Tumor Targeted Superantigen (TTS(Enhances CAR-T Cell Activity Against Solid Tumors *In Vivo*

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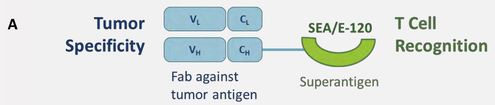
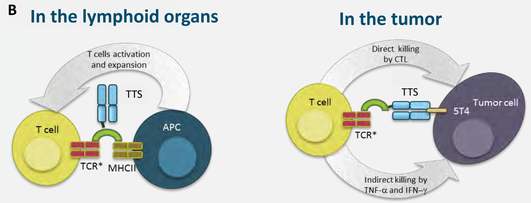
Background

Chimeric antigen receptor (CAR) T-cell therapy has shown limited efficacy against solid tumors primarily due to low trafficking to the tumor, restricted cell expansion, tumor antigen heterogeneity, and immunosuppressive tumor microenvironment. TTSs are fusion proteins that consist of genetically engineered superantigens linked to fragment antigen-binding moieties directed to tumor-associated antigens. It was previously shown that TTS turns "cold tumors hot" [1], induces long-term memory responses in preclinical models [2], and activates CAR-T cells, enhancing their anti-cancer efficacy *in vitro* [3]. Here we present new preclinical data demonstrating the synergistic anti-tumor effect of TTS in combination with CAR-T cells in the poorly immunogenic B16F10 tumor model, highlighting the potential of TTS to overcome existing limitations of CAR-T therapy.

Tumor Targeted Superantigen (TTS)- mode of action



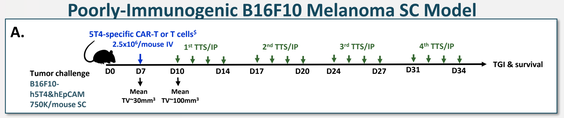


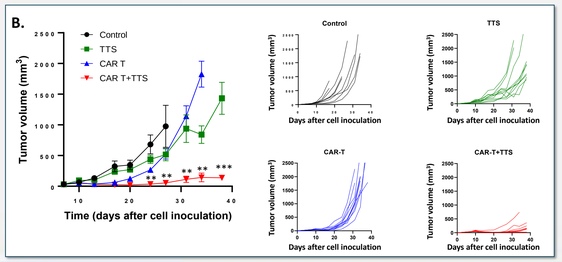
Selective T-cell (with *TCR Vβ6.4 in human and TCR Vβ3 in mice) activation and expansion outside of the immunosuppressive TME

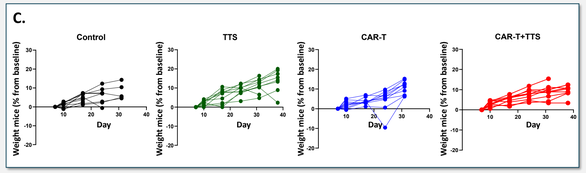
Activated T cells identify Superantigen bound to tumor surface and mount an antibacterial immune response

Figure 1. Tumor Targeted Superantigen (TTS). A. TTS is a fusion protein that consists of genetically engineered Superantigens (Sag) linked to Fragment antigen binding (Fab) moieties directed to tumor-associated antigens. TTS only binds and activates specific Vβ subsets of T cells (Vβ3 in mice and Vβ6.4 in humans for SEA and SEA/E-120-containing TTSs, respectively). **B.** TTS activates and expands specific Vβ subsets T cells within the lymphoid tissues. Once activated, these T cells subsequently migrate into the tumor microenvironment (TME), where they engage with TTS and initiate the destruction of cancer cells through targeted binding and immune-mediated cytotoxicity.

Synergistic Anti-tumor Activity of TTS and CAR-T in the Poorly-Immunogenic B16F10 Melanoma SC Model







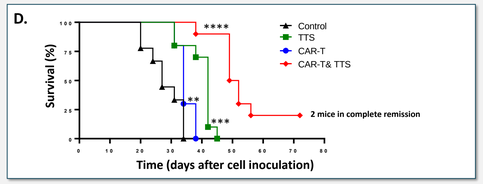


Figure 2: A. C57BL/6 mice were engrafted subcutaneously with B16F10 cells expressing both human 5T4 and EpCAM antigens. Seven days after tumor inoculation, mice were intravenously injected with murine CAR-T cells targeting the 5T4 antigen (h5T4-CAR-T) or control non-transduced T cells (NTD). Three days later, TTS targeting the hEpCAM antigen (C215Fab-SEA), was administered via intraperitoneal injection for four cycles of four consecutive days. **B.** The left graph shows the mean tumor volume (TV) of 7-10 mice/group ±SEM. CAR-T monotherapy group showed a transient effect on tumor volume and TTS monotherapy group showed also a modest effect, while combination therapy showed a synergistic effect on tumor volume. **p<0.005; ***p=0.0001 combination vs. control or monotherapies; multiple unpaired *t test*. Single plots are shown on the right. **C.** The combination treatment was well tolerated, no signs of adverse events and /or significant body weight loss (over 20%) were recorded. **D.** The combination treatment of CAR-T and TTS exhibited the most significant impact on survival with two mice in this group achieving complete remission. The median survival time: Control- 27 days; CAR-T- 34 days; TTS- 42 days; CAR-T+TTS – 50 days. n=9-10 mice/group. **p=0.0085; ***p=0.0004; ****p<0.0001, for treatments versus control; *Mantel- Cox test*

