# Novel Variant Ig Domain (vIgD) Proteins Generated Via Directed Evolution of IgSF Domains Have Therapeutic Efficacy in Animal Models of Graft Versus Host Disease

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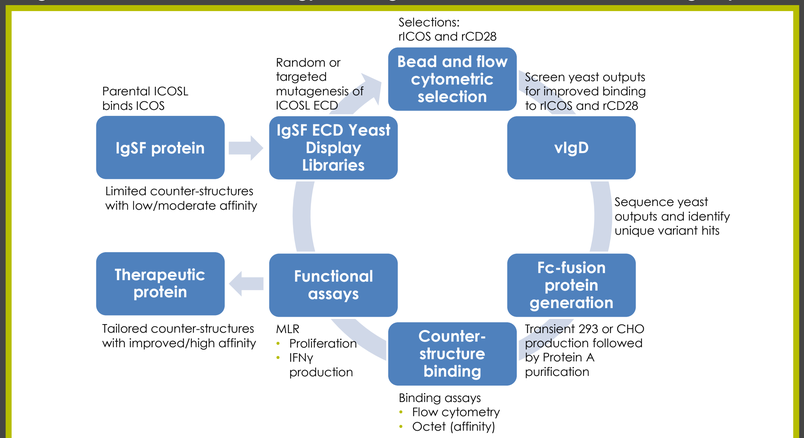
## Abstract

The immunoglobulin superfamily (IgSF) is a large, diverse family of proteins extensively targeted for treatment of cancers and autoimmune diseases. Most of the therapeutic strategies targeting this family have focused on high affinity antibodies binding a single receptor. Wild-type IgSF receptors typically exhibit low affinities for their counter-structures, limiting their utility in therapeutic modulation of immune responses. We have developed a novel variant Ig domain™ (vIgD™) directed evolution platform to affinity mature human IgSF extracellular domains. In this platform, libraries of mutagenized IgSF domains are selected for altered affinity to specific recombinant protein counterstructures. Fc fusion proteins incorporating the resulting engineered IgSF domains are then tested *in vitro* for their ability to either agonize or antagonize T cell responses.

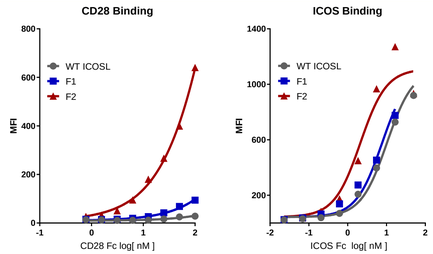
We successfully engineered the IgSF protein ICOSL, which binds to the costimulatory molecule ICOS on T cells and triggers T cell activation. We used our directed evolution platform to engineer a first-in-class single vIgD domain with higher affinity for ICOS and high affinity binding to the costimulatory molecule CD28, which ICOSL does not normally bind. Following the creation of dual ICOS/CD28 antagonism, the resulting domains were used to generate individual constructs encoding Fc-fusion proteins. These enhanced ICOS/CD28 dual antagonist Fc-fusion proteins were produced in HEK-293 cells and purified proteins were then tested in a FACS based binding assay on cells transfected with each costimulatory receptor showing up to a 2-3-fold increase in ICOS binding and high affinity binding to CD28. Various ICOS/CD28 dual antagonist Fc-fusion proteins were then tested in soluble format over a range of concentrations for functional activity in mixed lymphocyte reaction cultures (MLR) where allogeneic dendritic cells (DC) were used to stimulate purified human T cells. Most variants showed significant ability to inhibit the MLR response as read out measuring proliferation and cytokine production. The potency of the Fc-fusion proteins varied somewhat depending on their relative affinities for CD28 and ICOS, with all being superior to wild type ICOSL protein and many showing superiority to the CD28 blocking reagent belatacept (CTLA4-Fc). The level of inhibition correlated well with binding of the Fc-fusion proteins to the T cells in culture.

