Engineered Variant Domain Fusion Proteins Provide Checkpoint Inhibition and Tumor Antigen-Dependent CD28 Costimulation Resulting in Potent Anti-Tumor Immunity

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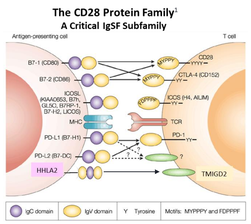
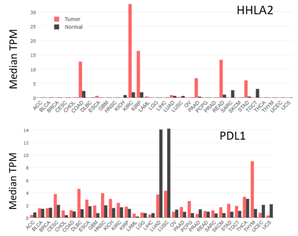
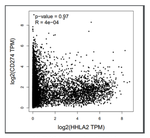
Background



- CD28 is a T cell costimulatory receptor that binds CD80 and CD86
- PD1 is an inhibitory CD28 family receptor that binds PDL1 and counteracts CD28 signaling
- Although PD1 blockade is an effective therapeutic approach in cancer, inadequate CD28 costimulation may contribute to anti-PD1/L1 refractoriness
- TMIGD2 is an inhibitory CD28 family receptor that binds HHLA2 and is a novel checkpoint inhibitor
- PD1 and/or TMIGD2 blockade coupled with CD28 costimulation may be an effective strategy to overcome resistance to checkpoint inhibitors
- Development of high affinity variants comprised as Fc fusion proteins that mediate tumor antigen (e.g., PDL1, HHLA-2)-specific CD28 costimulation represents a novel approach to enhance tumor immune responses

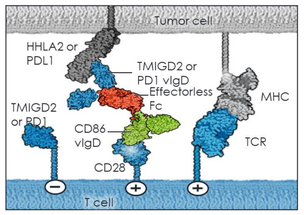
HHLA2 and PDL1 are Over-Expressed on a Subset of Tumors





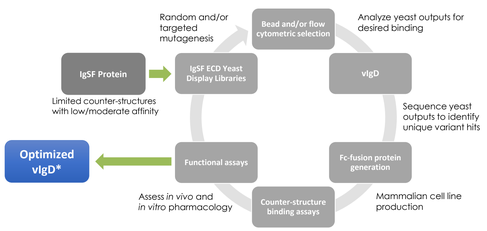
HHLA2 (left, top) and PDL1 (left, bottom) are over-expressed on a subset of tumor (red) vs. normal (black) tissues. Median TPM values for HHLA2 and PDL1 expression from TCGA RNAseq data are indicated. TPM levels for HHLA2 in all TCGA tumor samples were plotted against normalized PDL1 TPM levels (above), indicating a trend for mutually exclusive expression. Analysis performed with Gene Expression Profiling Interactive Atlas (GEPIA)[2].

Binding Model



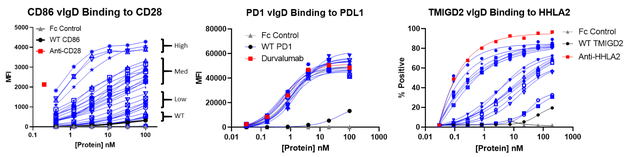
Model for TMIGD2-CD86 or PD1-CD86 binding to HHLA2 or PDL1, respectively, preventing its interaction with inhibitory receptors on T cells and simultaneously engaging CD28 to trigger costimulatory signals.

Model of vIgD Directed Evolution Platform



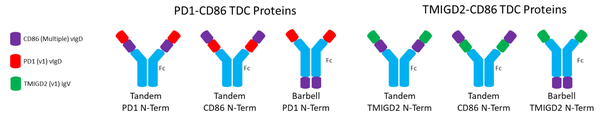
The platform utilizes yeast surface display and mutagenesis of IgSF proteins coupled with FACS and bead-based selections for variants with enhanced binding to appropriate IgSF family counter-structures as previously described.[3]

