# A synthetic biology approach to address the immunosuppressive tumor microenvironment: novel TGF-β switch receptors convert inhibitory signals into enhanced NK cell activity

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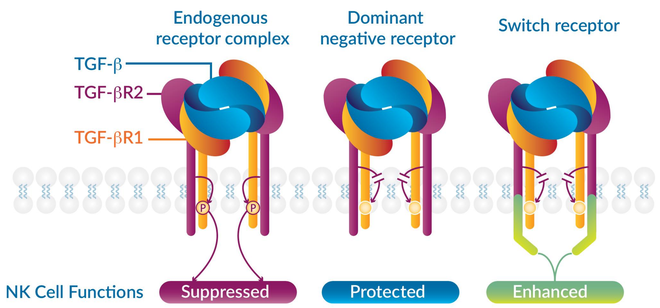
## INTRODUCTION

We are developing allogeneic Natural Killer (NK) cells engineered to effectively recognize antigen-expressing tumor cells through chimeric antigen receptors (CARs) and overcome transforming growth factor-beta (TGF-β)-mediated immunosuppression through chimeric switch receptors (SRs). The TGF-β pathway is integral to the establishment, persistence, and therapeutic resistance of tumors derived from epithelial cells, such as renal cell carcinoma, ovarian cancer and other solid tumors. Tumor microenvironment-derived TGF-β also inhibits immune responses to the tumor by inducing dysfunction in NK and cytotoxic T cells as well as promoting suppressive cells such as regulatory T cells. TGF-β reduces the anti-tumor therapeutic potential of NK cells by suppressing the expression of endogenous activating receptors and interfering with NK cell growth and function.

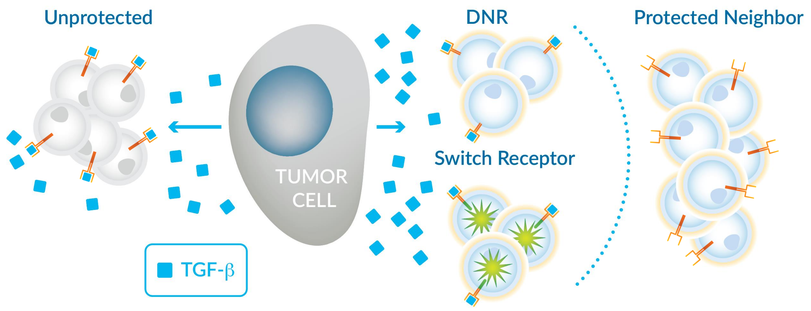
We reasoned that the ideal solution to enhancing immune cell function in TGF-β rich environments would be redesigning TGF-β signaling to confer three functions on the engineered cells: (a) protect the engineered cells from TGF-β induced dysfunction, (b) convert the TGF-β signal into an immune-stimulatory signal, and (c) locally reduce TGF-β signaling to other cells within the tumor microenvironment.

To directly counteract TGF-β's immunosuppression, we designed SRs that convert this inhibitory signal into NK cell activation signals. We replaced the endogenous Smad2/3 inhibitory signaling of TGF-β receptor 2 with ectopic signaling domains that stimulate costimulation, growth, survival, and other pathways. We then assessed the impact of TGF-β SR expression on the proliferation and survival, cytotoxic function, and cytokine production of engineered human NK cells.

