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CD80 vIgD-Fc Proteins Combine Checkpoint Antagonism and Costimulatory Signaling for Potent Anti-tumor Immunity

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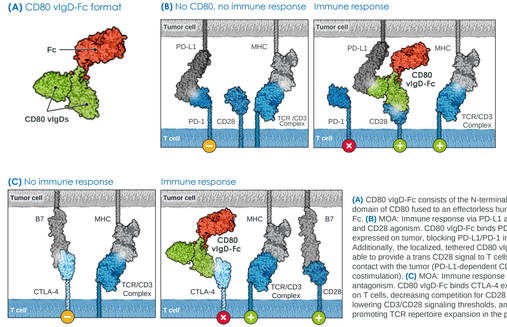
ABSTRACT

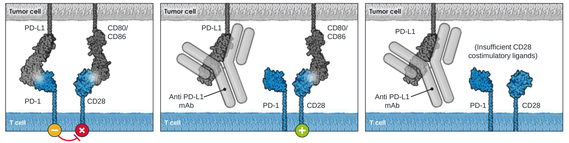
- **INTRODUCTION:** PD-1 pathway antagonists have revealed the importance of checkpoint pathways in regulating anti-tumor immunity, but an existing immune response is generally required for clinical efficacy. Specific T cell costimulation through CD28 is critical to this process, but the CD28 ligands CD80 and CD86 are often poorly expressed in the tumor microenvironment, accounting for a second critical mechanism of immune evasion by tumors. In contrast, PD-L1 expression has been found extensively in multiple tumor cell types. Novel therapeutics that combine PD-L1/PD-1 antagonism coupled with PD-L1 dependent CD28 agonism may therefore provide a more potent, yet safe immunotherapeutic approach.
- **EXPERIMENTAL PROCEDURES:** The variant Ig Domain (vIgD)™ platform has generated a diversity of human CD80 variants using yeast display affinity maturation and selections against all three CD80 counterstructures CD28, CTLA-4, and PD-L1. CD80 vIgDs were produced in a mammalian expression system as recombinant Fc fusion proteins and their binding properties were quantified by flow cytometry. Functional activity was determined *in vitro* by assessing responses from human primary T cells or an IL-2-luciferase Jurkat T cell reporter line stimulated with PD-L1-expressing artificial antigen presenting cells (aAPC). *In vitro* human T cell cytotoxicity assays with a human PD-L1-expressing tumor line were also performed. Anti-tumor activity was assessed *in vivo* with mice implanted with human PD-L1 transduced MC38 tumors.
- **DATA SUMMARY:** The human CD80 IgV fragment was found to be optimal for high affinity PD-L1 and CD28 binding. A large panel of CD80 vIgD-Fc variants demonstrated a range of binding towards CD28, PD-L1, and/or CTLA-4, and CD80 variants with high affinity for PD-L1 antagonized the PD-L1/PD-1 interaction. Some CD80 vIgD-Fc molecules agonized CD28 in a PD-L1 dependent fashion with increased luciferase activity in the Jurkat reporter assay as well as increased cytokine production by primary human T cells when stimulated with PD-L1 expressing aAPC *in vitro*. The same candidates also showed specific killing of human PD-L1 expressing tumor cells *in vitro* compared to the parental tumor line lacking PD-L1 expression. Importantly, selected CD80 vIgD-Fc caused significant tumor reduction in the MC38 *in vivo* tumor model.
- **CONCLUSION:** Engineered CD80 vIgD-Fc proteins that deliver a localized CD28 costimulatory signal to T cells while simultaneously antagonizing the inhibitory PD-L1/PD-1 pathway may provide a transformative mechanism of action to drive potent, tolerable anti-tumor immunity. Preclinical development of therapeutic candidates is underway.

Figure 1: CD80 vIgD-Fc are engineered for three mechanisms of action: PD-L1 and CTLA-4 antagonism combined with CD28 agonism



(A) CD80 vIgD-Fc consists of the N-terminal IgV domain of CD80 fused to an effectorless human IgG1 Fc. **(B)** MOA: Immune response via PD-L1 antagonism and CD28 agonism. CD80 vIgD-Fc binds PD-L1 expressed on tumor, blocking PD-L1/PD-1 interactions. Additionally, the localized, tethered CD80 vIgD-Fc is able to provide a trans CD28 signal to T cells making contact with the tumor (PD-L1-dependent CD28 costimulation). **(C)** MOA: Immune response via CTLA-4 antagonism. CD80 vIgD-Fc binds CTLA-4 expressed on T cells, decreasing competition for CD28 signaling, lowering CD3/CD28 signaling thresholds, and promoting TCR repertoire expansion in the periphery.