Variants of CD86, PD1, and TMIGD2 Display Increased Binding to Cognate Ligand



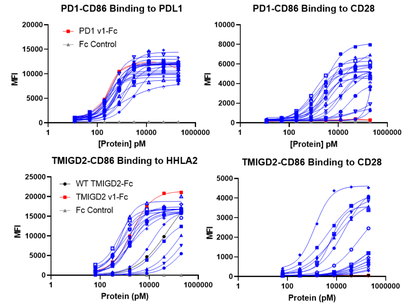
Variants of CD86, PD1 and TMIGD2 were identified as described above. Variants demonstrating increased binding to cognate ligands (CD28, PDL1, HHLA2) were subcloned into mammalian cell expression vectors and expressed and purified as Fc-fusion proteins. Variant IgSF domains (blue symbols/lines) were screened for binding to cells expressing each cognate ligand at indicated concentrations and compared to Fc control (gray triangle), wild-type Fc fusion protein (black circle) or target specific antibody (red square). High affinity PD1 and TMIGD2 variants were fused to CD86 variants with either high, medium or low CD28 binding (left panel) for further characterization.

Fusion Protein Engineering to Mediate Target-Dependent Costimulation Against Either PDL1+ or HHLA2+ Tumors



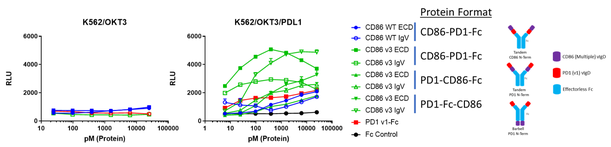
Selected variants from previous figure were engineered into fusion proteins with CD86 domains (purple) at either the N- or C-terminus or in the middle and with PD1 (red) or TMIGD2 (green) tumor localizing domains at either the N-terminus or in the middle. Proteins were engineered with an effectorless Fc domain (blue), expressed in HEK-293 cells and purified by protein A chromatography. A single high affinity variant of PD1 or TMIGD2 was paired with multiple CD86 domains and each was functionally tested for targeted costimulation potential. The resultant proteins were then tested for their capacity to provide Target Dependent Costimulation (TDC).

PD1-CD86 and TMIGD2-CD86 TDC Proteins Retain Binding Activity to Their Individual Counterstructures



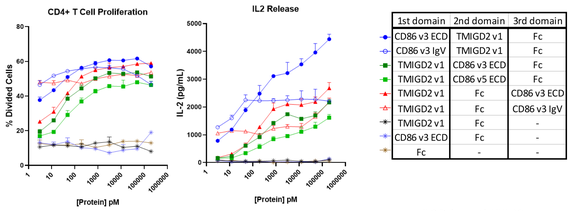
PD1-CD86 (Left) or TMIGD2-CD86 (Right) fusion proteins were tested for PDL1 (top left) or CD28 (bottom left) or HHLA2 (top right) or CD28 (bottom right) binding, respectively, at indicated concentrations. A single PD1 (PD1 v1) or TMIGD2 (TMIGD2 v1) domain was included while multiple CD86 domains were tested either as full ECD fusions (including both CD86 IgV and IgC domains), or with the IgV CD28 binding domain only. CD86 IgV only domains exhibited better binding than full ECD domains.

PD1-CD86 TDC Proteins Enhance Responses in Jurkat Reporter Cells in a PDL1-Dependent Manner



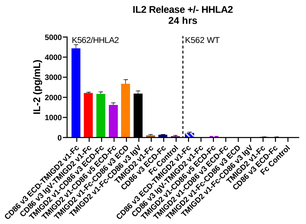
PD1+ Jurkat/IL-2 reporter cells were incubated 5 hr with PDL1+ (left) or PDL1+ (right) K562/OKT3 cells in the presence of indicated PD1-CD86 TDC proteins. Proteins utilized either a WT (blue) or variant (red, CD86 v3) CD86 domain. CD86 domains with full ECD (closed symbols) or CD28 binding IgV domain only (open symbols) were also evaluated.

TMIGD2-CD86 TDC Proteins Enhance Primary Human T Cell Responses in an HHLA2-Dependent Manner



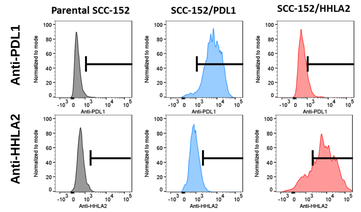
1st domain	2nd domain	3rd domain
CD86 v3 ECD	TMIGD2 v1	Fc
CD86 v3 IgV	TMIGD2 v1	Fc
TMIGD2 v1	CD86 v3 ECD	Fc
TMIGD2 v1	CD86 v5 ECD	Fc
TMIGD2 v1	Fc	CD86 v3 ECD
TMIGD2 v1	Fc	CD86 v3 IgV
TMIGD2 v1	Fc	-
CD86 v3 ECD	Fc	-
Fc	-	-

