Development of a Clinical *Ex Vivo* Assay for the Assessment of Therapeutic CD28 Costimulatory Pathway Engagement

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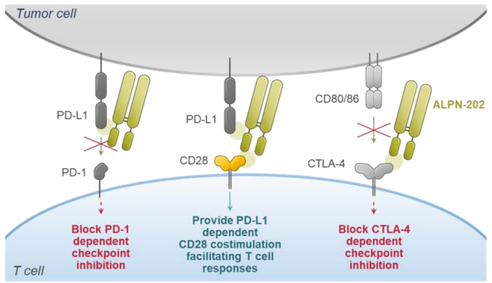
Abstract

Background: Preclinical evidence supports combining checkpoint inhibition (CPI) with T cell costimulatory agonism to improve the breadth and durability of anti-tumor responses relative to CPI alone. Currently, there are a number of therapeutic approaches combining costimulatory receptor agonists (e.g. CD28, 4-1BB, OX40L, etc.) with tumor targeting agents and/or CPI. Identification of a pharmacodynamically-justified therapeutic dose can be challenging because traditional duration of target occupancy does not necessarily correlate with immunological activity in the case of costimulatory molecules, and an 'always on' dose risks immune exhaustion. ALPN-202, a variant CD80 vIgD-Fc fusion protein that mediates PD-L1-dependent CD28 costimulation and inhibits the PD-L1 and CTLA-4 checkpoints, is in development for the treatment of multiple advanced malignancies. To assess clinical CD28 agonism in the context of ALPN-202 treatment, we developed a novel, *ex vivo* whole blood target-dependent costimulation (TDC) assay.

Methods: A TDC assay was developed using clinical samples from NEON-1 (NCT04186637), an ongoing dose escalation and expansion clinical trial of ALPN-202 for patients with advanced malignancies. The assay uses patient blood stimulated with paraformaldehyde-fixed, artificial antigen presenting cells (aAPC) expressing both cell-surface anti-CD3 and PD-L1. Pre-dose and end-of-infusion (EOI) blood was drawn from trial participants and co-cultured for 24 hours with the aAPCs in a pre-made assay plate. Plasma was collected and secreted IL-2 was quantified and used as a measure of PD-L1-dependent CD28 costimulation. Nonlinear regression was used to calculate area under the curve (AUC) for each condition and compared to a positive control (pre-dose blood stimulated with a fixed concentration of ALPN-202).

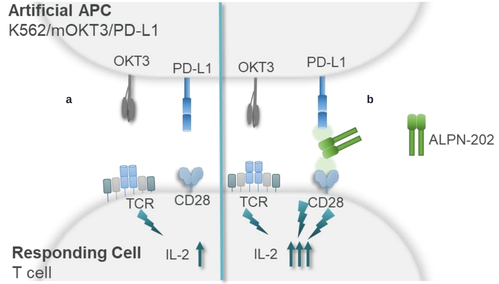
Results: Using the *ex vivo* TDC assay, ALPN-202 demonstrated PD-L1-dependent T cell costimulation at all dose levels tested to date in the NEON-1 clinical trial, consistent with preclinical assay development data.

Figure 1: ALPN-202 Mechanisms of Action



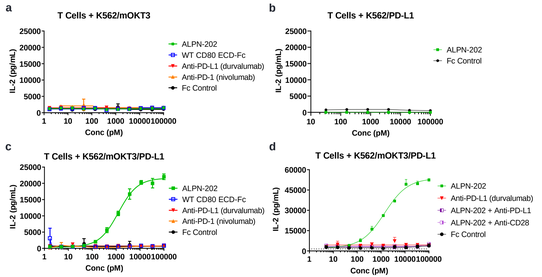
ALPN-202 binds PD-L1 on the tumor cell or APC, blocking PD-L1/PD-1 interactions. PD-L1-bound ALPN-202 provides a *trans* CD28 signal to T cells making contact with the tumor or APC (PD-L1-dependent CD28 costimulation). Additionally, ALPN-202 binds CTLA-4 expressed on Treg cells, preventing interaction with CD80/86.

