Cytokine affinity tuning using the *AlphaSeq* platform to generate targeted immuno-oncology therapeutics

A-ALPHA BIO

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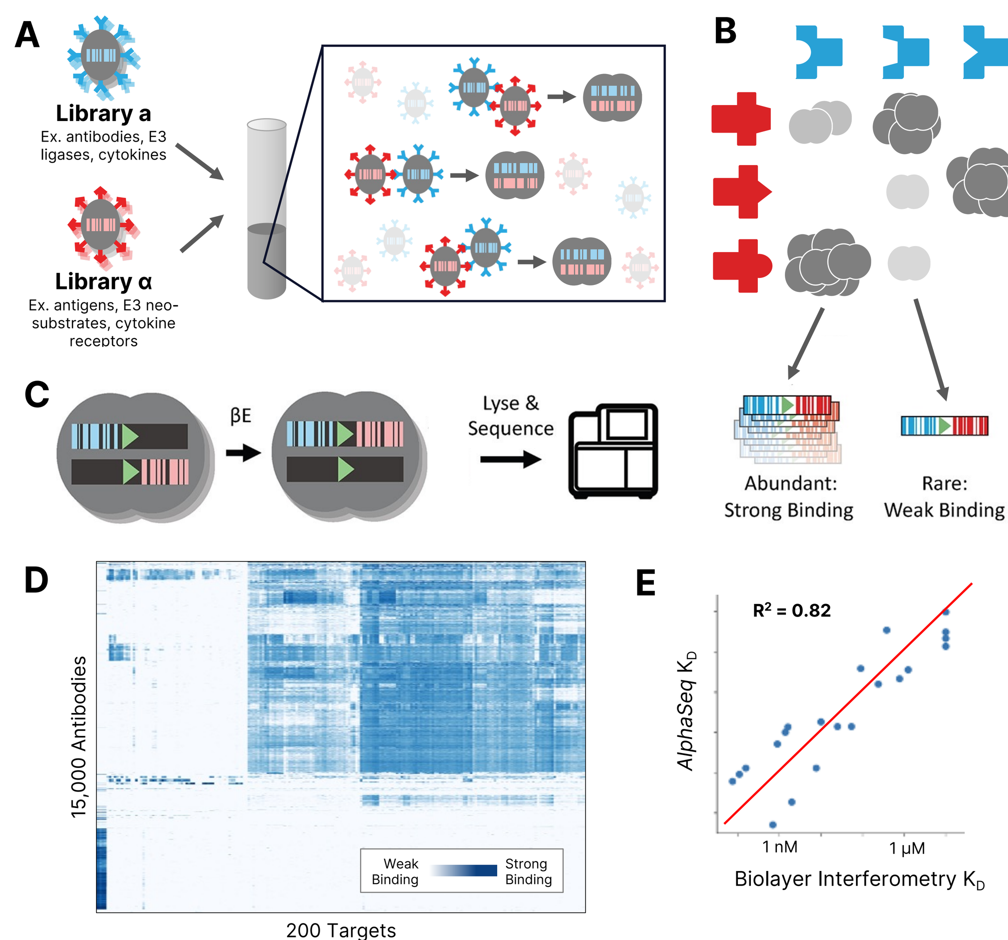
Abstract