K562/OKT3 target cells transduced to express HHLA2 were incubated with CFSE labeled primary human T cells for 72 hrs. Supernatants were collected for cytokine release and proliferation was evaluated by CFSE dilution. Graphs above show the percentage of divided CD4+ T cells vs protein concentration (left) and IL2 determined by ELISA (right). Responses were dependent on HHLA2 expression, because addition of TMIGD2-CD86 proteins to T cells cultured with K562/OKT3 cells lacking HHLA2 failed to enhance IL2 release (right).



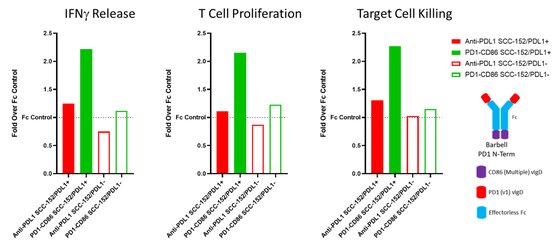
Development of a System to Evaluate TDC Driven Anti-Tumor Responses Using Antigen-Specific T Cells

Stimulated primary human T cells were infected with lentivirus encoding an E6 peptide reactive T cell receptor (TCR.) T cells were expanded over 10 days, phenotyped, and used in functional assays using different versions of the E6+ SCC-152 tumor target cell line.



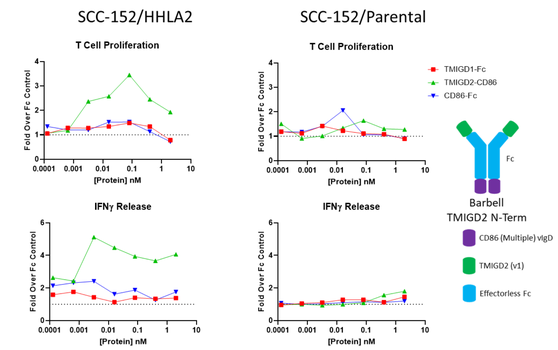
E6+ SCC-152 cells expressing either PDL1 or HHLA2 were generated by lentiviral transduction for evaluation of target dependence and function of TDC proteins.

A PD1-CD86 TDC Protein Enhances Antigen-Specific T Cell Responses in a PDL1-Dependent Manner



Cell trace violet (CTV) labeled E6 TCR T cells were incubated with SCC-152/PDL1+ (solid bars) or SCC-152/PDL1- (open bars) cells. A PD1-CD86 TDC barbell protein (green bars; cartoon on the right), the anti-PDL1 antibody durvalumab (red bars), or Fc control was added at 10 nM. Supernatants were collected at 48 hr for IFNγ analysis by Luminex (left) and at 72 hr for T cell proliferation analysis by CTV dilution (center) and target cell killing (right). Graphs show responses to PD1-CD86 TDC protein or durvalumab as fold over Fc control treated cultures (in triplicate). Responses to PD1-CD86 TDC protein were reproducibly more robust compared with durvalumab and when SCC-152 target cells expressed PDL1.

A TMIGD2-CD86 TDC Protein Enhances Antigen-Specific T Cell Responses in an HHLA2-Dependent Manner

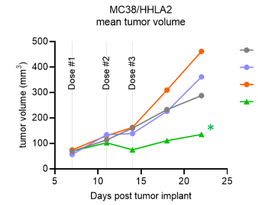


CTV labeled E6 TCR transduced T cells were incubated with SCC-152/HHLA2+ (left) or parental SCC-152 cells (right). A TMIGD2-CD86 TDC barbell protein (cartoon on right), TMIGD2 only, or Fc control was added at the indicated concentrations. Supernatants were collected at 48 hr for analysis of IFNγ content by Luminex (lower graphs) and at 72 hr for proliferation analysis by CTV dilution (upper graphs). TDC protein induced responses were reproducibly increased when target cells expressed HHLA2 (left graphs) compared to HHLA2- parental target cells (right graph)