To assess the function of the ICOS/CD28 dual antagonists *in vivo*, ICOSL vIgD-Fc proteins were tested in the human PBMC-NSG™ GVHD mouse model. This model monitors graft versus host disease generated by xenogeneic responses of T cells in human PBMCs transferred into NSG™ mice. Administration of the dual ICOS/CD28 antagonist Fc-fusion proteins significantly protected mice from the effects of xenogeneic T cell activation *in vivo* with treated animals showing dramatically enhanced survival and greatly reduced disease scores. The level of protection correlated with the potency of the molecules in the *in vitro* MLR assay with less potent molecules in MLR assays failing to protect while more potent molecules protected well. Belatacept was also effective in protecting animals from GVHD, but not as effective as the ICOS/CD28 dual antagonists produced by the vIgD platform. Collectively, these data demonstrate the vIgD directed evolution platform can generate IgSF proteins with altered affinities and improved binding properties. Variants engineered for desired immunological properties can effectively perturb T cell costimulation and affect T cell responses both *in vitro* and *in vivo* with the *in vitro* potency of the proteins correlating to *in vivo* potency. The dual ICOS/CD28 antagonists produced with the vIgD platform can be used therapeutically in disease settings where attenuation of T cell responses is beneficial, including GvHD and potentially other autoimmune diseases. Phase I enabling studies are in progress.

## Figure 1: Directed Evolution Strategy Selecting Protein Variants with Altered Binding Properties

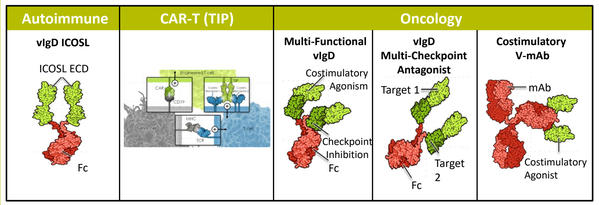



## Figure 2: Simultaneous Affinity Maturation of ICOSL Towards Two Receptors




**Dual-Ligand Affinity Maturation with a Single, Randomly-Mutated ICOSL vIgD Library**
Yeast were transformed with a single, randomly-mutated vIgD library and affinity matured by selection with CD28 and ICOS. Individual receptor binding to bulk yeast populations are shown, including two rounds of flow cytometric selection. Improvements to both ligands were noted after 1st selection (F1), and were further improved after 2nd selection (F2). MFI, mean fluorescence intensity.

## Figure 3: vIgDs can be Formatted Specifically for Various Therapeutic Applications




**vIgDs from Alpine's directed evolution platform may have multiple therapeutic formats**

- Fusion proteins (Fc or mAb) with antagonistic or tumor-localizing agonistic activity
- Cell-displayed (TIP)™ or secreted (SIP)™ versions for enhancement of adoptive cellular therapies

## Figure 4: CD28 and ICOS Costimulation in Graft Versus Host Disease

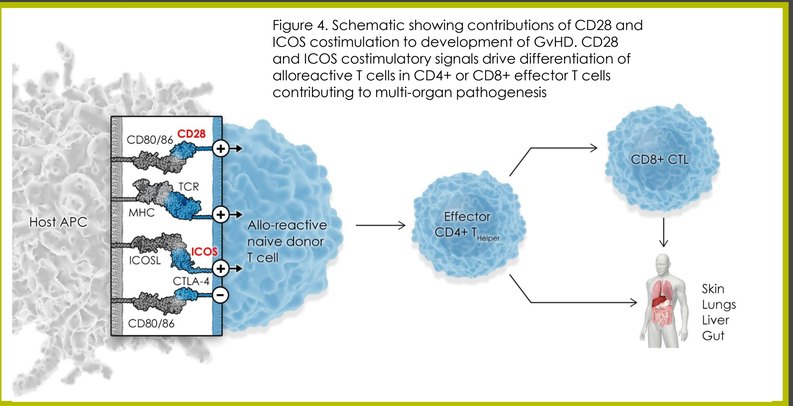



Figure 4. Schematic showing contributions of CD28 and ICOS costimulation to development of GvHD. CD28 and ICOS costimulatory signals drive differentiation of alloreactive T cells in CD4+ or CD8+ effector T cells contributing to multi-organ pathogenesis