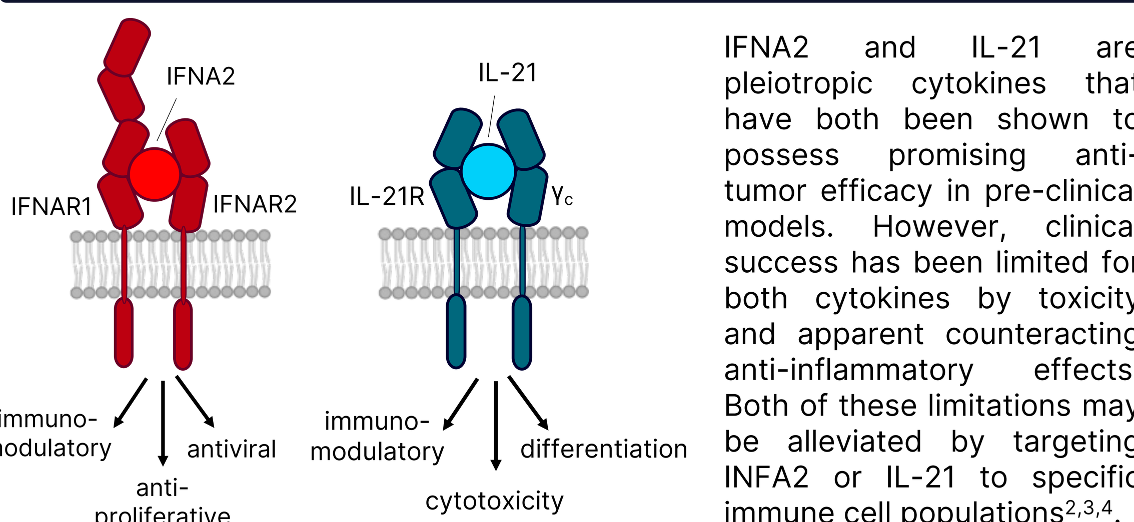
Despite promising anti-tumor effects, the potential of cytokine therapies has been hindered by dose-limiting toxicities and a narrow therapeutic index[1]. Next-generation cytokine therapies have been developed to drive conditional signaling specifically in the tumor microenvironment or targeted immune cell populations. Here we apply the *AlphaSeq* platform, which enables library-on-library screening of protein-protein interactions, to generate affinity-detuned cytokine therapeutic candidates and mouse-reactive surrogates for fusion to antibodies targeting immune cell subpopulations. Using IFNA2 and IL-21 as illustrative examples, we show that *AlphaSeq* can be used to identify hundreds of detuned cytokine variants with a wide range of affinities to the IFNAR2 and IL21R receptors, respectively. Candidate detuned cytokines were recombinantly expressed as Fc-fusion proteins and assayed for receptor binding and *in vitro* signaling, both of which showed strong correlation with *AlphaSeq*-derived affinity values. Finally, detuned IFNA2 candidates were fused to anti-CD8 antibodies to demonstrate cell population-specific signaling. We are continuing to deploy *AlphaSeq*'s potential for rapid, comprehensive, and multiplexed affinity determination to develop a portfolio of clinically relevant therapeutic immunocytokines.

AlphaSeq Platform

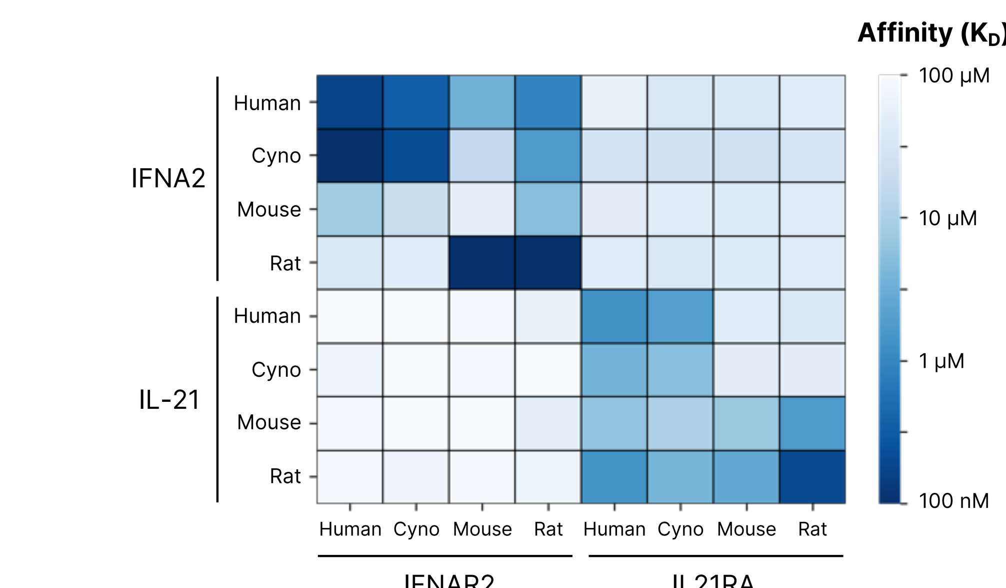


AlphaSeq uses synthetic biology and next generation sequencing to measure protein-protein interactions at a library-on-library scale. **(A)** Two libraries containing barcoded protein sequences for display on the yeast cell surface are mixed in liquid culture. Interactions between surface displayed proteins drive agglutination and cell fusion. **(B)** The number of fused cells with a given protein pair is dependent on protein interaction strength. **(C)** Recombination is induced with β-estradiol to consolidate DNA barcodes. Cells are then lysed and sequenced to count the abundance of each barcode pair and determine all protein interaction strengths. **(D)** Example *AlphaSeq* dataset measuring ~3 million interaction affinities in a single assay. **(E)** *AlphaSeq*-measured affinities strongly correlate with BLI-measured affinities.