References

[1] Modified from *Nat Rev Immunol* 2:116 (2002)
[2] Tang, Z. et al. (2017) GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res*, 10.1093/nar/gkx247.
[3] Levin et al (2019), *Front Immunol* 10: 3086
[4] Dillon et al (2020), B Cell Modulatory Variant TNF Receptor Domains (vTDs) Identified by Directed Evolution to Inhibit BAFF and APRIL, Alone or Combined with Variant Ig Domains (vIgD™) that Inhibit T Cell Costimulation, for the Treatment of Systemic Lupus Erythematosus and Other Severe Autoimmune Diseases, EULAR poster

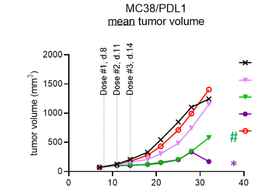
A TMIGD2-CD86 TDC Protein Enhances Anti-Tumor Responses in an MC38/HHLA2 Tumor Model



*p = 0.015 vs Fc Control + TACI vTD-Fc group by 2-way repeated measures ANOVA for 'treatment' effects

MC38/HHLA2* cells were implanted into flanks of C57Bl/6 mice. Because anti-drug antibody formation was previously encountered when administering these human proteins to mice, treatments for half of the animals included co-treatment with a B cell modulator (TACI vTD-Fc)[4] to reduce anti-drug antibody responses. Seven days post tumor implant, mice were staged into groups and half of each group (+/- TACI vTD-Fc) received 50 µg of the TMIGD2-CD86 barbell protein or Fc control as indicated. TMIGD2-CD86 had modest antitumor activity without administration of the B cell modulator, and greater activity in mice when TACI vTD-Fc was included. Notably, treatment with a B cell modulator did not affect growth of MC38/HHLA2* tumors.

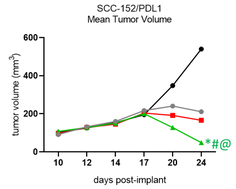
A PD1-CD86 TDC Protein Enhances Anti-Tumor Responses in an MC38/PDL1 Tumor Model, but a TMIGD2-CD86 TDC Protein Has No Effect



* p = 0.0002 and # p < 0.0001 vs Fc control by 2-way repeated measures ANOVA for 'treatment' effects

MC38/PDL1 cells were implanted into flanks of C57Bl/6 mice, and B cells were depleted in all mice with TACI vTD-Fc as described in above. Indicated doses of therapeutic proteins were administered on days 8, 11 and 14 and tumor growth was monitored over time. Treatment with a PD1-CD86 TDC protein attenuated tumor growth in a dose dependent manner. The TMIGD2-CD86 protein had no effect in this experiment, demonstrating its target dependence for therapeutic activity.

A PD1-CD86 TDC Protein Enhances Anti-Tumor Responses in a Humanized Tumor Model



*p = 0.0112, #p = 0.0091, @p = 0.0003 vs. TCR+ T cells only, TCR+ T cells + PD1, and Mock infected T cells groups, respectively, by 2-way repeated measures ANOVA for 'treatment' effects

PD1-CD86 enhances anti-tumor responses in a humanized tumor model. Four million SCC-152/PDL1 tumor cells were implanted into NOD/SCID/Gamma (NSG) mice on day 0. On day 13, 5 million E6 TCR transduced or mock infected T cells were administered by retro-orbital injection. Mice were treated with either 50 µg of PD1-CD86 barbell protein or a molar matched amount of an isolated PD1-Fc domain on days 14, 17 and 20 and tumor growth was monitored over time. Administration of E6 TCR transduced T cells alone had some impact on tumor growth compared with those receiving mock infected T cells, but tumor growth was further attenuated in mice treated with the PD1-CD86 barbell protein.

Conclusions

- **Variant immunoglobulin domains (vIgDs) can be engineered by directed evolution (e.g. from CD86, PD1, and TMIGD2) to comprise Fc fusion proteins capable of mediating tumor antigen (e.g., PDL1, HHLA-2)-specific CD28 costimulation**
- **These tumor target antigen-dependent costimulation proteins exert target antigen-dependent T cell costimulation *in vitro*, and potently suppress tumor antigen-positive tumors in syngeneic or humanized mouse models**
- **Tumor antigen-dependent costimulation using vIgD-based binding domains provides a novel and potentially highly effective therapeutic approach to safely target costimulatory molecules in the tumor microenvironment**