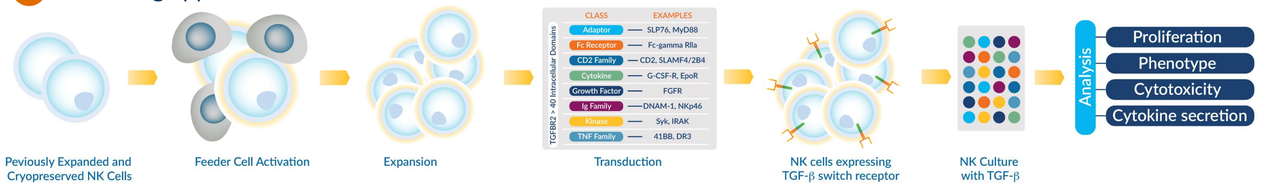
## 1 Redesigned TGF-β receptors protect and enhance immune cells




## 2 TGF-β switch receptors function by three beneficial mechanisms




## 3 Screening approach for TGFBR2-SR constructs




A panel of TGFBR2-SR constructs was designed for gammaretroviral transduction into NK cells. In addition to the SR constructs, a negative control construct consisting of truncated CD19 tag was also designed (dCD19). All constructs were bi-cistronic designs with the second element being an inert expression tag.

Previously expanded and frozen peripheral blood NK cells were thawed and activated with feeder cells. Packaging cells were transfected in a small scale 24-well format to produce gammaretroviral supernatants and transduce the activated NK cells.

Three days after transduction NK cells were counted and construct expression was analyzed by flow cytometry. Cells were then re-plated and were cultured for 5 days with the addition of recombinant human IL-2 and were treated with or without 10 ng/mL TGF-β1. Cytokines were replenished throughout the culture. On day 5, cells were analyzed and then re-plated for functional assays.

## 4 Classes of TGFBR2-SR designs convert TGFB to a growth factor for NK Cells

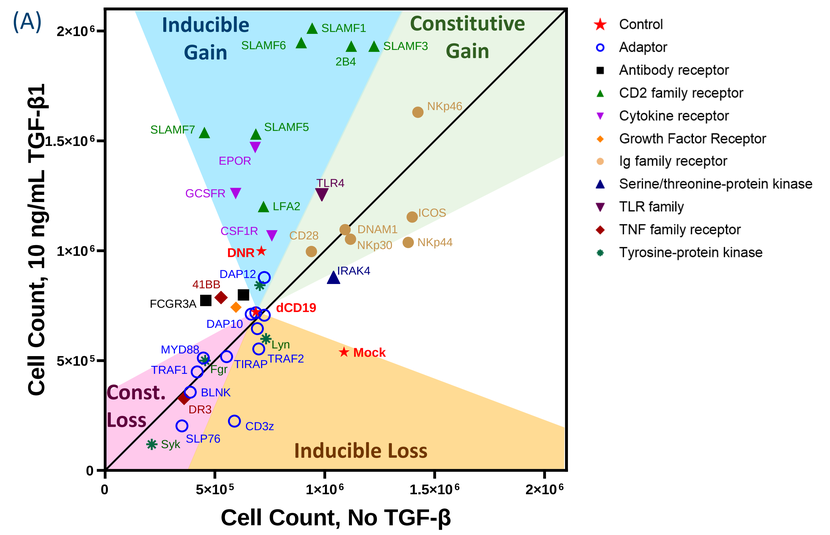



**Figure 4:** NK cells expressing TGFBR2-SR constructs were generated and conditioned for 5 days with 10 ng/mL TGF-β1. Cells were then counted to determine the impact of TGF-β conditioning on cell growth. (A) Total number of cells recovered from each culture, plotted as a comparison of cell number with or without TGF-β treatment. The location of constructs in different regions indicates inducible or constitutive behavior in response to TGF-β, and a gain or loss in function relative to the dCD19 tag control. (B) Waterfall plot showing the number of population doublings for each construct when cultured with 10 ng/mL TGF-β1.

