A-ALPHA BIO

Abstract #1087

Preclinical Development of Cis-Acting, Affinity Tuned IL-21 Immunocytokines for Cancer Immunotherapy

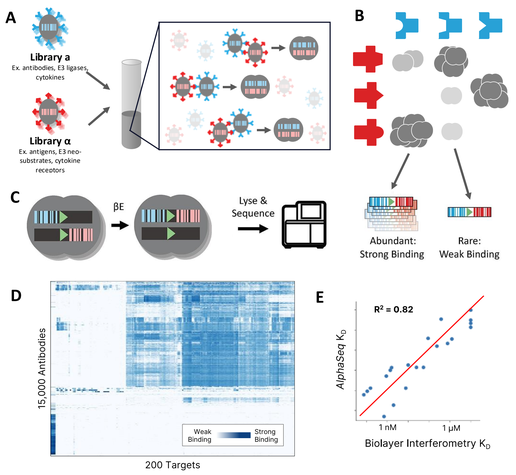
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Abstract

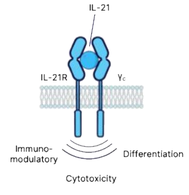
Although cytokine therapies have demonstrated curative effects in some cancer patients, clinical use remains limited due to inflammatory toxicity profiles accompanying systemic administration. Next generation cytokine approaches include specific targeting and conditional signaling, focusing on the tumor microenvironment or specific immune cell populations. Here, we share a novel approach for using the *AlphaSeq* platform to engineer IL-21 therapeutic candidates with increased stability and decreased affinity to the IL-21 receptor. These detuned candidates demonstrated improved analytics over parental IL-21 and strong cell-biased signaling when fused to a CD8-targeting antibody. While parental IL-21 led to dose-limiting toxicity in the murine MC38 tumor model, detuned IL-21 localized to either CD8+ cells or TIGIT+ cells elicited a strong anti-tumor response. In combination, the *AlphaSeq* lab platform and the *AlphaBind* ML platform allow the discovery and optimization of diverse biologics.

AlphaSeq Platform



AlphaSeq applies 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 depends 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.

IL-21 is a promising immuno-oncology cytokine

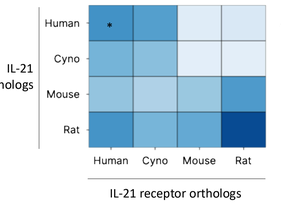


IL-21 is a pleiotropic cytokine with demonstrated anti-tumor properties and a unique mechanism of action

In addition to driving cytotoxic activity in CD8 cells via STAT3 activation, IL-21 supports stem-like phenotype for a more durable anti-tumor response.

However, poor stability, short half-life, and systemic toxicity have hindered the therapeutic potential of IL-21

AlphaSeq was used to measure pairwise interactions between several orthologs of IL-21 and IL-21R. The human cytokine bound the human and cynomolgus receptor, but not the rodent receptors. Surprisingly, the observed interaction affinity was ~1 μM, much weaker than expected, likely reflecting the known poor expression or stability of the cytokine.



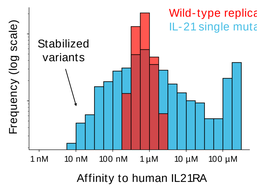
Engineering IL-21 stability using *AlphaSeq*

Although cytokines have a long history of recombinant expression, this class of proteins is known to have production and stability challenges. IL-21 possesses multiple biophysical liabilities, including an unusually high isoelectric point and low mammalian expression titers with resulting heterogeneous species. Additionally, fusing cytokines to antibodies for immunocytokine therapies can introduce further complications, such as cytokine payload clipping from the antibody fusion partner. For these reasons, we used the *AlphaSeq* platform to deeply explore human IL-21 sequence space and identify variants supporting manufacturing requirements.

Construct	ExpiCHO titer (μg/mL)	ASEC % Purity
IL-21_WT	54.0	55.5
Anti-CD8 mAb (control)	283.0	100.0

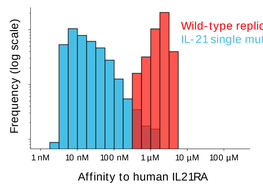
WT human IL-21 expressed as a C-terminal Fc fusion had low transfection titer and low purity following Protein A purification.

AlphaSeq identification of stabilized IL-21 single-mutants



An IL-21 site-saturation mutagenesis (SSM) library was built to identify stabilizing and detuning substitutions. Despite the weak measured affinity of the WT IL-21/IL-21R interaction (~1 μM), many single-mutant variants demonstrated enhanced affinity (highest affinity ~10 nM). Enhanced affinity substitutions were not confined to the IL-21R-binding interface, indicating that these substitutions likely increased cytokine expression or stability.

