Enhancing directly selected tumor-reactive TIL function through genetic modification

Emily Carron, Sowbarnika Ratliff, April Fraley, Justin Rahman, Niloufar Khojandi, Bryant Thompson, Mathew Thayer, Christophe Pedros, Larissa Pikor, and Barbara Sennino

Turnstone Biologics, San Diego, CA, USA

Abstract #350

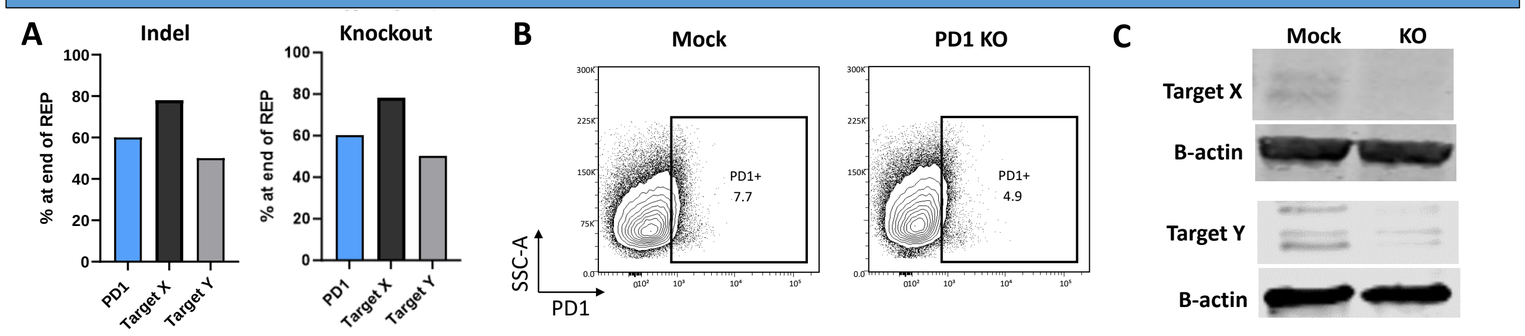
Background: Adoptive cell therapy (ACT) with tumor infiltrating lymphocytes (TIL) has emerged as a potential treatment of various types of solid tumors. However, responsiveness to TIL therapy can be limited by several mechanisms including tumor mediated-immune suppression, limited persistence, exhaustion, and toxicity associated with high dose IL2 treatment {1}. We believe the ability to genetically modify TIL has the potential to overcome these limitations, increase the activity of TIL therapy, and further expand ACT to a broad range of solid tumor types {2}. Herein, we demonstrate the process of genetically engineering directly selected tumor-reactive TIL using CRISPR-Cas and show that knocking out specific genes of interest can enhance TIL phenotype and function.

Methods: Following selection for tumor reactivity, patient-derived TIL underwent CRISPR-Cas mediated editing of the PDCD1 locus or other loci of interest followed by rapid expansion. At the end of production, knockout of gene targets of interest was confirmed by flow cytometry, western blot, and Inference of CRISPR Edits analysis. Gene edited and mock TIL were characterized *in vitro* by phenotyping, serial killing assays, intracellular cytokine staining, and autologous tumor reactivity assays.

Results: Using CRISPR-Cas, we were able to generate directly selected knockout TIL with gene editing efficiencies up to 95% with 70% efficiency at the PDCD1 locus. There was no significant change in rapid expansion or viability between gene edited and unedited samples. There was no improvement in memory phenotype in PD1 knockout TIL; however, ablation of other gene targets (Target X and Y) resulted in increased central memory populations. Polyfunctional cytokine secretion was improved in both PD1 knockout TIL and the other targets tested. In serial killing assays, PD1 KO TIL exhibited similar cytolysis as mock and unedited TIL while knockout against Target X exhibited enhanced cytolysis in the absence of cytokine support. Moreover, coculture of tumor cells with autologous selected tumor-reactive Target X knockout TIL led to increased tumor cell expression of cleaved-caspase 3 as compared to the selected tumor-reactive mock TIL.