IFNA2 and IL-21 background and *AlphaSeq* validation



IFNA2 and IL-21 are pleiotropic cytokines that have both been shown to possess promising anti-tumor efficacy in pre-clinical models. However, clinical success has been limited for both cytokines by toxicity and apparent counteracting anti-inflammatory effects. Both of these limitations may be alleviated by targeting INFA2 or IL-21 to specific immune cell populations[2,3,4].



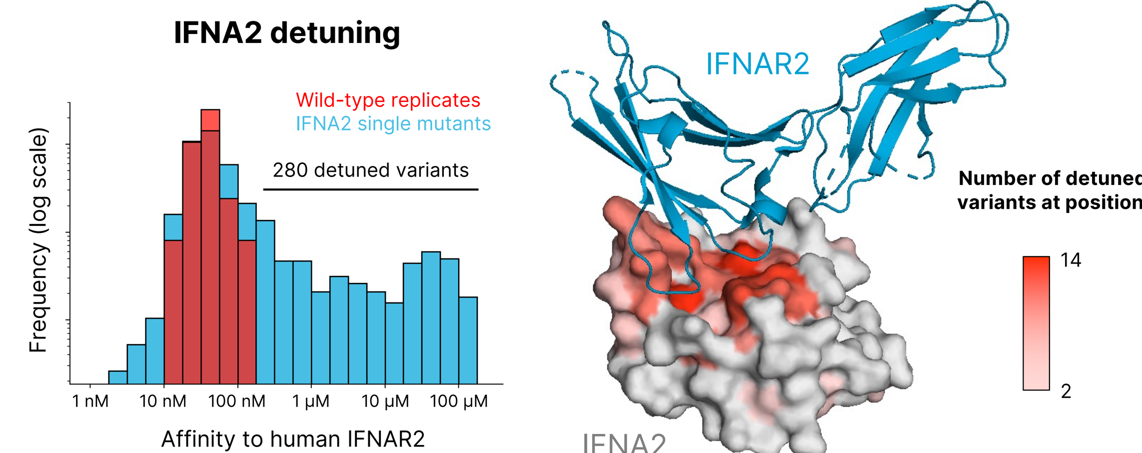
In a single *AlphaSeq* assay, the IFNA2/IFNAR2 and IL-21/IL21RA interactions were validated. In addition to the human cytokines and receptors, cytokine/receptor pairs were validated for cynomolgus monkey, mouse, and rat. For both IFNA2 and IL-21, the human cytokine showed strong cross-reactivity with the cyno receptor, but reduced cross-reactivity with the rodent receptors.

