Tumor-Localizing NKp30/ICOSL vIgD Fusion Proteins Direct Effective Dual CD28/ICOS T cell Costimulation to B7-H6+ Tumor Cells *In Vitro* and Tumors *In Vivo*

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Abstract

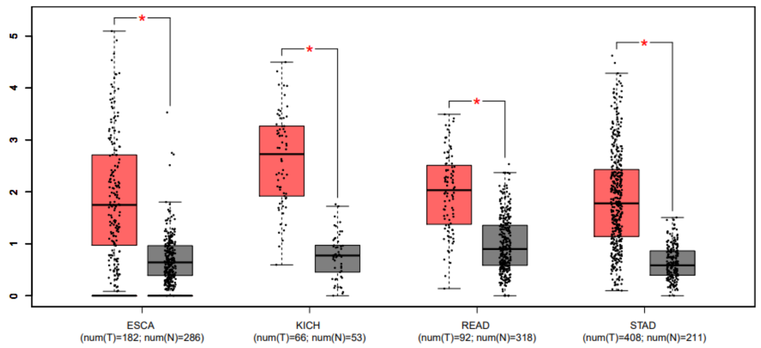
Background: Although checkpoint inhibitor therapies have significantly improved outcomes in multiple cancers, complete and durable responses remain infrequent, possibly attributable to a lack of adequate T cell costimulation and/or activating signals. Novel therapeutic proteins which confer T cell costimulation may be particularly effective anti-tumor therapies, particularly in combination with checkpoint inhibitors. But at the same time, localization of such costimulatory activity to tumors, such as via a tumor-specific targeting antigen, may be simultaneously important to maintain tolerability of such agonist therapeutics. B7-H6, a cell surface immunoglobulin superfamily (IgSF) member which binds the NKp30 receptor, appears to be expressed specifically in multiple tumor types, and may serve as such a tumor-specific antigen. Novel therapeutic proteins which localize costimulatory agonist domains to B7-H6 may therefore be capable of significant antitumor efficacy yet may be safely administered systemically by preferentially localizing agonist activity to the B7-H6 tumor microenvironment.

Methods: Using our platform technology, which is based on the directed evolution of IgSF members, NKp30/ICOSL variant immunoglobulin domain (vIgD) fusion proteins were created from NKp30 vIgDs with high affinity against B7-H6 and ICOSL vIgDs, which dually agonize the T cell costimulatory receptors ICOS and CD28. These tumor-localizing vIgD proteins were evaluated *in vitro* in T cell costimulation assays with target cells with or without B7-H6, and *in vivo* in a B7-H6+ CT26 mouse tumor model.

Results: NKp30/ICOSL vIgD-Fc fusion proteins conferred effective B7-H6-dependent costimulation to T cells *in vitro*, with enhanced T cell proliferation and cytokine production (IFNγ, TNFα, IL-2) in response to B7-H6-expressing but not B7-H6-negative target cells. Isolated ICOSL and NKp30 vIgDs alone were not efficacious. Importantly, NKp30/ICOSL vIgD-Fc fusion proteins demonstrated anti-tumor efficacy in a B7-H6+ mouse tumor model, especially when combined with a PD-1 inhibitor (Figure 1).

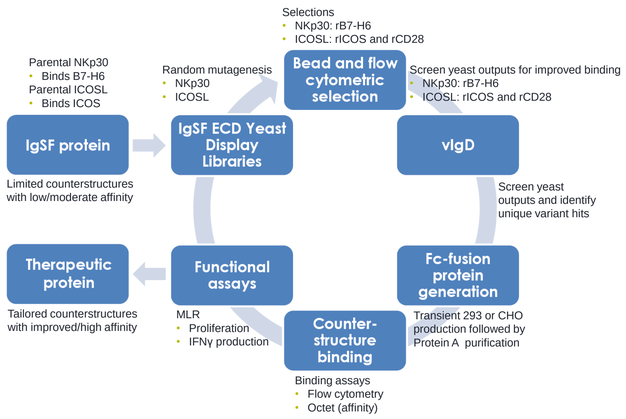
Conclusions: Tumor-localizing NKp30/ICOSL vIgDs confer potent T cell costimulation via CD28 and ICOS dependent upon the tumor antigen B7-H6 and elicit encouraging efficacy against B7-H6+ tumors *in vivo*, including in combination with PD-1 inhibitors. Such fusion proteins may provide particularly effective therapeutics for B7-H6+ tumors either as monotherapy or in combination with checkpoint blockade. These findings further suggest tumor-localized immunomodulation is possible and may improve cancer outcomes.