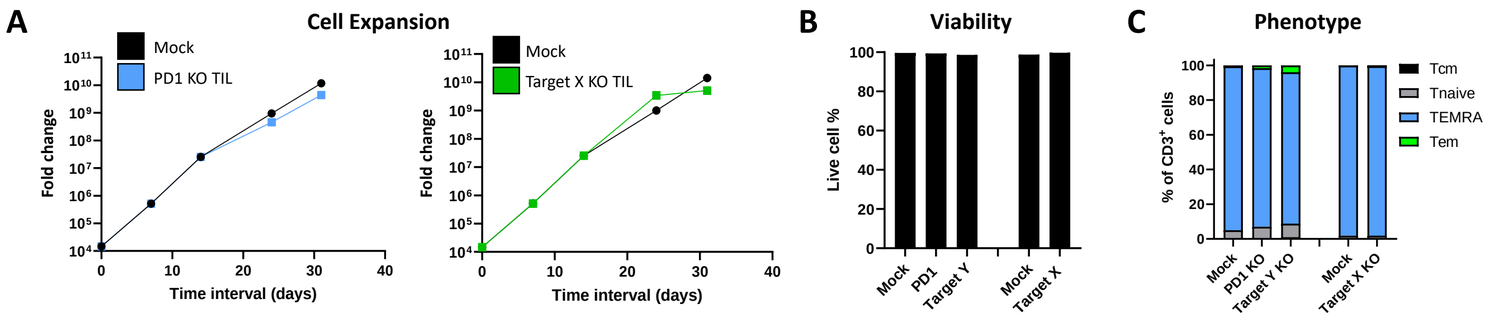
Conclusion: Collectively, these studies demonstrate that directly selected tumor-reactive TIL can be genetically engineered and expanded, and that CRISPR-Cas mediated-knockout of specific loci is a strategy for enhancing TIL phenotype and function.

Genetic deletion of PD1, Target X, or Target Y can be successfully achieved in selected TIL



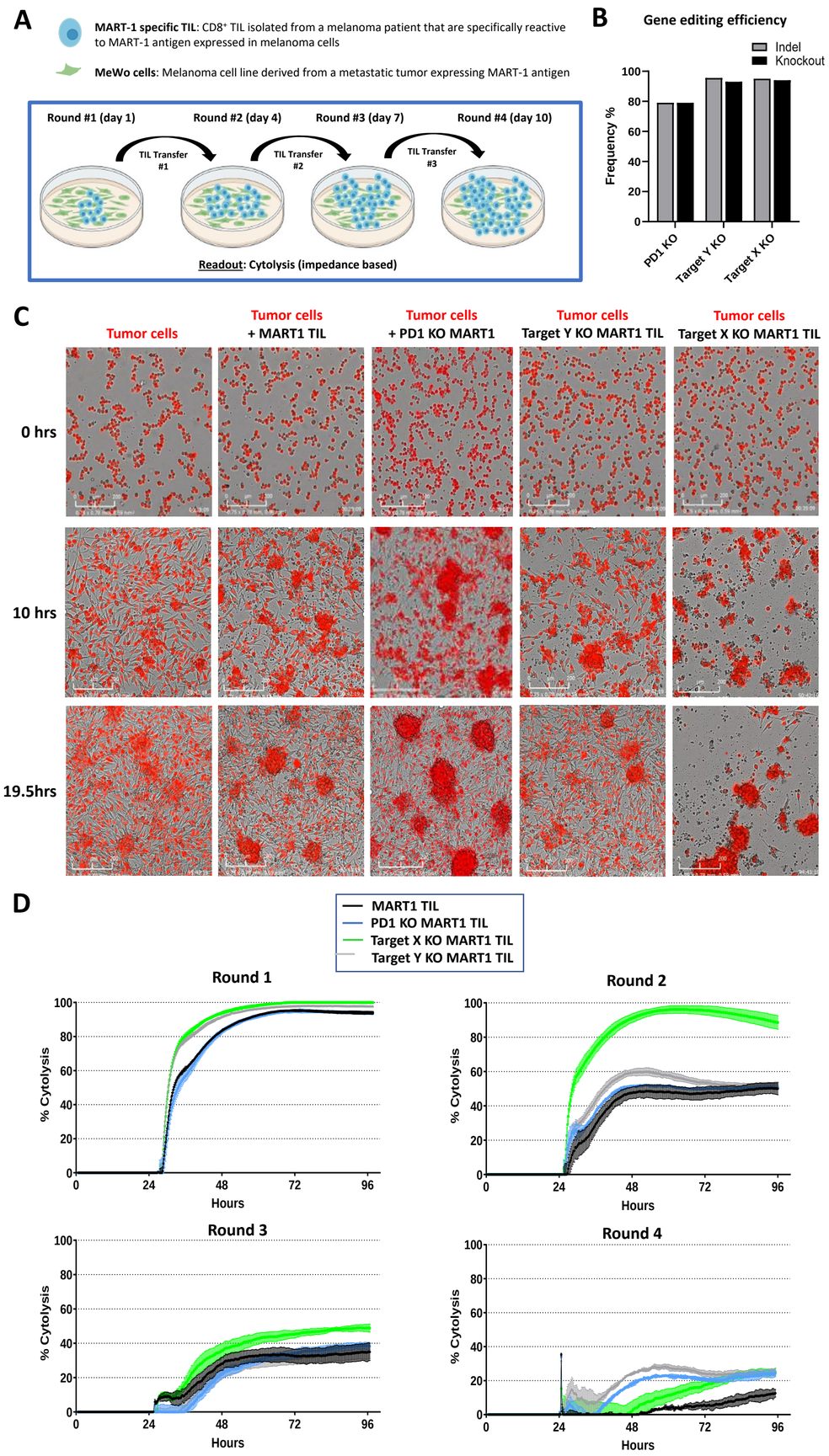
(A) Following disaggregation of primary tumor samples tumor-reactive TIL were directly selected by FACS based on differential expression of surface activation markers. Selected TIL underwent CRISPR-Cas mediated editing of the PDCD1 or Target X or Target Y locus followed by rapid expansion. Sanger sequencing was performed at the end of REP. Indel and Knockout frequency was assessed by Inference of CRISPR Edits analysis. **(B)** Confirmation of decreased PD1 protein expression in directly selected TIL at the end of REP. PD1 expression was measured within the $CD3^+$ population by flow cytometry. **(C)** Confirmation of decreased protein expression by sgRNA specific for Target X and Target Y using western blot analysis.