References

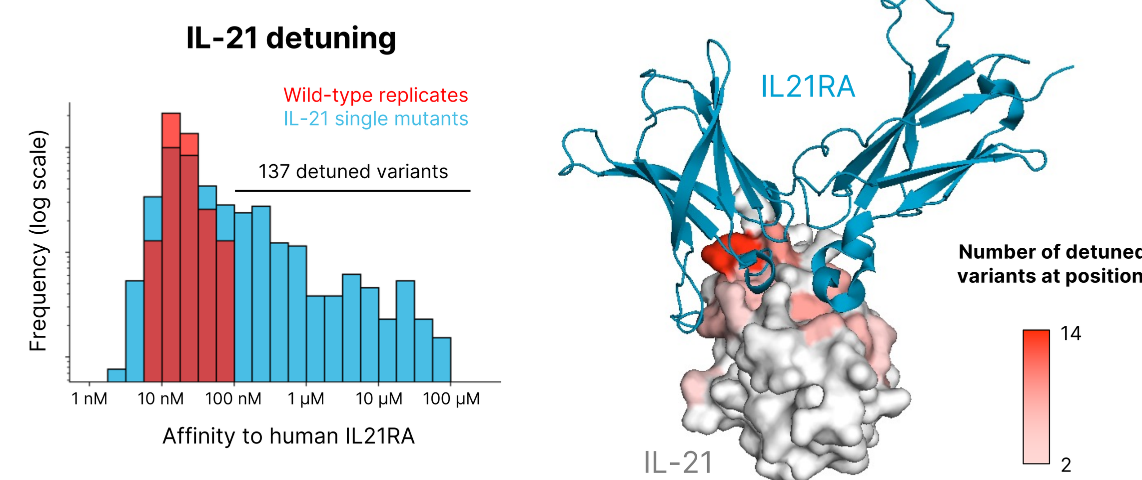
1. Deckers et al. Engineering cytokine therapeutics. *Nat. Rev. Bioeng.* (2023).
2. Boukhaled et al. Opposing roles of Type I interferons in cancer immunity. *Annu. Rev. Pathol.* (2021).
3. Liang et al. Targeting IFNα to tumor by anti-PD-L1 creates feedforward antitumor responses to overcome checkpoint blockade resistance. *Nat. Commun.* (2018).
4. Shen et al. Engineered IL-21 Cytokine Muteins Fused to Anti-PD-1 Antibodies Can Improve CD8+ T Cell Function and Anti-tumor Immunity. *Front. Immunol.* (2020).

AlphaSeq detuning of IFNA2 and IL-21

Site-saturation mutagenesis libraries were constructed of all IFNA2 and IL-21 single mutants. *AlphaSeq* was used to measure the affinity of each variant to human, cyno, rat, and mouse receptors simultaneously. Libraries were FACS sorted for expression such that low-expressing variants could be excluded from further analysis. Detuned variants to the human receptor were identified as those with decreased receptor affinity relative to wild-type cytokine.



280 detuned human IFNA2 variants were identified, spanning 3 orders of magnitude of affinity to human IFNAR2, from near-wild-type to background-level binding. Many IFNA2 detuning mutations were identified near the IFNAR2 binding interface, but detuning mutations distal to the binding interface were also identified.

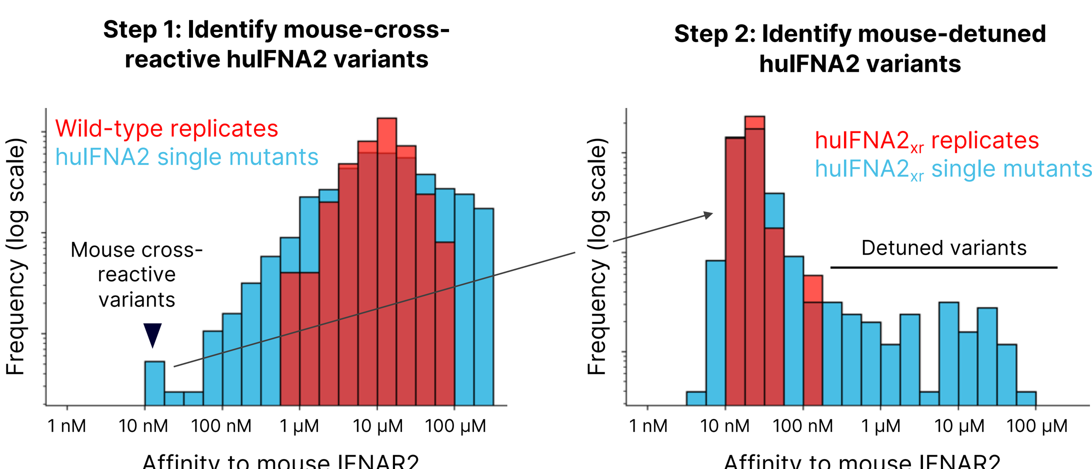


137 human IL-21 variants were identified with detuned affinity to human IL21RA. As with IFNA2, many, but not all, detuning substitutions were clustered at the IL21RA binding interface.