Figure 1: B7-H6 (NCR3LG1) is an IgSF protein overexpressed in some cancer types



Expression of B7-H6 in tumor samples compared to corresponding normal tissues: Expression of B7-H6 was evaluated in cancers and normal tissues using GEPIA and the Cancer Genome Atlas data. Shown are those cancer types where the expression in tumor tissue was significantly greater compared to corresponding normal tissues ($p < 0.01$), including esophageal carcinoma (ESCA), kidney chromophobe renal carcinoma (KICH), rectal adenocarcinoma (READ), and stomach adenocarcinoma (STAD).

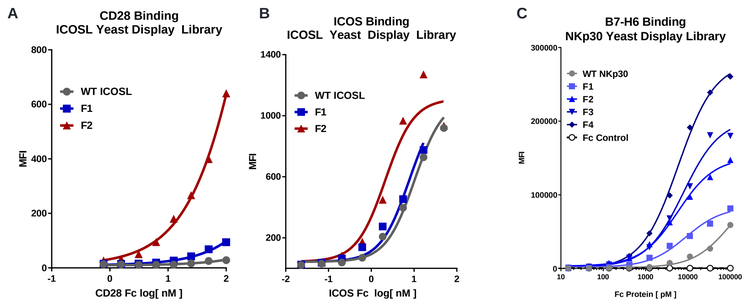
Figure 2: The vIgD platform was used to engineer enhanced binding by IgSF proteins NKp30 and ICOSL



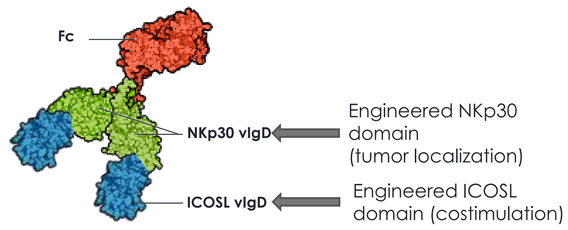
The vIgD platform utilizes yeast display and random mutagenesis of IgSF proteins coupled with FACS and bead-based selections for variants with enhanced binding to appropriate IgSF family counter-structures.

- ICOSL was selected for enhanced binding to ICOS and the non-cognate ligand CD28 generating a dual costimulatory receptor agonist
- NKp30 was selected for enhanced binding to its cognate ligand B7-H6 to drive tumor localization

Figure 3: Engineering NKp30 and ICOSL domains to provide B7-H6-dependent T cell costimulation



Dual-Ligand Affinity Maturation with a Single Random ICOSL vIgD Library: Yeast were transformed with an ICOSL vIgD library incorporating small numbers of random mutations and affinity matured by selection with recombinant CD28 and ICOS Fc-fusion proteins using bead and FACS-based sorting. Individual receptor binding of (a) CD28 and (b) ICOS to bulk yeast populations expressing ICOSL variants are shown, including output from two rounds of FACS selection. Improved binding to both ligands was noted after 1st selection (F1), with further improvements after a 2nd selection (F2). A similar strategy was used to select for NKp30 variants with enhanced binding to B7-H6 following multiple sorts (F1-F4) selecting for increased B7-H6 binding (c). MFI, mean fluorescence intensity.