Figure 2: Model for successful PD-1/L1 inhibition requiring CD28 signaling



In the tumor microenvironment (TME), PD-L1 engages PD-1 which suppresses T cells by antagonizing CD28 signaling, the most important of the T cell costimulatory molecules.[1]

1. Krummel & Allison, *J Exp Med* 1996;182:459-65.

Anti PD-1/L1 mAbs prevent PD-1-mediated suppression of CD28 signaling. Successful T cell activation likely requires sufficient endogenous CD28 ligands – but these are often poorly expressed in tumors, potentially explaining why only a minority of treated patients respond to checkpoint antagonism.

In the absence of sufficient CD28 ligands and costimulation, anti-PD-1/L1 treatment may be insufficient to promote anti-tumor immunity due to inadequate T cell activation.[2] Therapies combining checkpoint antagonism and CD28 costimulation could therefore improve anti-tumor responses and outcomes.

2. Kamphorst…Ahmed. *Science* 2017;355:1423-1427.

Figure 3: Directed evolution strategy with CD80 IgV domain using vIgD platform

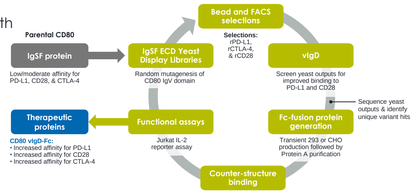
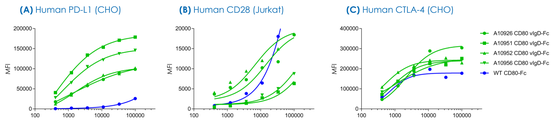
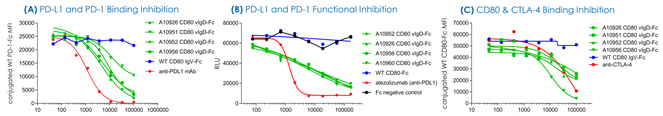


Figure 4: CD80 vIgD-Fc were successfully engineered to bind human PD-L1, CTLA-4, and CD28 with higher affinity than wild-type CD80



Indicated transfectant or cell line was plated and stained with titrated amounts of CD80 vIgD-Fc or control proteins. Bound protein was detected with fluorochrome conjugated anti-huFc and mean fluorescence intensity (MFI) was measured by flow cytometry.

Figure 5: CD80 vIgD-Fc antagonize PD-1 and CTLA-4 receptors

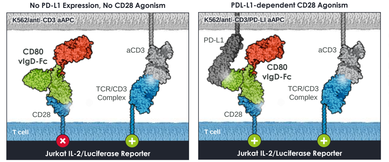


CHO cells stably expressing human PD-L1 were plated with titrated CD80 vIgD-Fc or controls for 30 minutes. Cells were washed one time before adding 100nM AF647-conjugated WT PD-1-Fc. The fluorescent competitor was incubated for another 30 minutes before two final washes and acquisition on a LSRII flow cytometer. MFIs were determined using FlowJo analysis software.

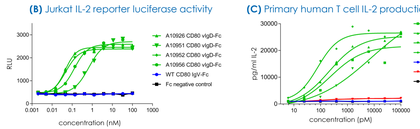
K562/OKT3/PD-L1 aAPC were pre-incubated with titrated CD80 vIgD-Fc or controls for 30 minutes before adding DiscoverX Jurkat/PD-1/SHP2 Pathhunter Enzyme Complementation Fragment Recruitment line for 2 hours. DiscoverX Bioassay Detection reagent was added for 1 hour and resulting luminescence was measured on a BioTek Cytation.

CHO cells stably expressing human CTLA-4 were plated with titrated CD80 vIgD-Fc or controls for 30 minutes. Cells were washed one time before adding 25nM AF647-conjugated WT CD80-Fc. The fluorescent competitor was incubated for another 30 minutes before two final washes and acquisition on a LSRII flow cytometer. MFIs were determined using FlowJo analysis software.

Figure 6: Functional assessment of PD-L1 dependent CD28 costimulation



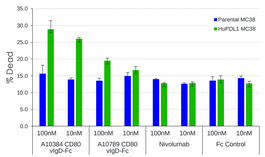
CD80 vIgD-Fc are functionally screened *in vitro* using a Jurkat T cell line which produces luciferase under the control of the IL-2 promoter. CD28 signaling, and subsequent IL-2 promoter activity, is dependent on trans ligand immobilization. In this assay, CD80 vIgD-Fc can only agonize CD28 in the presence of PD-L1. This provides an *in vitro* system to screen large numbers of variants for optimal activity.