## Figure 5: ICOSL vIgD-Fc, an ICOS/CD28 Dual Antagonist for Autoimmunity/Inflammation

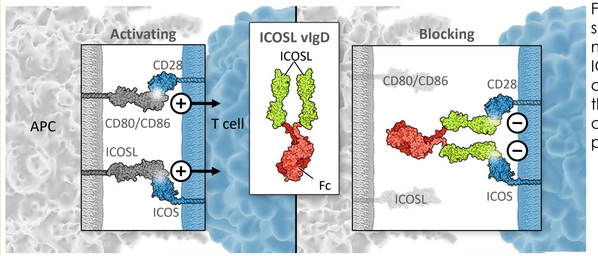
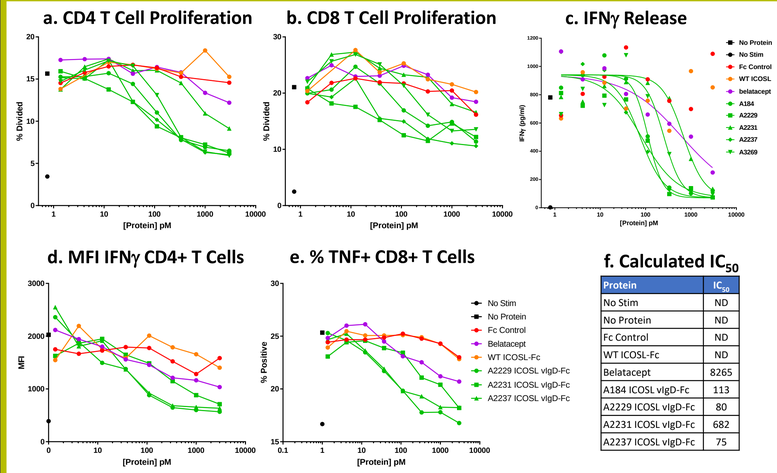



Figure 5. Schematic showing proposed mechanism of action for ICOSL vIgD-Fc with its capacity to inhibit both the CD28 and ICOS T cell costimulation pathways

## Figure 6: ICOSL vIgD-Fc Proteins Suppress T Cell Responses *in vitro*




**f. Calculated $IC_{50}$**

| Protein | $IC_{50}$ |
|---|---|
| No Stim | ND |
| No Protein | ND |
| Fc Control | ND |
| WT ICOSL-Fc | ND |
| Belatacept | 8265 |
| A184 ICOSL vIgD-Fc | 113 |
| A2229 ICOSL vIgD-Fc | 80 |
| A2231 ICOSL vIgD-Fc | 682 |
| A2237 ICOSL vIgD-Fc | 75 |

Figure 6. Soluble, engineered ICOSL vIgD-Fc proteins effectively attenuate T cell responses in a human mixed lymphocyte reaction (MLR). Monocyte derived dendritic cells from one donor were used to allogeneically stimulate T cells from another donor. Responses were measured by assessing (a) CD4 T cell proliferation, (b) CD8 T cell proliferation, (c) IFN-γ levels in culture supernatants, and (d-e) the percentage of CD4+ T cells that stained for intracellular IL-21 (d) or IL-4 (e). Proliferation results use CFSE dilution to show the percentage of divided cells versus protein concentration. IFN-γ levels are shown as pg/ml versus protein concentration. Intracellular cytokine staining is reported as (d) the MFI of CD4+ IFN-γ + T cells or (e) the percentage of CD8+ T cells that stained for TNF. (f) $IC_{50}$ values (pM) were calculated based on IFN-γ release data in (c) using GraphPad Prism. ND = could not be determined. Results shown are representative of at least three separate experiments performed with each protein.

