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# Engineering Off-the-Shelf T Cell Receptor Fusion Construct (TRuC™) T cells

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## Abstract

The natural T cell receptor (TCR) recognizes its cognate peptide antigen only when presented on human leukocyte antigen (HLA) molecules and requires HLA matching of α/β TCR-engineered T cells for cancer therapy. To bypass the need for HLA matching, we have previously described the generation of autologous T Cell Receptor Fusion Construct (TRuC™) T cells which are engineered to express a fusion protein that comprises an antibody-derived binder tethered to the CD3ε signaling subunit. Upon integration of the TRuC into the TCR, it recognizes tumor surface antigens independent of HLA and uses the complete receptor complex to trigger a comprehensive T cell response. Here, we report the engineering of off-the-shelf TRuC-T cells directed against CD19 and mesothelin (MSLN), respectively. To eliminate the alloreactivity of α/β T cells and reduce the risk of graft-versus-host-disease (GvHD), the TRAC locus is knocked-out. To enable the re-assembly of the TCR, the endogenous TCRα and β subunits are replaced with fusion proteins comprising antibody binders fused to the murine TCRα or β constant domains, or replaced with murine TCRα or β constant domains and a fusion protein comprised of antibody binder and CD3ε. Similar to their autologous counterparts, allogeneic CD19 and MSLN-targeting TRuC-T cells upregulate activation markers, secrete cytokines, and lyse tumor cells in an antigen-specific manner. Importantly, allogeneic TRuC-T cells lack alloreactivity as demonstrated in mixed lymphocyte reactions and clear tumors in NSG xenograft models with similar efficacy and persistence as our autologous anti-CD19 and anti-MSLN TRuC-T cells, but without signs of GvHD. To reduce host rejection and enhance the persistence of the allogeneic TRuC-T cells, MHC class I expression on the surface of the TRuC-T cells was down-regulated by means of RNAi. In summary we have engineered allogeneic TRuC-T cells that maintain the signaling properties of the TCR complex with comparable efficacy as donor-matched autologous TRuC-T cells; moreover, these T cells have the potential to persist in an allogeneic host by diminishing the risk of rejection by the host.

## Allogeneic TRuC-T Cell Platform

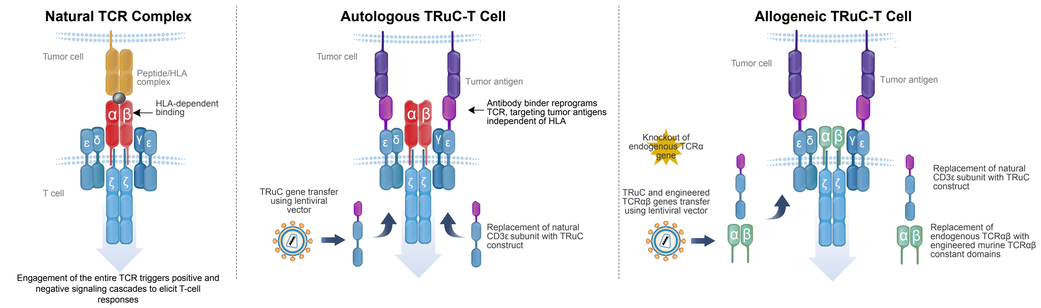


**Figure 1:** Schematic representations of autologous and allogeneic TRuC-T cells in comparison with the endogenous T cell receptor (TCR). By fusing an antibody binding domain to the CD3ε subunit of the TCR, TRuC-T cells reprogram TCRs to target specific cancer antigens in an HLA-independent manner. Our novel, allogeneic TRuC-T cell platform additionally requires integration of non-alloreactive TCRαβ subunits which reduces GvHD risk, yet maintains the full functionality of the host TCR. Multiple configurations of allogeneic TRuC-T cells are utilized where the antibody binding domain can also be fused to the engineered TCRαβ subunits instead of CD3ε (as shown).

