NKp46 engaging Bicycle NK-TICA® drives tumor targeted cytotoxicity

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Abstract #
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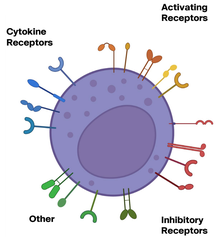
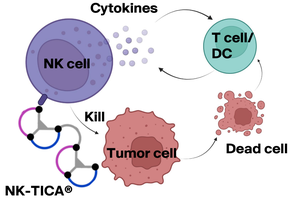
ABSTRACT
Natural killer (NK) cells are immune cells that can detect and eliminate tumor cells and bridge innate to adaptive immune responses. Bicycles are small (ca. 1.5 kDa), chemically synthetic, structurally constrained peptides discovered via phage display and optimized using structure-driven design and medicinal chemistry approaches. We have now applied this technology to identify Bicycles that bind specifically to a key activating receptor, NKp46. We term this new class of fully synthetic molecules Bicycle® natural killer- tumor-targeted immune cell agonists (NK-TICAs) and herein we will describe their *in vitro* evaluation.

INTRODUCTION
We have developed a novel, fully synthetic tumor binding and NKp46 binding NK-TICA® molecule that is capable of inducing NK cell activation in the presence of tumor. As an immunotherapeutic agent, Bicycle's NK-TICA® molecules are positioned to engage NK cells in a tumor antigen dependent manner to kill and drive adaptive immunity in solid tumors.

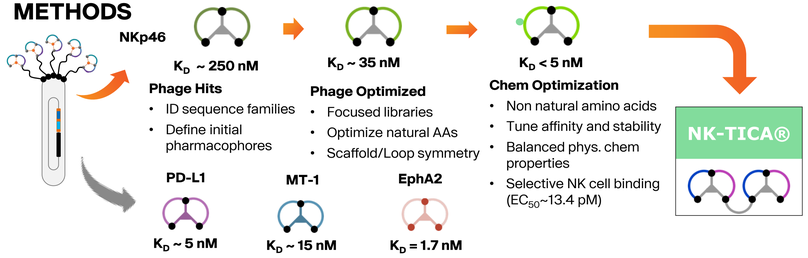


◀ LEFT
Surface receptors expressed on human NK cells (based on Chiossone *et al.*, 2018). NK cells emanate from the bone marrow, patrol the body, last for several days, and can kill by direct contact-dependent cytotoxicity or signaling through death receptors. These innate cells use receptors to read the surface of cells for signs of stress, transformation, viral infection, or decoration with antibodies.

RIGHT ▶
Recent work suggests a role of NK cell activation in situ to catalyze the development of anti-tumor immunity via release of tumor antigens (kill) and activation of DCs/T cells (cytokines) (Wang *et al.*, 2021).



NK cell activation results in tumor cytotoxicity and activation of other immune cells by cytokine release

METHODS


Using our unique phage display screening platform, we identified high affinity, selective binders to NKp46. For proof-of-concept studies, NKp46 binding Bicycles were conjugated with an EphA2-binding *Bicycle®*. The EphA2 and PD-L1 binding *Bicycle®* is specific and potent with ~1.7 nM and ~5 nM, respectively, evaluated by SPR (Upadhyaya *et al.*, 2021). The MT-1 *Bicycle®* is specific and potent with ~15 nM evaluated by SPR (Lani *et al.*, 2017). The resulting bifunctional NK-TICA® were then tested in primary human cell-based functional models.

RESULTS
We have developed a novel modular compound with high affinity and selectivity to NK cell receptors with specific tumor targeting potential. We demonstrate potent, selective binding of our Bicycles to receptor-expressing cells and the capability of the bifunctional molecule to induce primary human NK cell function *in vitro*.

Figure 1. NK-TICA® selectively binds primary NK cells
Binding of NKp46 *Bicycles* was measured by flow cytometry. The fluorescently labelled (AF488-tagged) NKp46 Bicycle bound only to NK cells in purified PBMC. Non-binding NK-TICA® control (D-amino acids ablating affinity to the target) demonstrated no binding above background in both CD56+ NK (Figure 1) and CD3+ T cells and other immune cell populations (data not shown).