K562 artificial APC (aAPC) were transduced to stably express transmembrane versions of anti-human CD3 and human PD-L1. The aAPC were plated in the presence of a Jurkat reporter line with IL-2 promoter driven luciferase expression. After 5 hours culture, Bio-glo luciferase substrate was added, and Relative Luciferase Units (RLU) determined by a Cytation Multi-Mode plate reader.

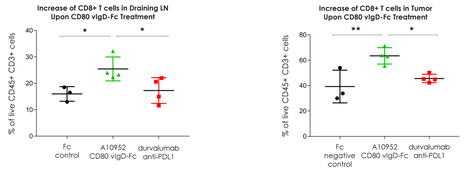
K562/OKT3/PDL1 aAPC cells were mitomycin-c treated and cocultured with primary human pan T cells in the presence of titrated CD80 vIgD-Fc or controls. Culture supernatants were harvested at 24 hours and human IL-2 levels were measured by ELISA.

Figure 7: CD80 vIgD-Fc improve *in vitro* cytotoxicity of HuPD-L1 transduced MC38 cells



MC38 tumor lines were treated with Mitomycin-C and plated with human pan T cells labeled with CFSE at a 1:5 ratio. CD80 vIgD-Fc or controls were added at 100nM or 10nM and cultured with cells for 72 hours. Cells were then harvested and stained with 7-AAD viability dye. After acquiring samples on a LSRII flow cytometer, the percentage of dead cells was calculated using FlowJo analysis by gating on 7-AAD+ cells in the CFSE- gate.

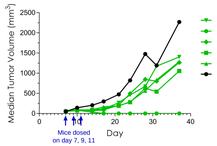
Figure 8: CD80 vIgD-Fc expand CD8 T cell percentages in tumor and lymph node



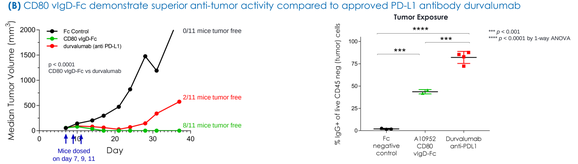
In a separate study performed as shown in Figure 9, 72 hours post 2nd dose of Fc control, CD80 vIgD-Fc or durvalumab, tumors and tumor draining lymph nodes (LN) from 3 or 4 mice/group were harvested and processed to single cell suspensions. Cells were stained with anti-CD45, CD3, and CD8 to assess T cell populations.

* $p < 0.05$, ** $p < 0.01$ by 1-way ANOVA

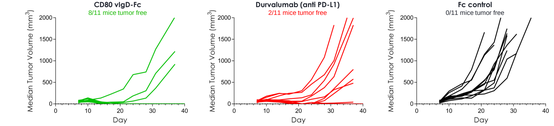
Figure 9: CD80 vIgD-Fc demonstrate potent anti-tumor immunity *in vivo*

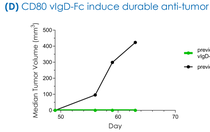


C57BL/6 mice were subcutaneously implanted with 1.5 million human PD-L1 transduced MC38 cells on day 0, with 11 mice per group staged with mean 50mm^3 tumor volume on day 7. On days 7, 9, and 11, mice received 100ug of the indicated CD80 vIgD-Fc by IP injection. Tumor volumes were measured through day 37.



(B) In the same study, 11 animals were treated with durvalumab. On days 7, 9, and 11, mice received 100ug of clinical grade durvalumab by IP injection. Tumor volumes were measured through day 37. Tumor exposure was determined by staining isolated tumor cells and staining with an anti-human IgG secondary. Statistical differences between groups were calculated using GraphPad Prism by 2-way ANOVA (repeated measures) analysis.





On day 49, the eight tumor-free animals previously treated with A10952 CD80 vIgD-Fc were re-challenged with 1.5 million huPDL1 transduced MC38 cells, as were two additional naive C57BL/6 mice (non-treated) to demonstrate viable cell tumor growth. Tumor volumes were measured through day 63.

SUMMARY AND CONCLUSIONS

- CD80 vIgD-Fc are dual PD-L1/CTLA-4 antagonists with PD-L1-dependent, CD28 costimulation
- CD80 vIgD-Fc demonstrate superior tumor clearance and increased CD8 T cell tumor infiltration *in vivo* compared to durvalumab, an FDA-approved anti PD-L1
- This potential novel mechanism of action may represent a new, best-in-class immunotherapy for cancer patients