## Figure 7: ICOSL vIgD-Fcs Protect from GvHD *in vivo*

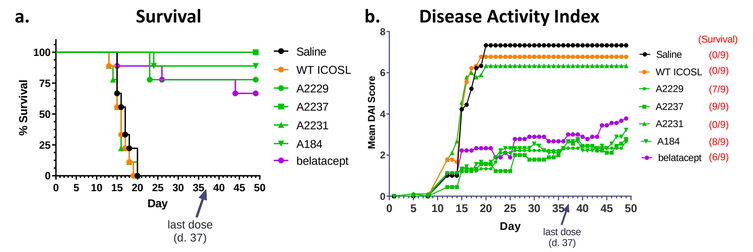



Figure 7. ICOSL vIgD-Fc proteins suppress immune responses *in vivo*. An acute model of graft-versus-host-disease (GvHD) was performed by adoptively transferring human PBMC into immunodeficient NSG mice (n=9/group). High affinity ICOSL vIgD-Fc proteins significantly (a) prolonged survival and (b) reduced mean disease activity index (DAI; a score of overall health and weight loss). Administration of high affinity ICOSL vIgD-Fc proteins protected from effects of GVHD at levels comparable to or better than belatacept, but wild-type ICOSL-Fc or a lower affinity ICOSL vIgD-Fc fusion protein (A2231) were not effective in protecting from GVHD in this model. (a) By log-rank test, belatacept and high affinity ICOSL-Fc proteins significantly prolonged survival as compared to saline and WT ICOSL-Fc ($p < 0.001$); ICOSL vIgD-Fc A2237 prolongs survival as compared to belatacept ($p = 0.065$). (b) By 2-way repeated-measures ANOVA, belatacept and high affinity ICOSL vIgD-Fc proteins significantly reduce DAI scores as compared to saline and wild-type ICOSL-Fc ($p \leq 0.001$); ICOSL-Fc A2229 and A2237 reduce DAI scores as compared to belatacept ($p = 0.053$ and $p = 0.035$, respectively).

## Figure 8: ICOSL-vIgD Prevent T Cell Activation *in vivo* and Preserve Naïve T Cell Phenotype

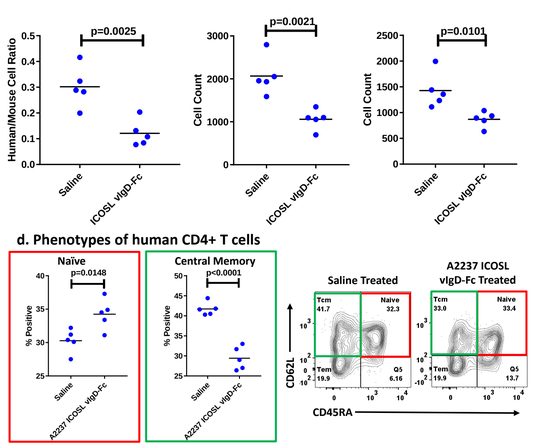



Figure 8. T cells from blood samples taken on day 6 from GvHD mice either treated with saline or A2337 ICOSL vIgD-Fc were stained for human CD4, CD8, CD62L and CD45RA and mouse CD45 to evaluate effects of treatment on T cell expansion and memory cell development. Treatment with ICOSL vIgD-Fc reduced (a) the ratio of human cells to mouse cells and (b) human CD4+ and (c) CD8+ T cell numbers in the blood. (d) The distribution of naïve and memory cell populations were also assessed based on expression of CD45RA and CD62L using the gating strategy shown. Treatment with ICOSL vIgD-Fc increased the percentage of cells retaining a naïve phenotype and reduced the number of cells with a central memory profile (Tcm). p values based on Student's t-test.

## Summary and Conclusions

- A variant Ig domain (vIgD) platform has been developed to generate novel immunomodulatory IgSF-based protein therapeutics with increased affinity and multiplicity of ligand binding, translating into superior preclinical efficacy *in vitro* and *in vivo*
- ICOSL vIgD-Fcs demonstrate novel, high affinity binding to CD28 and ICOS and can inhibit these costimulatory pathways
- ICOSL vIgD-Fcs demonstrate superior efficacy to belatacept (CTLA4-Ig) *in vitro* in human MLR assays including inhibition of T cell proliferation and cytokine production, including many of the cytokines induced in a GVHD response
- ICOSL vIgD-Fcs protected better than belatacept in a humanized GVHD *in vivo* model
- The vIgD therapeutic platform has broad potential to enhance the activity of biologics in treatment of human disorders driven or subject to modulation by IgSF proteins, including autoimmunity, cancer and infectious diseases
- Preclinical development of ICOSL vIgD-Fc (ALPN-101) is underway to support clinical studies

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