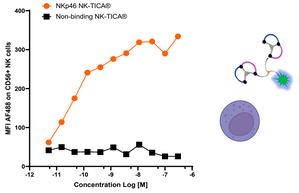
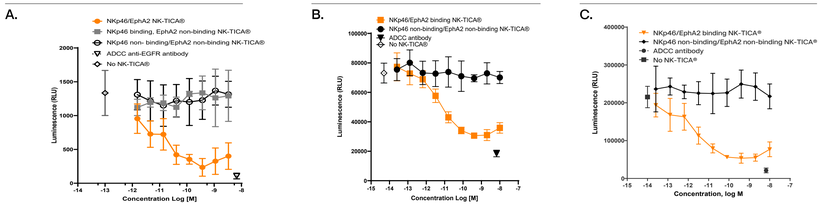
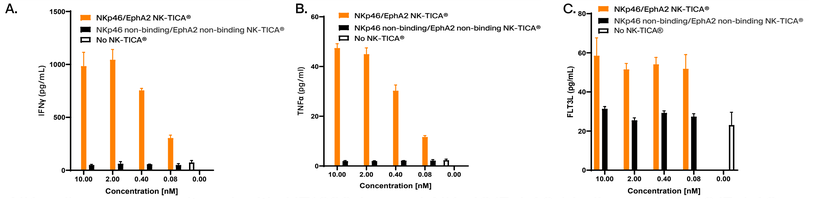
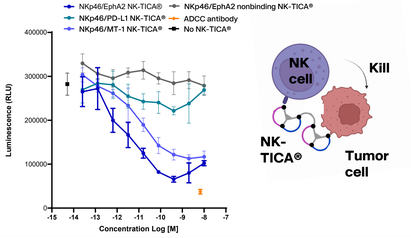


Figure 2. NK-TICA® enhances NK killing across multiple EphA2+ cell lines


NK cells specifically kill EphA2+ cell lines: HT1080 (~60000 EphA2-PE/cell, Figure 2A,B) and A431 (~43000 EphA2-PE/cell, Figure 2C) in the presence of EphA2 binding Bicycle® NK-TICA®. Without EphA2 binding, the NK-TICA® NKp46(nb)/EphA2(nb) and NKp46(b)/EphA2(nb) did not enhance tumor killing compared to NKp46 /EphA2 NK-TICA® (EC_{50}~2.pM). ADCC-capable anti-EGFR antibody was used as positive control. Luminescence for no NK-TICA® is shown at 10^{-14}M.

Figure 3. NK-TICA® enhances NK cytokine production in the presence of EphA2+ cell line


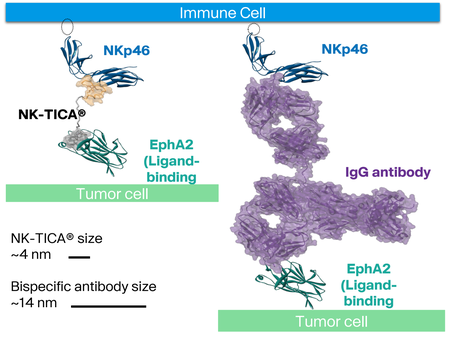
NK cells were co-cultured with HT1080-luc and NKp46/EphA2 binding or NKp46/EphA2 non-binding NK-TICAs. Cytokines in supernatants at 4hr (IFNγ, Figure 3A and TNFα, Figure 3B) and 48hr (FLT3L, Figure 3C), measured by MesoScale Discovery™ multiplex assay.

Figure 4. NK cells can be directed to kill tumor cells by NKp46 NK-TICAs employing multiple different tumor antigens: EphA2, MT-1 and PD-L1


NK cells co-cultured with HT1080-luc cells in the presence of NKp46 NK-TICAs of varying tumor binding arms: EphA2, MT-1, or PD-L1. ADCC-capable anti-EGFR antibody was used as positive control. Luminescence values for no NK-TICA® addition is arbitrarily shown as 5 x 10^{-15} M.

Modeling of NK-TICAs in complex with NKp46 and EphA2 in comparison to a bispecific antibody
Graphical model demonstrating the approximate size of an NK-TICA® and relative spacing when bound simultaneously to tumor target EphA2 and NKp46.

Shown for comparison is the size and relative bound spacing for a bispecific antibody (IgG) binding EphA2 and NKp46 at the same epitopes. (PDB: 6rw2, 6iap, 1hzh, Gauthier et al.2019).



CONCLUSIONS
- Building on success with CD137 Bicycle® TICAs, the Bicycle platform has now been successfully applied to build prototype NK cell engagers
- NK-TICAs drive NK cell-mediated tumor cell killing and cytokine production *in vitro* and as such have the potential to catalyze the development of durable anti-tumor immunity in tumor types not well served by current therapies

REFERENCES
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2. Lani et al. PEGS-Boston (2017)
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4. Wang et al. Oncogene. 40:717–730 (2021)
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