PD1 KO does not impact selected TIL cell yield, viability or memory phenotype



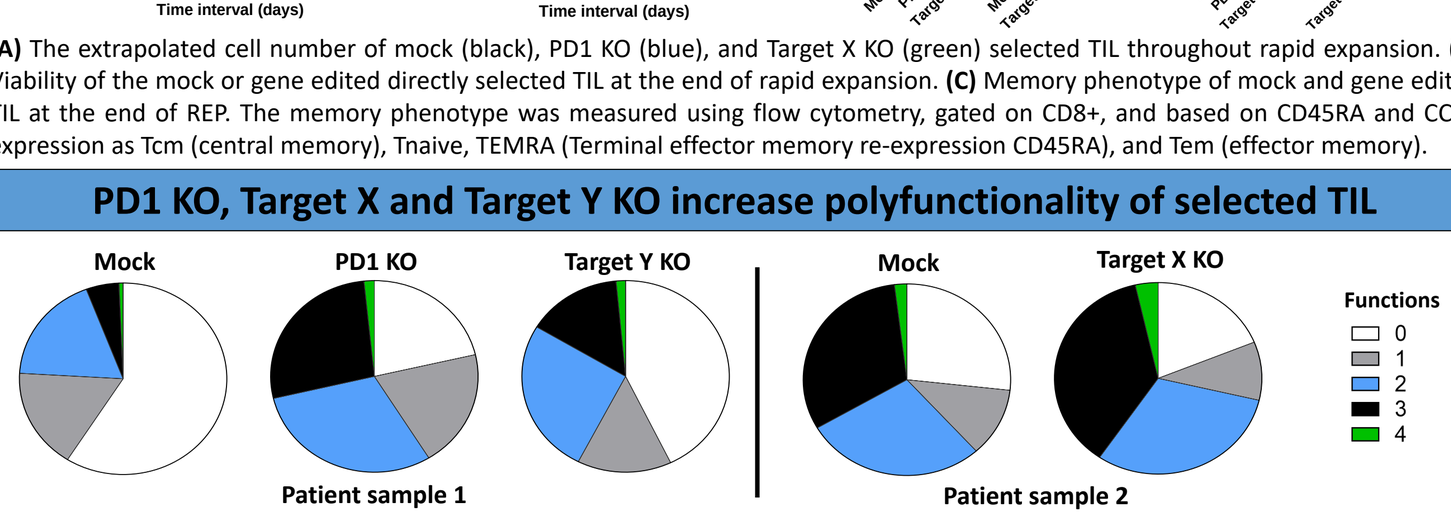
(A) The extrapolated cell number of mock (black), PD1 KO (blue), and Target X KO (green) selected TIL throughout rapid expansion. **(B)** Viability of the mock or gene edited directly selected TIL at the end of rapid expansion. **(C)** Memory phenotype of mock and gene edited TIL at the end of REP. The memory phenotype was measured using flow cytometry, gated on CD8+, and based on CD45RA and CCR7 expression as Tcm (central memory), Tnaive, TEMRA (Terminal effector memory re-expression CD45RA), and Tem (effector memory).