## Allogeneic TRuC-T Cell Process

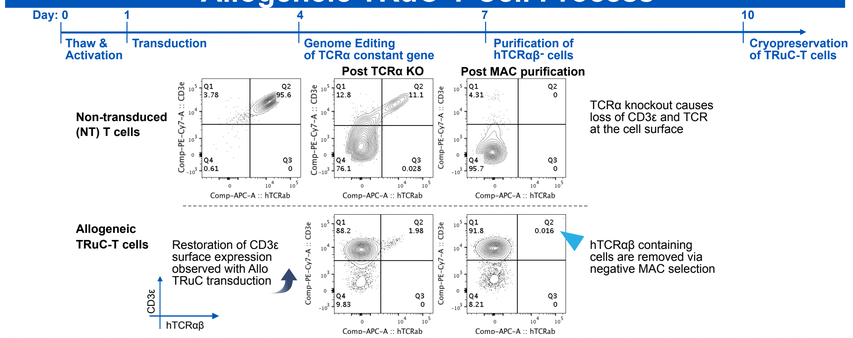


**Figure 2:** Schematic of allogeneic TRuC generation process and corresponding flow cytometry plots demonstrating changes in surface staining for CD3ε and TCRαβ. Cells are thawed and activated on Day 0 and transduced with Allogeneic TRuC lentiviruses on Day 1. TCRα constant domain is targeted by Crispr/Cas9 on Day 4 to delete endogenous TRCα. Subsequently, loss of CD3ε and TCRαβ staining is observed for NT controls (top row). High CD3ε levels are restored for Allo TRuC-T cell constructs (bottom row). Cells are expanded and are purified on Day 7 by negative MAC selection to remove residual cells expressing human TCRαβ. Cells are expanded through Day 10 and are cryopreseved.