## 5 TGFBR2-SRs potentiate target-driven NK cell functionality

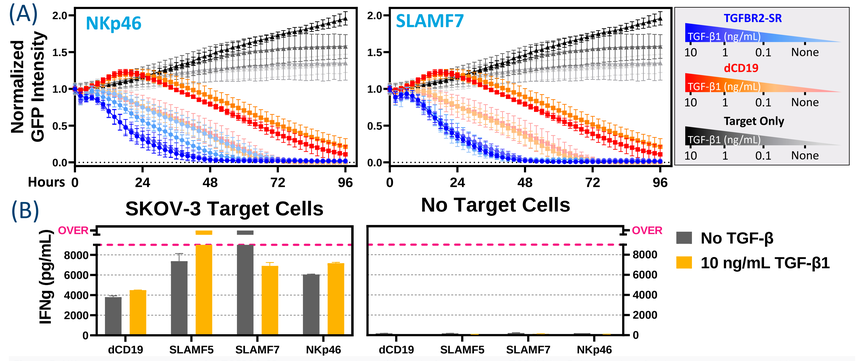



**Figure 5:** NK cells were transduced with TGFBR2-SR constructs and conditioned with or without TGF-β1 for 5 days as described above, then re-plated for functional assays. (A) NK cells were conditioned +/- TGF-β at 3 different concentrations of 10 ng/mL, 1 ng/mL, or 0.1 ng/mL. These NK cells were placed into a long-term spheroid killing assay run on an Incucyte® Live-Cell Analysis System (Sartorius). NK cells were added to wells containing pre-formed SKOV-3-GFP spheroids at an approximate E:T ratio of 5:1. Additional IL-2 (100 IU/mL) and TGF-β1 was added at the start of the assay, with the TGF-β1 concentration matching that of the preconditioning period. GFP intensity was measured every 2 hours and normalized to T0. (B) NK cells were added to SKOV-3 target cells at a 3:1 E:T ratio and cultured for 24 hours. Secreted interferon gamma (IFNg) was measured by ELISA (Meso Scale Diagnostics)

## 6 TGFBR2-SR constructs protect against TGF-β by multiple mechanisms

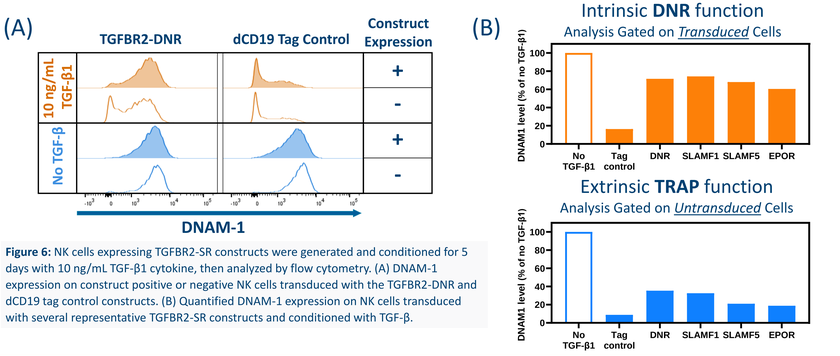



**Figure 6:** NK cells expressing TGFBR2-SR constructs were generated and conditioned for 5 days with 10 ng/mL TGF-β1 cytokine, then analyzed by flow cytometry. (A) DNAM-1 expression on construct positive or negative NK cells transduced with the TGFBR2-DNR and dCD19 tag control constructs. (B) Quantified DNAM-1 expression on NK cells transduced with several representative TGFBR2-SR constructs and conditioned with TGF-β.

## CONCLUSIONS

**We successfully executed a screen in primary NK cells discovering functional switch receptors that convert inhibitory TGF-β into beneficial signals.**

- **TGFB-SRs convert TGF-β into a growth factor**
  Diverse classes of TGFBR2-SRs reversed TGF-β-mediated inhibition and promoted NK cell expansion in vitro
- **TGFB-SRs enhance NK Cell functional responses**
  With SR engineering, chronic TGF-β stimulation strengthened NK cell tumor killing and target-induced cytokine secretion
- **TGFB-SRs include dominant negative activity**
  TGFBR2-SRs protected from suppressive TGF-β signaling

**Switch receptors offer a flexible and rich design strategy for creating therapeutic NK cells capable of robust functioning within hostile tumor microenvironments.**