Parallel IFNA2 and IL-21 mouse surrogate development

With the goal of moving quickly into mouse syngeneic tumor modeling, detuning against the mouse IFNAR2 and IL21RA receptors was pursued in parallel to the human receptors. Due to different species cross-reactivity profiles, different approaches were taken for IFNA2 and IL-21:

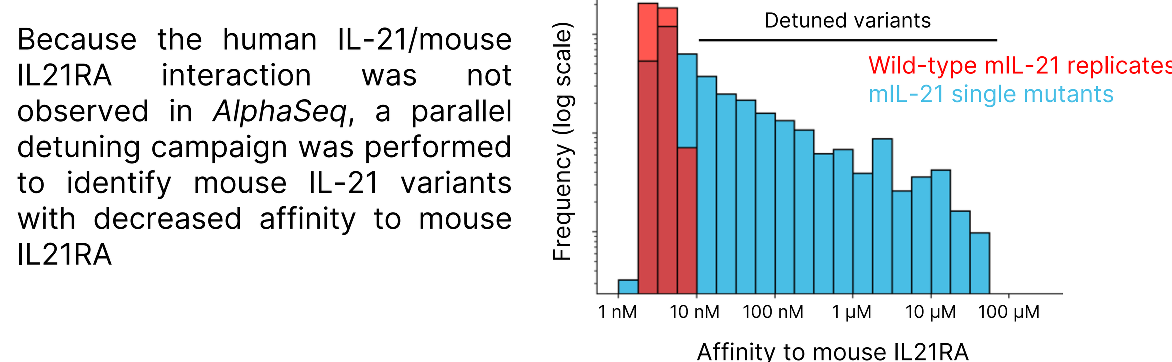
IFNA2: engineer human cytokine for mouse cross-reactivity



Wild-type human IFNA2 binds weakly (~10 μM) to mous[e] but individual IFNA2 point mutants confer strong cros[s] One such cross-reactive variant (hulFNA2$_{xr}$) was use[d] iteration SSM library to identify hulFNA2 variants d[...] IFNAR2.

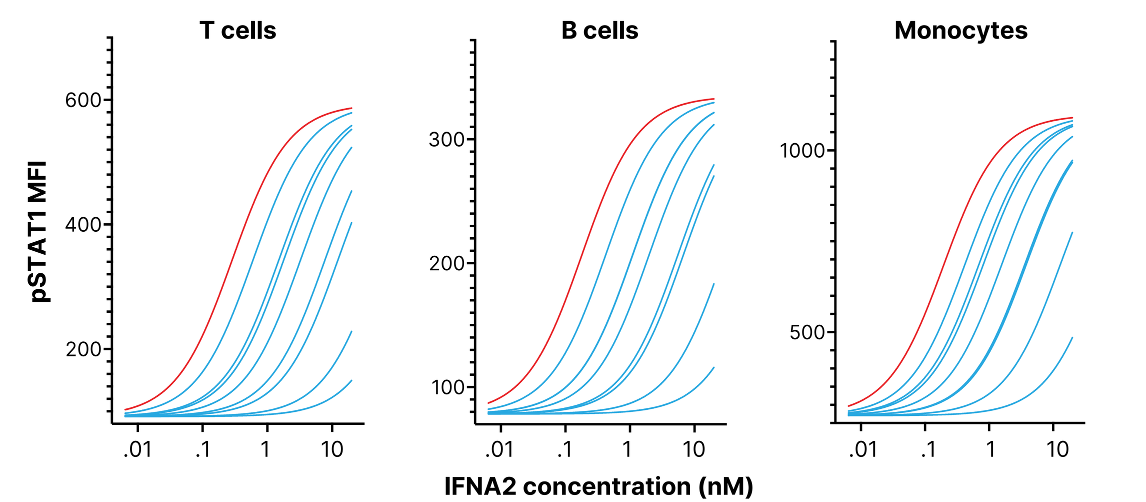
IL-21: parallel detuning of mouse c[...]

Because the human IL-21/mouse IL21RA interaction was not observed in *AlphaSeq*, a parallel detuning campaign was performed to identify mouse IL-21 variants with decreased affinity to mouse IL21RA

