A novel multi-specific antibody targeting PD-L1-overexpressing cancers that stimulates antigen-committed CD8+ T cells through concomitant engagement of a T cell costimulatory receptor

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Targeting PD-L1-overexpressing cells with therapeutic antibodies is a clinically validated strategy for the treatment of various solid tumors. In order to increase efficacy, PD-1/PD-L1 blocking agents are currently being applied in combination with additional immune checkpoint modulators (ICMs). However, such combination therapies are often associated with considerable treatment-related adverse events, resulting in a narrow therapeutic window and thereby limiting treatment efficacy. To maximize potency and improve the safety of ICM combination approaches, we sought to design a multi-specific molecule bearing two ICM domains that depletes PD-L1-overexpressing cancer cells via selective recruitment and stimulation of tumor-reactive effector T cells in the tumor microenvironment (TME). The molecule consists of three monovalent antibody variable-domain fragments (Fvs) specific for PD-L1, human serum albumin and a T cell costimulatory receptor fused in a single-chain (PD-L1/HSA/Costim tri-specific scDb-scFv). The monovalent and Fc-less structure of the molecule ensures that clustering of the costimulatory receptor on effector cells can only arise when the molecule concomitantly binds to PD-L1 on the surface of target cells. The addition of a half-life-extending anti-HSA domain, meanwhile, not only enables convenient dosing but also should promote delivery of the molecule to the TME. Here, we demonstrate the successful production of a novel multi-specific antibody that potently blocks PD-L1/PD-1 signaling and elicits T cell costimulation solely in the presence of cells that overexpress PD-L1, as confirmed using a transgenic Jurkat reporter T cell-line in co-cultures with HT29 and HCC827 cancer cell-lines. We show that, *in vitro*, the molecule costimulates T cells considerably more selectively and potently than a first-generation, clinical-stage, anti-costim IgG (Costim IgG-C), even when combined with an anti-PD-L1 IgG (PD-L1 IgG). Furthermore, the PD-L1xCostim scDb-scFv is incapable of stimulating T cells in the absence of primary T cell activation. Finally, in two HCC827 NCSLC xenograft models using humanized mice, the PD-L1xCostim scDb-scFv was better tolerated, slowed tumor growth and induced intratumoral CD8+ T cell proliferation to a greater extent than monospecific IgG variants of the anti-PD-L1 and anti-Costim domains. These data support the hypothesis that the therapeutic window for a promising, clinically validated ICM-based treatment strategy can be broadened by combining multiple ICM domains in an Fc-less, monovalent multi-specific molecule that selectively forms immunological synapses between cancer cells and activated effector cells.

PD-L1xCostim scDb-scFv designed for improved risk-benefit profile

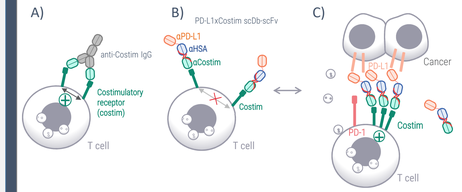


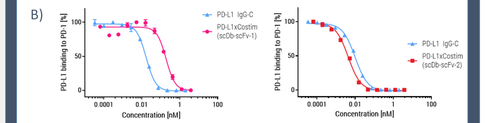
Figure 1. A) Cross-linking of T cell costimulatory receptors (Costims) with bivalent IgGs triggers systemic activation of T cells that may lead to immune-related adverse events (irAEs). **B)** Monovalent multi-specific molecules do not intrinsically trigger Costim clustering and signaling, unless **C)** clustering of the molecules is triggered by binding to tumor cells over-expressing PD-L1; binding of the PD-L1xCostim scDb-scFv to PD-L1 expressing cancer cells supports the formation of an immunological synapse, triggers hyper-clustering of costimulatory receptors on the T cell, and simultaneously blocks PD-1/PD-L1 immune-suppression in TME.

High affinity binding to PD-L1 and potent inhibition of PD-1/PD-L1 signaling

A)

Protein	k_a (1/Ms)	k_d (1/s)	K_D (M)
PD-L1 IgG-C	5.49E+05	< 1.00E-05	<1.82E-11
PD-L1xCostim scDb-scFv-1	1.32E+06	1.22E-04	9.24E-11
PD-L1xCostim scDb-scFv-2	3.75E+06	< 1.00E-05	<2.67E-12

Inhibition of PD-1 / PD-L1 interaction in competiton ELISA:



Blockade of PD-1/PD-L1 signaling in reporter T cells:

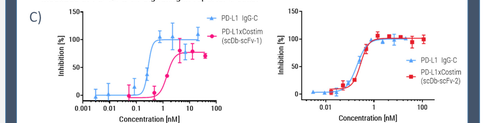


Figure 2. Binding affinity and neutralization potency of anti-PD-L1 molecules. **A)** Affinity of anti-PD-L1 molecules to human PD-L1 was determined by SPR, applying a multi-cycle kinetics protocol. Curves were fitted using a 1:1 binding model. **PD-L1xCostim scDb-scFv-2 demonstrated superior binding affinity to human PD-L1 compared to PD-L1xCostim scDb-scFv-1. B)** Neutralization of PD-1/PD-L1 interaction was assessed by competition ELISA. In this ELISA, 4ug/ml of PD-1 was coated overnight, incubated with biotinylated PD-L1 and competitor molecules followed by detection using streptavidin-HRP. **PD-L1xCostim scDb-scFv-2 showed superior PD-L1 neutralization potency than the avelumab analogue anti-PD-L1 IgG-C and PD-L1xCostim scDb-scFv-1. C)** the potency of PD-L1/PD-1 signaling blockade by anti-PD-L1 molecules was assessed in a transgenic NFAT-Luciferase reporter Jurkat cell-line. In this assay, PD-1 signaling is induced by co-cultivation of reporter Jurkat cells with PD-L1 and T-cell receptor activator expressing CHO cells. PD-L1 inhibition by anti-PD-L1 molecules results in elevated reporter gene activity. For analysis, data were normalized to PD-L1 IgG-C. **In this assay, the monovalent PD-L1xCostim scDb-scFv-2 showed similar PD-L1 neutralization potency as the bivalent PD-L1 IgG-C.**

Costim signaling occurs exclusively in proximity of PD-L1 expressing cells

Costim signaling in reporter T cells in presence of:

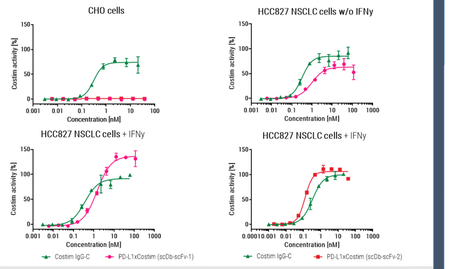


Figure 3. Costim activation in luciferase reporter Jurkat cells by PD-L1xCostim scDb-scFv molecules in presence and absence of PD-L1 expressing cells. PD-L1 expressing HCC827 NSCLC cancer cells (stimulated with IFNγ to express PD-L1) or CHO control cells were cultivated together with reporter Jurkat T cells tjat express luciferase upon Costim signaling. PD-L1xCostim molecules were added in increasing concentrations and in presence of 25 mg/ml HSA. After 24 h of incubation, Costim signaling activity in Jurkat cells was assessed by measurement of luciferase activity. **In presence of PD-L1 expressing HCC827 cancer cells, PD-L1xCostim scDb-scFv molecules induced luciferase activity more efficiently than the Costim-IgG-C, whereas in the absence of PD-L1 (CHO cells) only Costim-IgG-C induced Luciferase expression. Thus, in contrast to Costim IgG-C, the bispecific monovalent PD-L1xCostim scDb-scFv triggers Costim signaling exclusively in the proximity of PD-L1-expressing cells.**

Costim signaling only occurs in combination with TCR stimulus and is more pronounced with PD-L1xCostim scDb-scFv than with the combination of PD-L1 IgG and Costim IgG

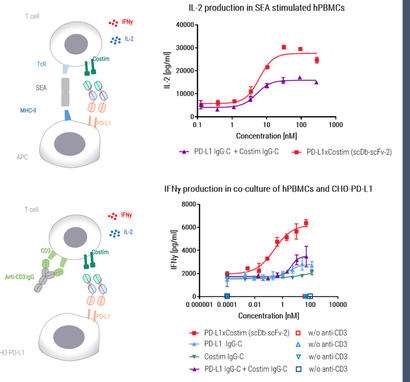


Figure 4. A) Freshly isolated human PBMC were stimulated with 10 ng/ml Staphylococcal enterotoxin A (SEA) to induce T cell activation and target gene expression (PD-L1 and Costim). Subsequently, PD-L1xCostim scDb-scFv-2 or the combination of PD-L1 IgG-C and Costim IgG-C was added to the culture. IL-2 secretion was assessed by ELISA. **The PD-L1xCostim scDb-scFv-2 was more potent to stimulate IL-2 production in PMBCs when compared to the combination of PD-L1 IgG-C and Costim IgG-C. B).** PBMCs from healthy donors were incubated for 3 days in presence of an agonistic anti-CD3 antibody. Human PD-L1 expressing CHO cells and serial dilutions of PD-L1 IgG-C, Costim-IgG-C, PD-L1 IgG-C/Costim-IgG-C combination or PD-L1xCostim scDb-scFv-2 were added to the culture. IFNγ secretion was assessed by ELISA. **The PD-L1xCostim scDb-scFv-2 more potently induced IL-2 (not shown) and IFNγ production than PD-L1 IgG-C, the Costim IgG-C, or the combination of the two. In absence of anti-CD3 antibodies, IL-2 and IFNγ levels were comparable to basal cytokine secretion at all concentrations tested, confirming that TCR signaling or CD3 engagement is required for productive Costim signaling.**