## AntiCD19 Targeting Allogeneic TRuC-T Cells

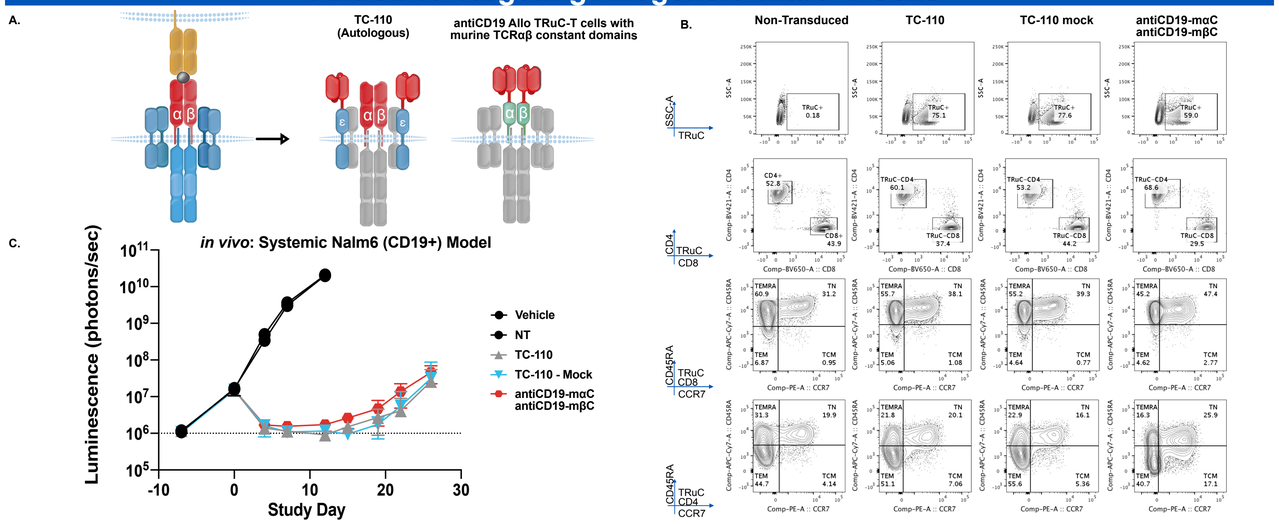


**Figure 3:** (A) Schematic representation of the endogenous T cell receptor compared to TCR2's autologous (TC-110) and allogeneic TRuC-T cells targeting CD19. AntiCD19 Allo TRuCs are comprised of antiCD19 (FMC63) scFVs fused to the murine constant domains of TCRα and TCRβ. (B) Flow cytometry plots characterizing surface marker expression on TRuC-T cells at Day 10 of the expansion process. Samples included non-transduced (NT) controls, TC-110, TC-110 mock (undergo mock electroporation and mock MAC purification) and antiCD19-mαC antiCD19-mβC (allogeneic TRuC). Top row of plots show transduction efficiency by staining for cell surface TRuC expression. Second row of plots compared CD4 and CD8 cell ratios in TRuC+ cells. Third and fourth row of plots compare memory phenotype in TRuC+ CD8+ and CD4+ cells, by staining for CD45RA and CCR7. (C) Summary plot of luminescence signal (tumor growth) from *in vivo* study comparing autologous and allogeneic antiCD19 targeting TRuC-T cells. On Day -7, animals were injected IV with CD19+ Luciferized Nalm6 cells. On Day 0 TRuC-T cells were injected and luciferase signal was measured over time. Overall allogeneic TRuC-T cells targeting antiCD19 have similar cell phenotypes and have comparable *in vivo* efficacy to autologous TC-110 TRuC-T cells.