TTS- C215Fab-SEA, a mouse version of TTS; NTD = non-transduced T cells; TGI= tumor growth inhibition.

$only 3% of CAR-T and T cells are with Vβ3 TCR (reactive to TTS).

Immune Monitoring Experiment

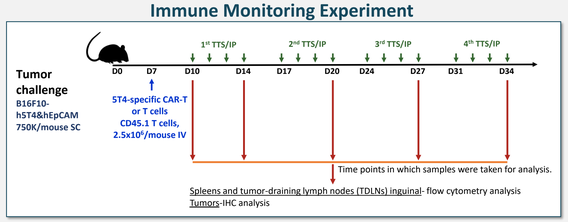
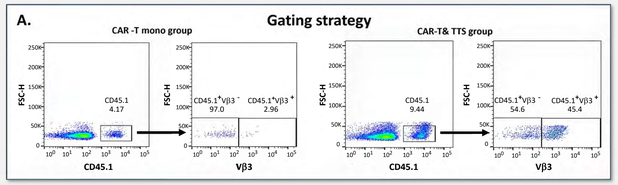


Figure 3: Schematic representation of the immune monitoring experiment. The immune monitoring procedures followed the treatment protocol outlined in Figure 2. To track the transferred CAR-T cells or T cells, CD45.1 T cells were administrated into mice with CD45.2 immune cells. The red arrows indicate the time points at which samples were collected for analysis. Spleens and tumor draining lymph node (TDLN) were extracted for flow cytometry analysis and tumors were preserved for IHC.

CAR-T Cells were Enriched 10-fold for vβ3 CD4 and CD8 T cells Following TTS activation



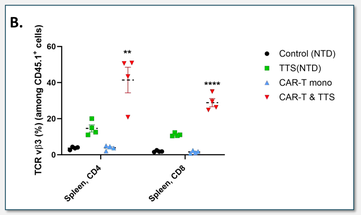


Figure 4. Following the 1st TTS cycle splenocytes were analysed for the following markers: CD3, CD4, CD8, CD45.1 and vβ3. **A.** Gating strategy: CD45.1 T cells were further analysed for vβ3 percentage. **B.** Analysis of vβ3 percentage for the four treatment groups. The percentage of vβ3 CD4 and CD8 was around 3% for control and CAR-T monotherapy groups without TTS stimulation, considered the baseline. Upon TTS stimulation vβ3 subset percentages increased. Notably, CAR-T stimulated with TTS showed a ten-fold increase in the percentage of CAR-T that were vβ3⁺, reaching around 30%. These vβ3 CAR-T cells have dual tumor-killing potential via CAR and TTS engagement and can be effectively directed to the tumor by TTS. Mean ±SE. **p<0.0 1, ****p<0.0001 vs. CAR-T monotherapy, *t* test analysis. n= 4.
NTD=non transduced T cells

TTS Induced Prolonged and Enhanced CAR-T Cells Activity in Spleen and TDLN

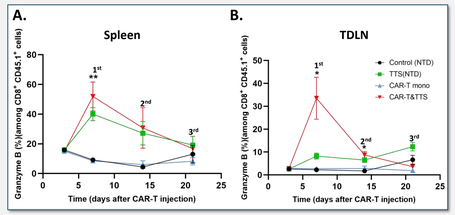


Figure 5. Transferred CD45. 1, CD8 CAR-T and CD8 T cells were analysed 3 days following injection to mice and following each TTS cycle for their activity as quantified according to Granzyme B expression. In the CAR-T monotherapy group, CAR-T cells activity was low in both spleen (**A**) and tumor-draining lymph nodes (TDLN) (**B**). Notably, in the combination group, the activity of CAR-T cells was significantly increased following TTS treatment both in the spleen (**A**) and TDLN (**B**). Mean ±SE. *p<0.05; **p<0.01 vs. CAR-T monotherapy, *t* test analysis; n=3-4. 1st, 2nd and 3rd - TTS treatment cycles.