Figure 2: T cell activation with artificial APCs and ALPN-202



T cells are co-cultured with a K562 aAPC expressing membrane anchored anti-CD3 (mOKT3) and PD-L1 in the target-dependent costimulation (TDC) assay. (**a**) In the absence of ALPN-202, costimulation does not occur and IL-2 production is at baseline levels. (**b**) When ALPN-202 is present and anchored to PD-L1 on the aAPC, ALPN-202 induces a CD28 costimulatory signal, resulting in additional IL-2 production.

Figure 3: T cell activation by ALPN-202 requires simultaneous engagement with PD-L1 and CD28

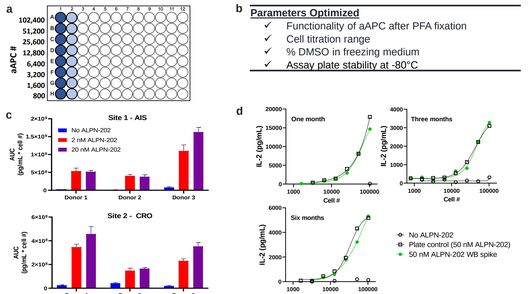


Primary human T cells were co-cultured 24 hours with test articles and K562 aAPC expressing membrane anchored (**a**) anti-CD3 (mOKT3), (**b**) PD-L1, or (**c, d**) both. (**a**) In the absence of PD-L1 to anchor ALPN-202 on the aAPC, no increase in IL-2 was detected above background. (**b**) Similarly, in the absence of TCR stimulation, ALPN-202 did not induce IL-2 production despite presence of PD-L1. (**c**) When both mOKT3 and PD-L1 were present on the aAPC, ALPN-202 induced a strong dose-dependent costimulatory signal well above that of WT CD80 ECD-Fc or PD-(L)1 blockade alone. (**d**) The costimulatory activity of ALPN-202 was inhibited when combined with blocking antibodies to either PD-L1 or CD28, confirming the requirement for dual binding to induce costimulation.

References:

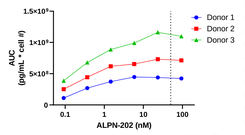
- Moser, J et al (2021), First-in-human dose escalation of ALPN-202, a conditional CD28 costimulator and dual checkpoint inhibitor, in advanced malignancies. *J Clin Oncol* 39:15_suppl, 2547-2547
- Food and Drug Administration (2005). Guidance for industry: estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. *Center for Drug Evaluation and Research (CDER)*, 7.

Figure 4: Paraformaldehyde-fixed aAPCs in pre-made assay plates enable consistency between testing sites



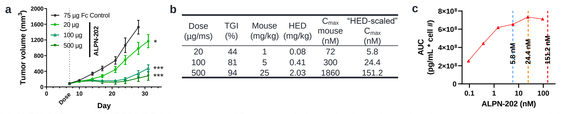
(**a**) Pre-made assay plates contain a serial dilution of PFA-fixed aAPC (K562/mOKT3/PD-L1) and either vehicle control (Column 1, dark blue) or 50 nM ALPN-202 (Column 2, light blue). (**b**) Several parameters were considered for optimization of assay performance and ease of use at multiple testing sites. (**c**) Assay plates prepared at AIS were shipped to CRO on dry ice and tested using healthy donor whole blood spiked with varying concentrations of ALPN-202. Samples were plated in triplicate to confirm inter-plate reliability and testing was performed at both AIS and CRO to confirm inter-site reliability. (**d**) Assay plates were stored at -80ºC and tested at one, three, and six months using healthy donor whole blood either with or without a 50 nM ALPN-202 spike. A different blood donor was used at each timepoint.
PFA – paraformaldehyde; AIS – Alpine Immune Sciences; CRO – Contract Research Organization

Figure 5: TDC assay response across a range of ALPN-202



During preclinical assay development, whole blood from three healthy human donors was incubated overnight with a serial dilution of ALPN-202. The following day, samples were added to assay plates and incubated for 24 hr. Plasma was collected and quantified for IL-2. IL-2 results were plotted by aAPC cell number and fit with a nonlinear regression curve. Area under the curve (AUC) values were calculated and plotted by ALPN-202 serial dilution, as shown. Responses from all three donors across a range of ALPN-202 treatment showed near-maximum IL-2 response at 50 nM ALPN-202 (*dotted line*), which was chosen as positive control in pre-made assay plates (Figure 4).

