encoding CAR and driver differentiate into CAR-TaNKs from tertiary lymphoid structures at the site of injection.
- CAR TaNKs formed subcutaneously can efficiently migrate to tumors and induce potent anti-tumor immunity against significant tumor burden.
- CAR TaNKs do not cause broad-spectrum increases in serum cytokine levels in humanized mice.
- Should clinical studies confirm that CAR TaNKs can indeed be consistently manufactured, released, and dosed without deep lymphodepletion and achieve clinically meaningful responses with lower CRS and ICANs burden, this would represent a significant advancement towards reducing the cost and complexity of adoptive cellular therapy.

References

1. Locke F. et al. Mol Ther. 2017 Jan 4;25(1):285-295. doi: 10.1016/j.ymthe.2016.10.020
2. Kochenderfer et al. J Clin Oncol. 2015 Feb 20;33(6):540-9. doi:10.1200/JCO.2014.56.2025
3. Orentas R et al. Blood. 2013 Feb 14; 121(7): 1165–1174
4. Mahnke YD et al. Eur J Immunol. 2013 Nov;43(11):2797-809
5. Hinrichs CS et al. Blood. 2011 Jan 20;117(3):808-14

Acknowledgements

We thank HistoWiz for tissue and histology support, Jackson Laboratories and Crown Bio for in vivo support, and Stanford Laboratories for serum cytokine analysis. We also thank Greg Wade and our colleagues at EXUMA Biotech for helpful comments and suggestions.