Target X KO MART1 reactive TIL exhibit enhanced tumor cytolysis compared to PD1 KO or Target Y KO MART1 TIL



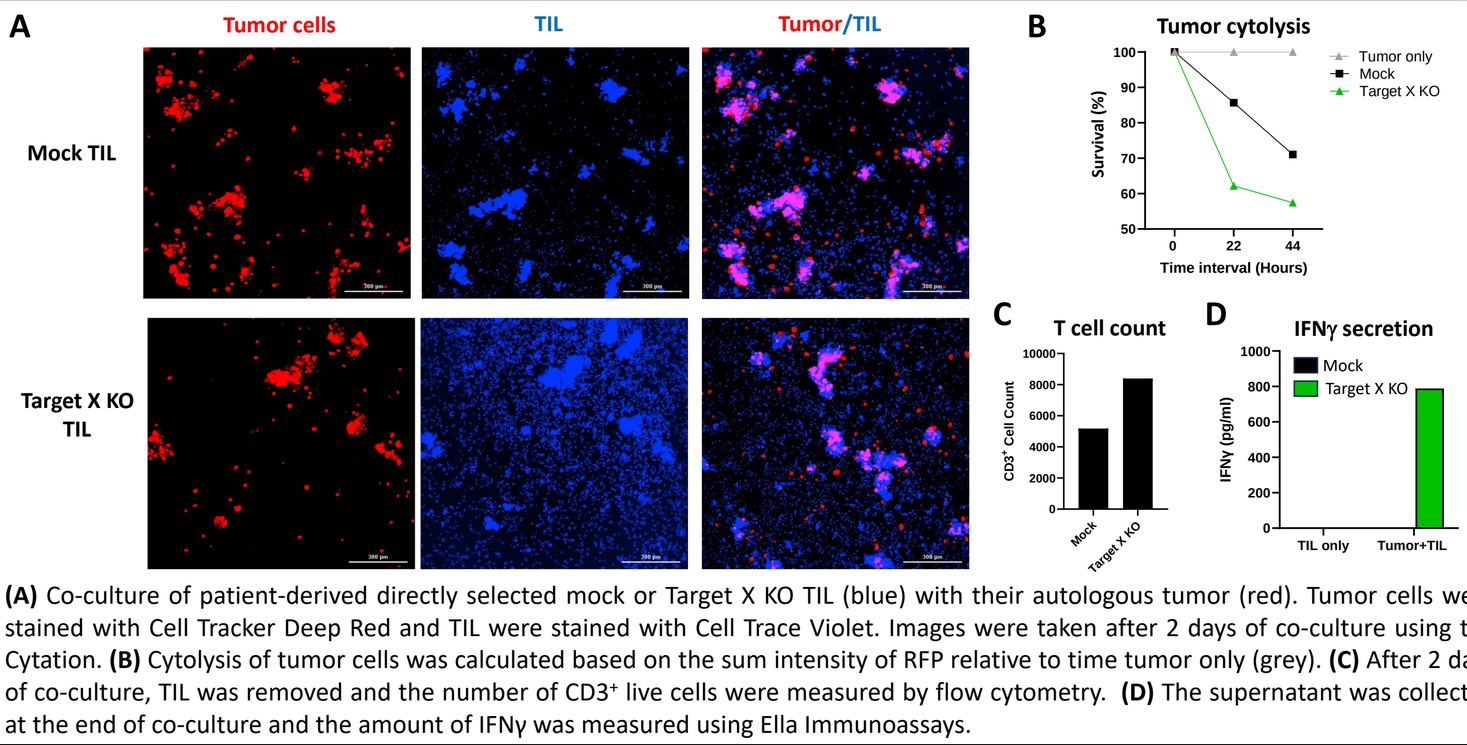
(**A**) Schematic of MART1 reactive TIL serial killing assay. MeWo tumor cells expressing the MART1 antigen (green) were plated on day 0 and MART1 reactive TIL (blue) were added 24 hours later. After 72 hours of co-culture, TIL were collected and re-cultured with MART1 expressing tumor cells seeded 24 hours prior. This was repeated 3 times for a total of 4 rounds. **(B)** Indel and Knockout frequency using Sanger sequencing and Inference of CRISPR Edits analysis in MART1 reactive TIL at the end of Rapid Expansion. **(C)** eSITE images of MART1 reactive TIL at time 0, 10 hrs, and 19.5 hrs from round 2 of co-culture of MART1 reactive TIL with MART1 antigen expressing tumor cells (red). **(D)** Graphs showing the tumor cell cytolysis through round 1-4 of coculture with mock (black), PD1 KO (blue), Target X KO (green), or Target Y MART1 reactive TIL (grey) of the serial killing assay in the absence of exogenous cytokine addition