Schematic Diagram of Tumor Localizing vIgD-Fc Protein: ICOSL vIgD domains engineered for increased binding to ICOS and CD28 were fused with an NKp30 vIgD selected for increased binding to the tumor antigen B7-H6 and an Fc domain. Four different ICOSL vIgD with different binding properties were used to generate multiple forms of the protein

Figure 4: NKp30/ICOSL vIgD-Fc fusion proteins bind expected targets on transfected cells

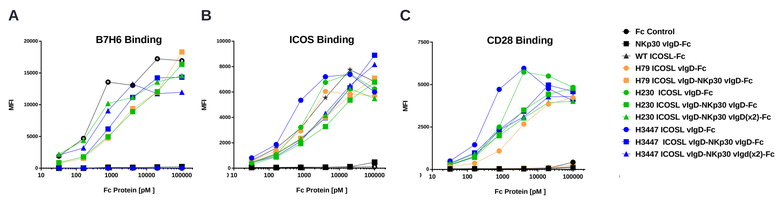
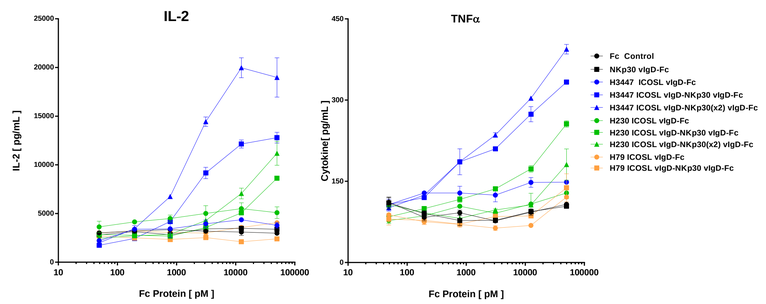


Table 1. EC50 Values (pM) for Indicated Ligands				
Protein	ICOSL Domain	B7H6	CD28	ICOS
ICOSL H230-Fc	H230	ND	835	929
ICOSL H230-NKp30-Fc	H230	10496	1267	8765
ICOSL H230 IgV-NKp30(X2)-Fc	H230	328.8	1010	1648
ICOSL H3447-Fc	H3447	ND	253	405
ICOSL H3447-NKp30-Fc	H3447	1077	1086	44981
ICOSL H3447-NKp30(X2)-Fc	H3447	550	880	5616
ICOSL H79-Fc	H79	ND	2606	ND
ICOSL H79-NKp30-Fc	H79	ND	ND	5171
WT ICOSL-Fc	WT	ND	ND	1295
NKp30-Fc	-	332	ND	ND
Fc Control	-	ND	ND	ND

Engineered ICOSL vIgD domains with varying affinities for CD28, ICOS and CTLA4 were combined with either one or two engineered NKp30 vIgD domains plus an effectorless Fc domain to generate Fc-fusion proteins. These cells were tested for binding to HEK-293 cells transiently transfected with (a) B7-H6, (b) ICOS, or (c) CD28 with binding detected over a range of input protein concentrations using a fluorochrome conjugated anti-Fc antibody and FACS analysis. EC_{50} values (pM) for binding were determined from these curves and are presented in Table 1. ND=Not Determined.

Figure 5: NKp30/ICOSL vIgD-Fc fusion proteins drive cytokine secretion in primary human T cells



Primary human T cells were incubated for 3 days with K562 cells expressing a single chain Fv fragment of the anti-CD3 antibody OKT3 and B7-H6 plus the indicated concentration of NKp30/ICOSL vIgD-Fc fusion proteins or controls. Supernatants were collected after three days and evaluated for IL-2 and TNFα content. Points represent mean determinations of triplicate wells +/- SEM. Results shown are representative of experiments done with at least two donors.