AlphaSeq identification of further stabilized IL-21 multi-mutant variants

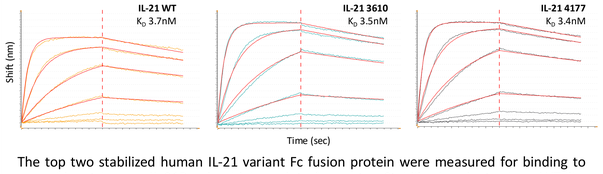


Using training data from above SSM IL-21 binding assay, we constructed a library of variants with up to 4 mutations from WT. The most stabilized variants bound with an affinity of ~2 nM. A subset of stabilized variants were selected for recombinant protein production and characterization.

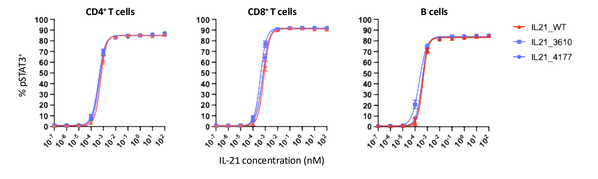
Construct	ExpiCHO titer (μg/mL)	ASEC % Purity
IL-21_WT	54.0	55.5
IL-21_2988	68.6	48.2
IL-21_3610	189.9	91.5
IL-21_4177	114.0	90.2
IL-21_4219	97.4	57.2
IL-21_0910	186.1	82.0
IL-21_4818	88.3	57.4
IL-21_4687	47.1	15.4

A subset of hits from *AlphaSeq* screening were expressed as Fc fusion proteins. 3 out of 7 selected variants had increased titer and purity as Fc fusion proteins.

Stabilized IL-21 variants demonstrate equivalent activity to WT IL-21

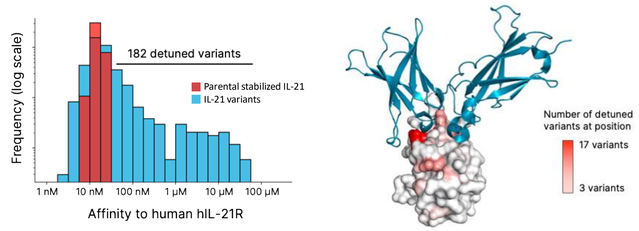


The top two stabilized human IL-21 variant Fc fusion protein were measured for binding to recombinant human IL-21R using biolayer interferometry. Stabilized variants show WT-like affinities.



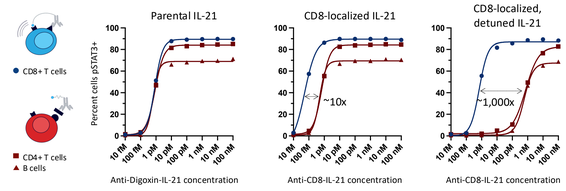
Human PBMC were stimulated with WT IL-21 Fc fusion protein or stabilized variants and STAT3 phosphorylation was quantified. Stabilized IL-21 variants signaled similarly to WT IL-21.

Tuning IL-21 affinity using *AlphaSeq*



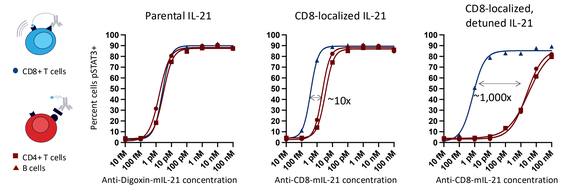
SSM libraries were constructed in several stabilized IL-21 backgrounds. *AlphaSeq* was used to measure affinities to human, cyno, rat, and mouse receptors simultaneously. The distribution of SSM affinities for one stabilized parental sequence is shown. This yielded a spectrum of hundreds of detuned IL-21 variants spanning several orders of magnitude of affinity. IL-21 positions with multiple identified substitutions that led to reduced IL-21R affinity were mapped on to the IL-21:IL-21R co-crystal structure. Detuning substitutions mostly cluster at the IL-21R binding interface, but some distal mutations also led to detuning.

Engineering cell-biased immunocytokines using detuned IL-21 variants



Stabilized and detuned IL-21 variants identified in *AlphaSeq* were formatted as immunocytokines by fusing to an anti-CD8 localizing antibody and assayed for activity in huPBMCs. Targeting non-detuned IL-21 to CD8 cells led to a modest increase in activity on targeted cells. Targeting the detuned IL-21 variant to CD8 cells led to dramatic cell-biased activity only on CD8 cells.