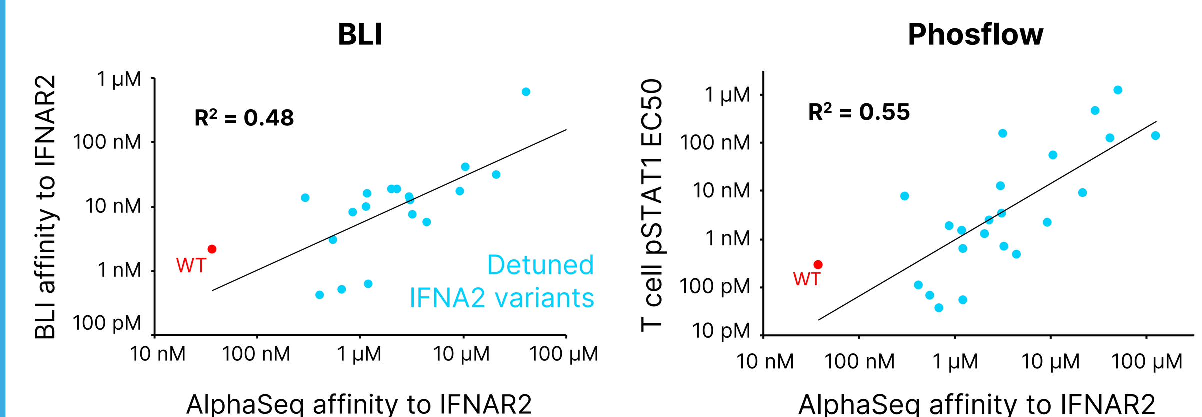


Recombinant IFNA2-Fc fusion proteins exhibit a wide range of detuning *in vitro*

A set of IFNA2 variants spanning 3 orders of magnitude of affinity to IFNAR2 were selected for mammalian expression as Fc fusion proteins. BLI was used to measure IFNA2-Fc variant affinity to IFNAR2, and a pSTAT1 Phosflow assay was used to characterize IFNA2-Fc variant potency in activating human PBMCs.



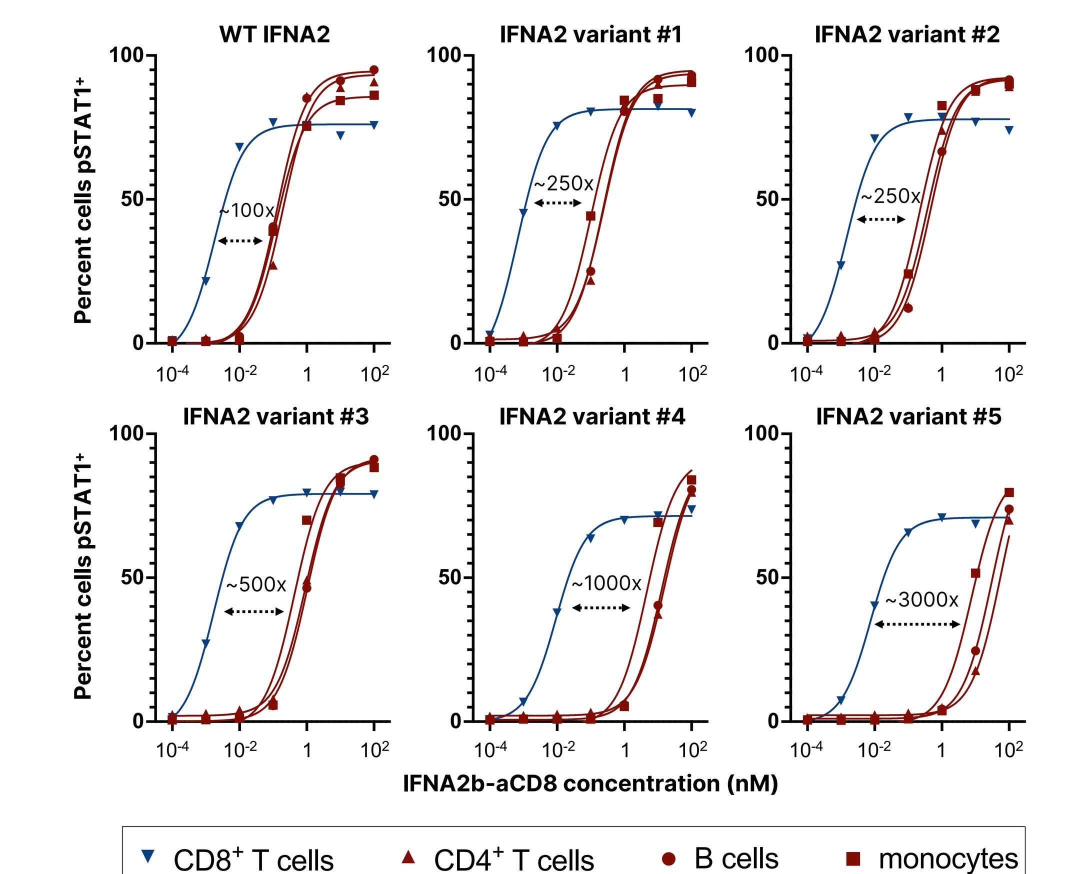
IFNA2-Fc fusions induced STAT1 phosphorylation with a wide range of potencies across several immune cell subsets.