PD1 KO, Target X and Target Y KO increase polyfunctionality of selected TIL



The polyfunctionality of mock, PD1 KO, Target Y KO, and Target X KO $CD8^+$ selected TIL was assessed in response to anti-CD3/CD28 stimulation. Polyfunctionality was based on $CD8^+$ IFNγ, TNFα, IL2, and CD107a. The frequency of each marker was assessed after 5 hrs by flow cytometry and calculated as the frequency of $CD8^+$ cells expressing 1, 2, 3, or 4 proteins. Comparison was performed using two different patient samples.

Target X KO selected TIL show increased tumor cytolysis, expansion, and IFNγ secretion



(A) Co-culture of patient-derived directly selected mock or Target X KO TIL (blue) with their autologous tumor (red). Tumor cells were stained with Cell Tracker Deep Red and TIL were stained with Cell Trace Violet. Images were taken after 2 days of co-culture using the Cytation. **(B)** Cytolysis of tumor cells was calculated based on the sum intensity of RFP relative to time tumor only (grey). **(C)** After 2 days of co-culture, TIL was removed and the number of $CD3^+$ live cells were measured by flow cytometry. **(D)** The supernatant was collected at the end of co-culture and the amount of IFNγ was measured using Ella Immunoassays.

Conclusions

- Genetic deletion of PD1, Target X, or Target Y can be successfully achieved in directly selected tumor-reactive TIL with no detrimental effect on TIL expansion, viability or phenotype.
- PD1 KO, Target X and Target Y KO directly selected tumor-reactive TIL have increased polyfunctionality in response to polyclonal stimulation.
- In a proof-of-concept MART1 reactive TIL serial killing assays, Target X KO TIL have increased tumor cytolysis compared to mock TIL, PD1 KO TIL and Target Y KO TIL.
- The directly selected Target X KO TIL product display increased cytotoxicity, proliferation, and IFNγ secretion against autologous tumor cells as compared to the directly selected mock TIL and may therefore represent a viable approach to further expand the utility of TIL therapies across variety of solid tumor indications.

References

1. Zhao, Y., Deng, J., Rao, S., Guo, S., Shen, J., Du, F., Wu, X., Chen, Y., Li, M., Chen, M., Li, X., Li, W., Gu, L., Sun, Y., Zhang, Z., Wen, Q., Xiao, Z., & Li, J. (2022). Tumor infiltrating lymphocyte (TIL) therapy for solid tumor treatment: Progressions and challenges. *Cancers*, *14*(17), 4160.
2. Weinstein-Marom, H., Gross, G., Levi, M., Brayer, H., Schachter, J., Itzhaki, O., & Besser, M. J. (2021). Genetic modification of tumor-infiltrating lymphocytes via retroviral transduction. *Frontiers in Immunology*, *11*.