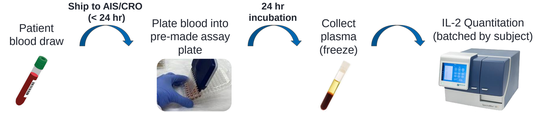
Figure 6: Activity in TDC assay correlates with preclinical anti-tumor efficacy



Dose (µg/ms)	TGI (%)	Mouse (mg/kg)	HED (mg/kg)	C_{max} mouse (nM)	"HED-scaled" C_{max} (nM)
20	44	1	0.08	72	5.8
100	81	5	0.41	300	24.4
500	94	25	2.03	1860	151.2

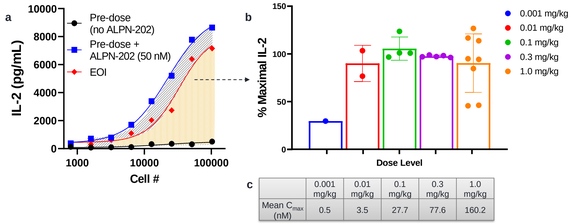
(**a**) ALPN-202-treated MC38/hPD-L1 tumors (n = 12 per group) showed a dose-dependent increase in anti-tumor activity (**b**) Median tumor growth inhibition (TGI) data is from Day 24 post implant. PK results from this tumor study demonstrated serum ALPN-202 exposure increased with increasing dose. Human equivalent dose (HED) conversion was performed according to FDA guidance document (mouse to human, divide by 12.3). (**c**) "HED-scaled" C_{max} is overlaid on a representative healthy donor response in the TDC assay. An increase is observed between the lowest and middle dose in both TGI and TDC assay response. Less difference is observed between the middle and highest doses, indicating near-maximum activity in both TGI and TDC assay response.

Figure 7: Whole Blood TDC assay procedure



Whole blood drawn from trial participants is shipped overnight to AIS or CRO. Upon receipt, assay plate is thawed and whole blood is added to each assay well and plate incubated at 37ºC. After 24 hr, plasma is collected and frozen until ready for IL-2 analysis performed at AIS.

Figure 8: Predicted TDC assay activity observed with human exposure to ALPN-202 monotherapy



c

	0.001 mg/kg	0.01 mg/kg	0.1 mg/kg	0.3 mg/kg	1.0 mg/kg
Mean C_{max} (nM)	0.5	3.5	27.7	77.6	160.2

(**a**) Human IL-2 is quantified using a mix-and-read HTRF assay (Cisbio). IL-2 values are interpolated from a standard curve and plotted by aAPC cell number (representative data shown). AUC values calculated in GraphPad Prism are used to compare pre-dose and end-of-infusion (EOI) responses. (**b**) To normalize across subjects, response at EOI is divided by response in pre-dose 50 nM ALPN-202 control condition and presented as % Maximal IL-2. ALPN-202 provides target-dependent costimulation across multiple doses. (**c**) Preliminary observed mean C_{max} following the first dose in human blood shows dose-dependent increases.

Summary and Conclusions

- We have developed a novel *ex vivo* assay to assess induction of PD-L1-dependent CD28 costimulation in a clinical therapeutic setting.
- This assay has been successfully employed to monitor controlled PD-L1-depdendent costimulation by the CD28 agonist therapeutic candidate ALPN-202 in the ongoing NEON-1 clinical trial (NCT04186637).
- This type of cell-based, *ex vivo* TDC assay could be adapted more broadly to assess costimulatory receptor engagement, particularly target-dependent costimulation, of other costimulatory agonist therapeutic targets in clinical development.
- See also Poster 497: A Study of ALPN-202, a PD-L1-dependent CD28 Costimulator and Dual Checkpoint Inhibitor, in Combination with Pembrolizumab in Patients with Advanced Malignancies (NEON-2)

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