AlphaSeq measurements of IFNA2/IFNAR2 interaction affinity were predictive of both BLI-measured IFNA2-Fc/IFNAR2 affinity and IFNA2-Fc signaling potency in human PBMCs.

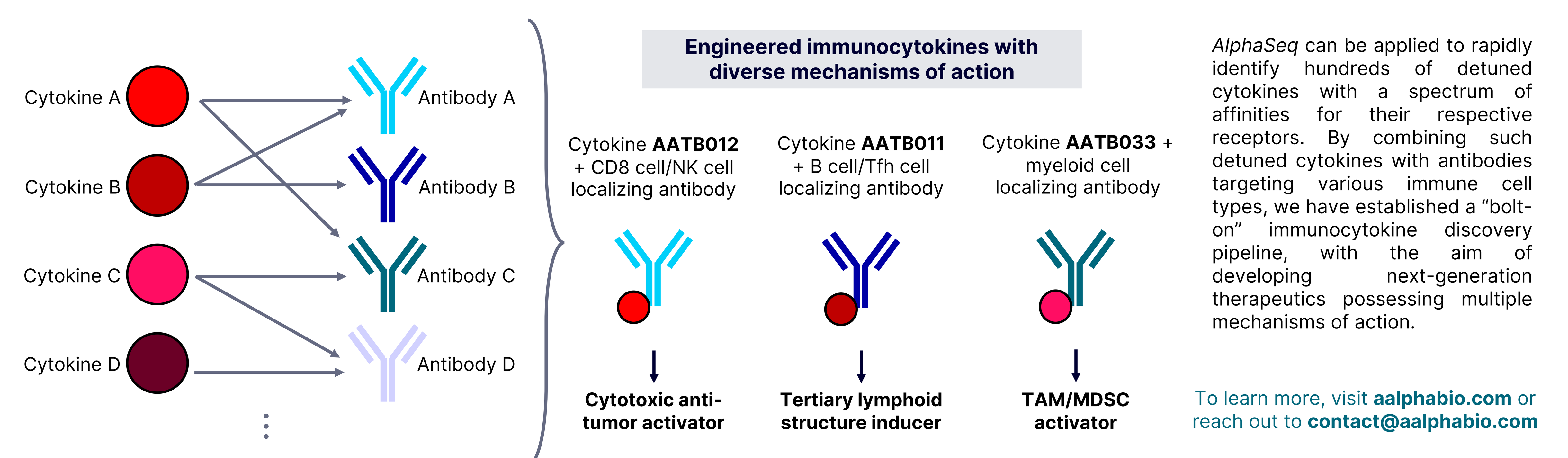
Cell-type specific signaling achieved with IFNA2-aCD8 immunocytokine

As a proof-of-concept demonstration of cis-targeted, cell-type-specific signaling, IFNA2 variants were fused to an anti-CD8 antibody in scFv-Fc format, generating immunocytokine IFNA2-aCD8 variants with a range of affinities to IFNAR2. These molecules were tested for cell-biased signaling in a pSTAT1 Phosflow assay in human PBMCs.



While wild-type IFNA2-aCD8 is biased by ~100-fold toward CD8⁺ T cells, detuned variant IFNA2-aCD8 molecules were identified with up to ~3,000-fold biased signaling in targeted vs. untargeted cells.

Summary and "bolt-on" immunocytokine discovery pipeline



AlphaSeq can be applied to rapidly identify hundreds of detuned cytokines with a spectrum of affinities for their respective receptors. By combining such detuned cytokines with antibodies targeting various immune cell types, we have established a "bolt-on" immunocytokine discovery pipeline, with the aim of developing next-generation therapeutics possessing multiple mechanisms of action.

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