## Anti-Mesothelin Targeting Allogeneic TRuC-T Cells

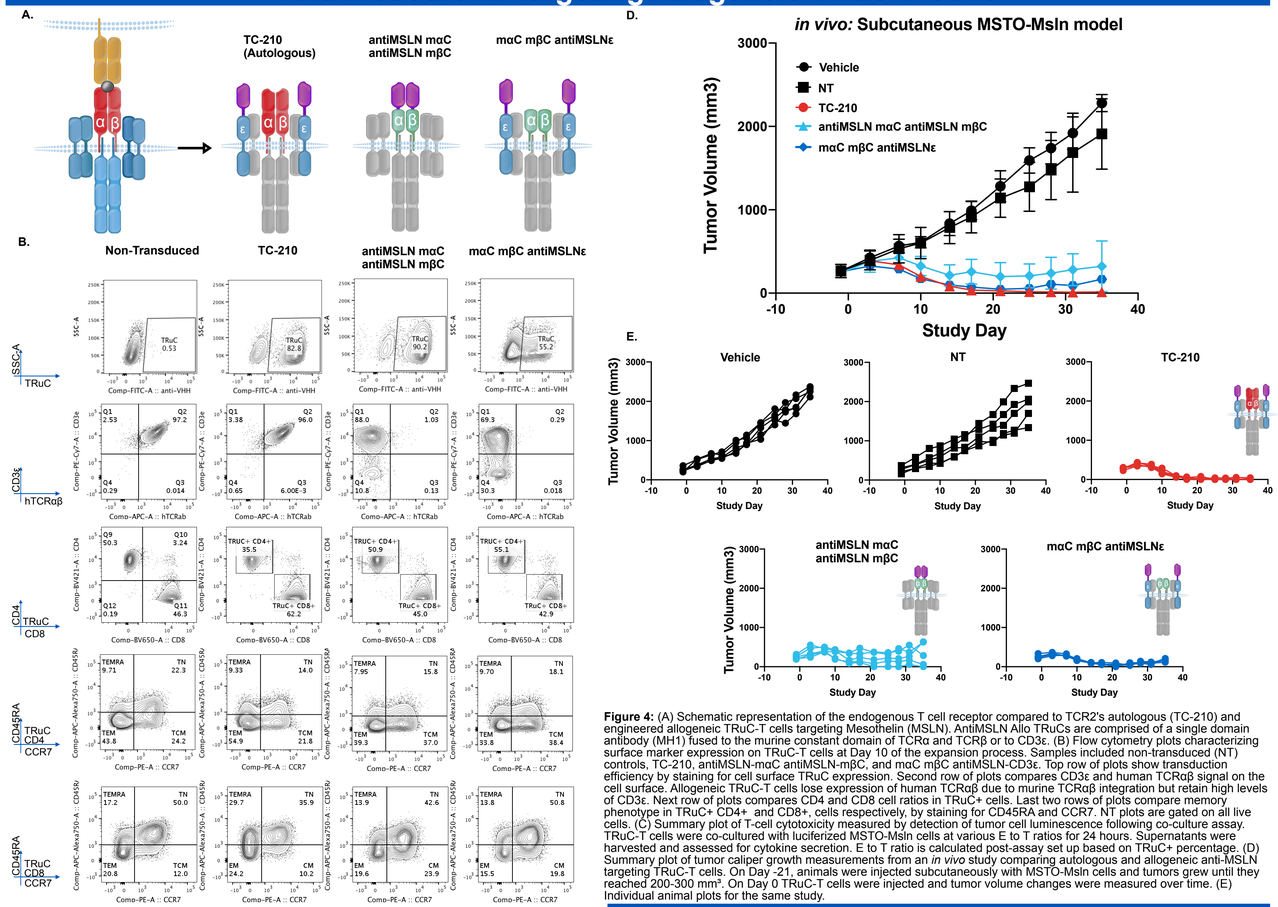


**Figure 4:** (A) Schematic representation of the endogenous T cell receptor compared to TCR2's autologous (TC-210) and engineered allogeneic TRuC-T cells targeting Mesothelin (MSLN). AntiMSLN Allo TRuCs are comprised of a single domain antibody (MH1) fused to the murine constant domain of TCRα and TCRβ or to CD3ε. (B) Flow cytometry plots characterizing surface marker expression on TRuC-T cells at Day 10 of the expansion process. Samples included non-transduced (NT) controls, TC-210, antiMSLN-mαC antiMSLN-mβC, and mαC mβC antiMSLN-CD3ε. Top row of plots show transduction efficiency by staining for cell surface TRuC expression. Second row of plots compares CD3ε and human TCRαβ signal on the cell surface. Allogeneic TRuC-T cells lose expression of human TCRαβ due to murine TCRαβ integration but retain high levels of CD3ε. Next row of plots compares CD4 and CD8 cell ratios in TRuC+ cells. Last two rows of plots compare memory phenotype in TRuC+ CD4+ and CD8+, cells respectively, by staining for CD45RA and CCR7. NT plots are gated on all live cells. (C) Summary plot of T-cell cytotoxicity measured by detection of tumor cell luminescence following co-culture assay. TRuC-T cells were co-cultured with luciferized MSTO-Msln cells at various E to T ratios for 24 hours. Supernatants were harvested and assessed for cytokine secretion. E to T ratio is calculated post-assay set up based on TRuC+ percentage. (D) Summary plot of tumor caliper growth measurements from an *in vivo* study comparing autologous and allogeneic anti-MSLN targeting TRuC-T cells. On Day -21, animals were injected subcutaneously with MSTO-Msln cells and tumors grew until they reached 200-300 mm³. On Day 0 TRuC-T cells were injected and tumor volume changes were measured over time. (E) Individual animal plots for the same study.