Figure 6: NKp30/ICOSL vIgD-Fc fusion proteins costimulate proliferation of primary human T cells

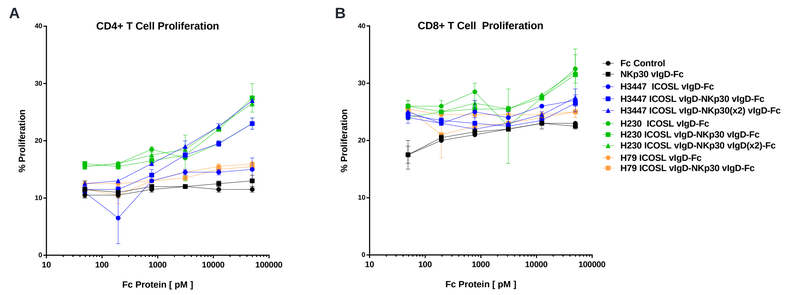
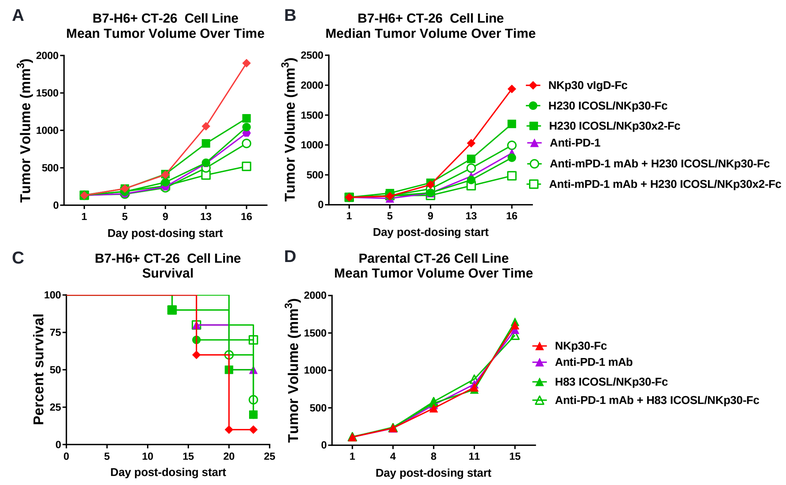


Figure 6: CFSE labeled primary human T cells were incubated for three days with K562 cells expressing a single chain Fv fragment of the anti-CD3 antibody OKT3 and B7-H6 plus the indicated concentration of NKp30/ICOSL vIgD-Fc proteins or control proteins. Proliferation of (a) CD4+ and (b) CD8+ T cells was read out from the percentage of cells in each population that had diluted CFSE. Points represent the mean percentage of cells that had divided in three triplicate wells +/- SEM and values are plotted versus protein concentration

Figure 7: NKp30/ICOSL vIgD-Fc fusion proteins inhibit growth of B7-H6+ CT-26 tumor cells *in vivo* as monotherapy and with anti PD-1 antibody



Balb/c mice were implanted with either (a-c) 3 x 10^5 CT-26 tumor cells that had been transduced with a B7-H6 encoding lentiviral vector or (d) parental CT-26 cells. Tumor size was monitored by caliper biweekly. Animals were treated with 5 mg/kg of the indicated Fc-fusion protein(s) three times over three weeks or with an anti-PD-1 antibody two times over a two week interval. Treatment of all groups was initiated when mean tumor volumes of all animals reached 100 mm^3. Graphs show (a) mean or (b) median tumor growth or (c) survival plots for CT-26/B7-H6 implanted mice. (d) Parental CT-26 cells that lack B7-H6 expression similarly implanted and treated were unaffected by any treatments indicating effects of reagents were dependent on B7-H6.

Summary and Conclusions

- B7-H6 is a novel tumor antigen expressed at significantly higher levels in some tumor types compared to corresponding normal tissues, including esophageal, rectal and stomach adenocarcinomas, and kidney chromophobe renal cell carcinoma
- Tumor-localizing NKp30/ICOSL vIgD-Fc proteins confer potent T cell costimulation *in vitro*
- NKp30/ICOSL vIgD-Fc proteins show promising effects on B7-H6+ tumors *in vivo* especially in combination with a PD-1 antagonist
- The vIgD platform has great potential to enable the development of therapeutic proteins that target different tumor types and effector immune cell populations via alternative and varied localizing and activating domains
- Such tumor-localizing vIgDs (TLVs), developed via directed evolution in the vIgD platform, may be capable of generating many effective therapeutic options to improve cancer outcomes