Introduction: Immunotherapy with the combination of monoclonal antibodies (mAbs) that block PD-1 (nivolumab) and CTLA-4 (ipilimumab) has shown clinical benefit beyond that observed with either mAb alone. We have developed PD-1 x CTLA-4 bispecific proteins aimed at inducing antitumor immunity through simultaneous blockade of both checkpoint molecules. Two proteins, a tetravalent PD-1 x CTLA-4 bispecific DART™ protein (bivalent both PD-1 and CTLA-4) and a trivalent PD-1 x CTLA-4 TRIDENT™ protein (bivalent for PD-1 but monovalent for CTLA-4) were engineered. The TRIDENT protein was designed to promote CTLA-4 blockade through avidity when bound to PD-1 on T cells coexpressing both receptors, thus favoring PD-1 over CTLA-4 in cells that do not coexpress both molecules.

Methods: Proteins were engineered from humanized PD-1 and CTLA-4 mAb sequences and demonstrated favorable biophysical properties. Binding assays were performed by ELISA or flow cytometry. Coengagement of PD-1 and CTLA-4 was assessed in an enzyme-fragment complementation assay (DiscoverX). T-cell activation was tested in reporter cells, staphylococcus enterotoxin B-stimulated PBMCs or PBMCs that coexpress PD-1 and TRIDENT proteins showed a comparable increase in TCR-driven luciferase gene upon TCR stimulation.

Results: Both DART and TRIDENT proteins showed equivalent potency in binding immobilized recombinant PD-1 or PD-1-expressing cell lines, inhibition of PD-1 interaction with PD-L1 or PD-L2 as well as reversal of PD-1/PD-L1-mediated T-cell signal inhibition. In all assays, both formats showed reporter signals that were comparable to those of the precursor PD-1 mAb. With respect to CTLA-4, the DART protein showed a minor potency loss in binding to CTLA-4 expressing cells, inhibition of CTLA-4/B7 interaction and reversal of T-cell signal inhibition compared to the precursor mAb. The TRIDENT protein, however, showed substantial lower potency than the DART protein in all CTLA-4 assays, consistent with the monovalent nature of the CTLA-4 arm. Importantly, in cells that coexpress both receptors, DART and TRIDENT proteins showed comparable coengagement of PD-1 and CTLA-4, as shown by enzyme-fragment complementation, suggesting that anchoring through PD-1 compensates for the decreased CTLA-4 avidity of the TRIDENT molecule when both target receptors are expressed. Similarly, in T-cell coengagement assays, both DART and TRIDENT proteins showed a comparable increase in IFN-γ response that recapitulated that observed with the combination of the individual parental mAbs.

Conclusions: Both PD-1 x CTLA-4 DART and TRIDENT bispecific proteins block PD-1 and CTLA-4 pathways, with the TRIDENT protein demonstrating a PD-1-biased binding preference, consistent with its design intended to reduce CTLA-4 blockade in the absence of PD-1 coexpression. Both molecules showed comparable T-cell activation activity. Further development of bispecific PD-1 x CTLA-4 molecules for cancer treatment is warranted.