## Mitigation of Host Rejection by B2M Knockdown

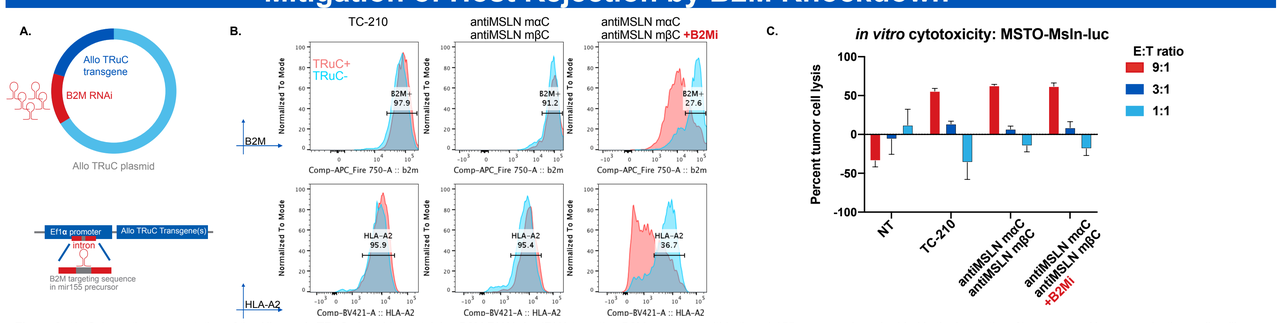


**Figure 6:** (A) Schematic representation of the Allogeneic TRuC plasmids containing B2M RNAi. A miRNA targeting B2M sequence within the mir155 precursor is embedded in the intron the ef1α promoter. Upon transduction, cells will express both the Allogeneic TRuC transgene and miRNA targeting B2M. (B) HLA-A2+ Donor T cells were transduced with TC-210 (autologous anti-MSLN TRuC), anti-MSLN mαC anti-MSLN mβC, and anti-MSLN mαC anti-MSLN mβC + B2Mi and expanded in the Allogeneic TRuC generation process. Histogram plots showing surface expression of B2M and HLA-A2 in TRuC+ and TRuC- demonstrated B2M and MHCI expression loss in TRuC+ cells in the anti-MSLN mαC anti-MSLN mβC + B2Mi cells. (C) Bar graph of T-cell cytotoxicity measured by detection of tumor cell luminescence following co-culture assay. TRuC-T cells normalized by TRuC surface expression were co-cultured with luciferized MSTO-Msln cells at various E to T ratios for 24 hours and cytotoxicity was measured by loss of tumor cell luminescent signal. Allogeneic TRuC-T cells containing B2Mi have equivalent efficacy to standard Allogeneic TRuC-T cells without B2Mi integration and to TC-210.

## Allogeneic TRuCs Do Not Induce GvHD

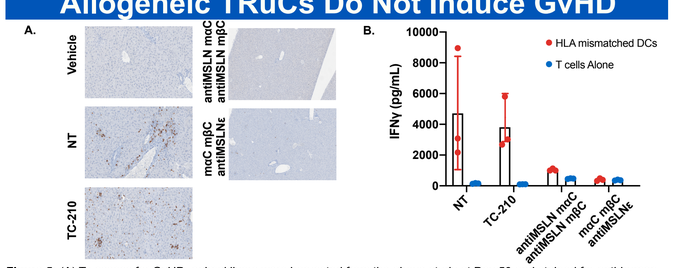


**Figure 5:** (A) To assess for GvHD, animal livers were harvested from the above study at Day 50 and stained for anti-human CD7 to look for TRuC-T cell infiltration into the liver. (B) Allogeneic TRuC-T cells were co-cultured with HLA donor mismatched *in vitro*-derived dendritic cells (DCs) for 72 hours. Supernatants were harvested and cytokine secretion analysis was performed.

## Conclusions

- Flexible allogeneic TRuC formats based on CD3ε or TCRα and β antibody fusions
- Equivalent preclinical anti-tumor activity of anti-CD19 and anti-MSLN allogeneic TRuC-T cells and their respective clinical development candidates
- *In vitro* proof-of-concept of B2M/MHC class I knock-down using miRNA encoded in TRuC transfer plasmid