Enhanced CAR-T Cell Proliferation in the Spleen and TDLN Following TTS Treatment

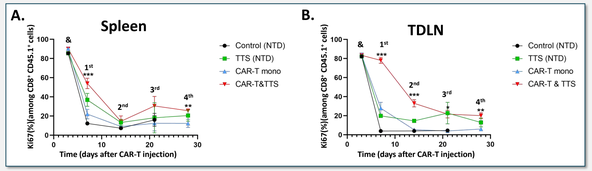


Figure 6. The proliferation of the transferred CD45. 1, CAR-T and T cells was assessed by Ki-67 expression 3 days following cells injection to mice and after each TTS cycle. Initially, after injection into mice, both CAR-T and T cells exhibited robust proliferation, primarily attributable to the in-vitro CAR-T preparation protocol. This proliferation was quantified in the spleen (A) and tumor-draining lymph nodes (TDLN) (B).

However, as time progressed, the proliferation of these transferred CAR-T and T cells declined significantly due to the absence of additional activation stimuli. Remarkably, in the combination group, CAR-T cell proliferation experienced a substantial and prolonged increase following TTS treatment, compared to CAR-T monotherapy, as observed in both the spleen (A) and TDLN (B). Mean ±SE. *p<0.05; **p<0.01 ***p<0.001 vs. CAR-T monotherapy, *t* test analysis; n=3-4. &= 3 days following CAR-T injection. 1st, 2nd and 3rd - TTS treatment cycles.

CD8 CAR-T cells were Significantly Enriched in TDLN following TTS Treatment

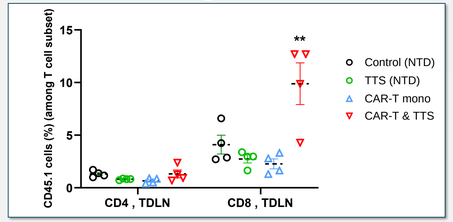


Figure 7. The enrichment of the transferred CD45.1 cells, including both CAR-T and T cells, was closely monitored within the tumor-draining lymph nodes (TDLN) after the first TTS cycle. The results are illustrated for the four distinct treatment groups. Remarkably, CD8 CAR-T cells were significantly enriched in TDLN following TTS treatment as compared to CAR-T monotherapy group. This resulted also in an increased CD8/CD4 ratio. Mean ±SE. **p<0.01 vs. CAR-T monotherapy, *t* test analysis. n=4
NTD=non transduced T cells

Following TTS treatment, vβ3 CAR-T Cells were Significantly more Active and Proliferative Compared to CAR-T Cells from Other TCR Subsets

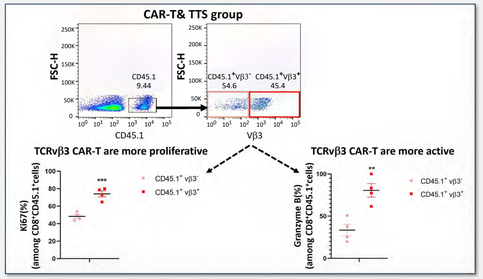


Figure 8. CD45.1 CAR-T cells were gated, according to vβ3 expression, to vβ3 CAR -T cells and to CAR-T cells from other TCR subsets. Each of these subsets underwent an evaluating of their proliferation by Ki67 expression and activity levels through Granzyme B analysis. Analysis showed that vβ3 CAR -T cells exhibited significantly higher levels of activity and proliferation when compared to CAR-T cells from other TCR subsets. These findings strongly suggest the specificity of TTS activation within the vβ3 CAR-T cell population. Mean ±SE. **p<0.01, ***p<0.001 vs. CAR-T monotherapy, *t* test analysis. n=4. NTD=non transduced T cells

Conclusion

Our preclinical results demonstrate the potential of TTS to enhance CAR-T cell activity against solid tumors. The synergistic effect observed in the B16F10 tumor model suggests that TTS can effectively address the limitations of current CAR-T therapy. The ex-vivo analysis further elucidates the positive impact of TTS on CAR-T cell persistence, proliferation, and activity. These results provide valuable insights for the development of improved strategies combining TTS with CAR-T therapy, paving the way for enhanced treatment options in solid tumors.

The 5T4-targeted TTS, naptumomab estafenatox, is currently being evaluated in clinical studies in combination with durvalumab [NCT03983954] and docetaxel [NCT04880863].

References

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2. Azulay M, Shahar M, Shany E, et al. Tumor-targeted superantigens produce curative tumor immunity with induction of memory and demonstrated antigen spreading. J Transl Med. 2023 Mar 26;21(1):222.
3. Sagi Y, Shahar M, Pinsker M. 576 Tumor Targeted Superantigen (TTS), Naptumomab Estafenatox (NAP), enhances CAR-T cells potency and can boost CAR-T efficacy against solid tumors. *Journal for ImmunoTherapy of Cancer* 2021;9:doi: 10.1136/jitc-2021-SITC2021.576