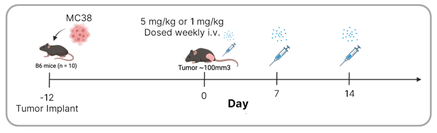
Parallel development of murine surrogate immunocytokine



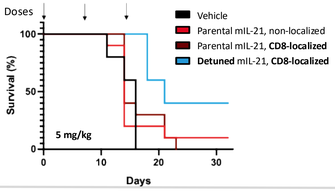
In parallel to the engineering of human IL-21 immunocytokines, a murine surrogate was developed with similar binding properties to the detuned human variant. Cell-biased activity was tested in mouse splenocytes, with the detuned surrogate showing a similar cell-biased activity to the human molecule.

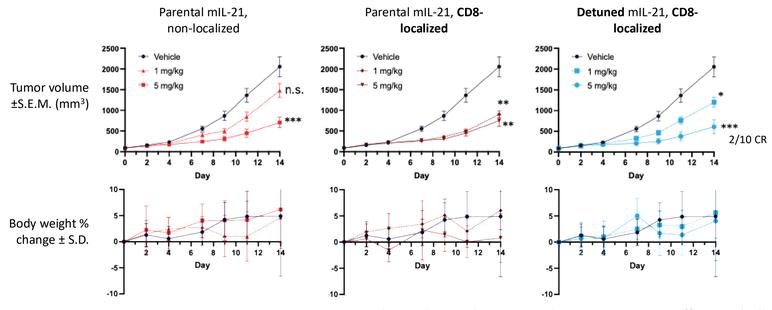
Mouse preclinical MC38 syngeneic tumor modeling

CD8-IL-21 immunocytokine efficacy modeling



To test the hypothesis that delivery of detuned IL-21 to CD8 cells would provide strong anti-tumor efficacy with minimal toxicity, we compared activity of non-localized mIL-21 control, CD8-localized mIL-21 and detuned CD8-localized mIL-21 in the MC38 tumor model.





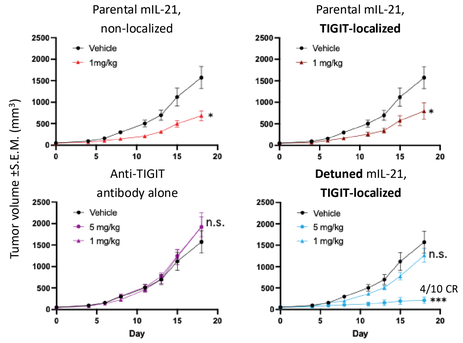
Number of animals euthanized following third dose

	Parental mIL-21, non-localized	Parental mIL-21, CD8-localized	Detuned mIL-21, CD8-localized
5 mg/kg	6/8	6/10	0/10
1 mg/kg	1/10	1/10	0/10

Non-detuned IL-21 demonstrated strong anti-tumor efficacy, which was enhanced by localizing to CD8 cells. Despite being well tolerated after two doses, both non-detuned molecules drove severe toxicity after administration of the third dose. In contrast, the detuned, CD8-localized IL-21 had equivalent anti-tumor effects as the parental molecule, without toxicity. This combination of high efficacy and low toxicity led to higher overall survival.

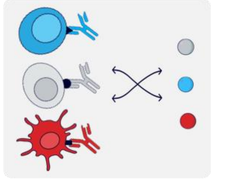
TIGIT-IL-21 immunocytokine efficacy modeling

To test the ability of IL-21 to drive anti-tumor activity via focused delivery to antigen-experienced cells, an MC38 efficacy study was performed using IL-21 localized to the checkpoint molecule TIGIT. The proprietary anti-TIGIT antibody was optimized using the *AlphaBind* ML platform for maximal affinity and cross-reactivity to human and mouse TIGIT. Because of the observed toxicity of the high dose non-detuned IL-21 molecules in the CD8 study, these agents were only delivered at the low dose. Detuned, TIGIT-localized IL-21 yielded a similar anti-tumor response as the equivalent CD8-localized molecule.



Significance calculated as two-way ANOVA comparison of tumor volume curves to control through last day all animals were on study, with Dunnett multiple comparison correction. * p < 0.05, ** p < 0.01, *** p < 0.0001

AlphaSeq and *AlphaBind* enable modular engineering of cytokines and antibodies



For more information on our platforms, pipeline, and partnerships, visit www.aalphabio.com

Or contact us at contact@aalphabio.com