PD-L1xCostim scDb-scFv effectively increases CD8+;PD1+ T cells in TME and reduces growth of HCC827 NCSLC in hPBMC substituted NOG mice

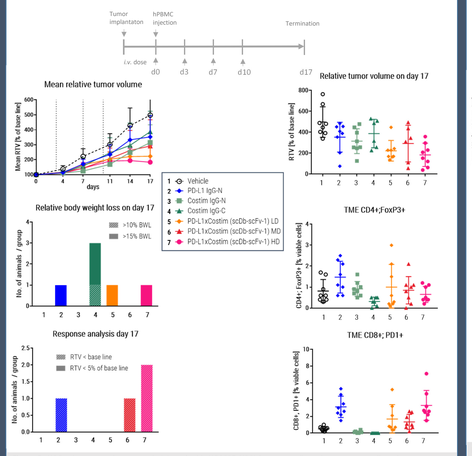
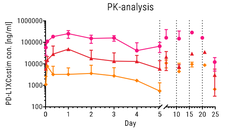


Figure 5. NOG mice were subcutaneously injected with HCC827 NSCLC cancer cells and tumors were allowed to grow to an average of 80 to 100 mm³ prior to randomization into treatment groups (n=8 each). Mice were engrafted with fresh human PBMCs intravenously and treatment commenced 1 hour later. Mice were treated with Vehicle, proprietary PD-L1 IgG-N (0.2 mg), proprietary Costim IgG-N (0.2mg), competitor analogue Costim IgG-C (0.2mg), PD-L1xCostim scDb-scFv-1 at 3 different dose levels (0.08 mg, 0.4 mg and 2 mg) on day 0, 3, 7 and 10 (dotted vertical lines). Tumor growth and body weight was recorded twice weekly. Tumors were harvested on day 17 and 18 post treatment and assessed for infiltration of human T cells by flow cytometry. **PD-L1xCostim scDb-scFv-1 therapy resulted in stronger reduction of tumor growth and higher response rates than therapy with PD-L1 IgGs or Costim IgGs. This correlated with a higher frequency of activated T cells (CD8+, PD1+) in the TME. Costim IgG-C also induced greater body weight loss (BWL) than PD-L1xCostim scDb-scFv-1 .**

Convenient dosing schemes enabled by serum half-life-prolonging effect of mouse cross-reactive serum albumin binding domain contained in the PD-L1xCostim scDb-scFv



Molecule	Dose/mouse/occasion [mg]	Route	$T_{1/2}$ [h]	AUC_{0-d5} [mg/ml*h]	Ratio AUC higher/lower dose
PD-L1xCostim scDb-scFv-2	0.5	*i.p.*	41.7	16'978'964	
	0.1	*i.p.*	36.8	2'475'621	6.9
	0.02	*i.p.*	38.4	331'269	7.5

Figure 6. Pharmacokinetic analysis to quantify PD-L1xCostim scDb-scFv-2 in serum samples from animals in HCC827 xenograft study using human CD34+ hematopoietic stem cell substituted NOG mice (see Fig 7, above). ELISA plates were coated overnight with Costim and serial dilutions of PD-L1xCostim scDb-scFv-2 were added to yield a calibration curve. Bound PD-L1xCostim scDb-scFv-2 was detected with biotinylated human PD-L1 and streptavidin poly-HRP. PD-L1xCostim scDb-scFv-2 concentrations in diluted serum samples were interpolated from the calibration curve. Pharmacokinetic parameters were estimated by means of PK solver software add-in using a non-compartmental approach. **Half-lives of 41.7 hours, 36.8 hours and 38.4 hours were determined by analyzing the first elimination phase after dosing for the PD-L1xCostim scDb-scFv-2 LD, MD and HD groups, respectively.**

PD-L1xCostim scDb-scFv leads to stronger CD8+ T cell infiltration into TME, higher response rates, and better tolerability than combination of PD-L1 IgG and Costim IgGs

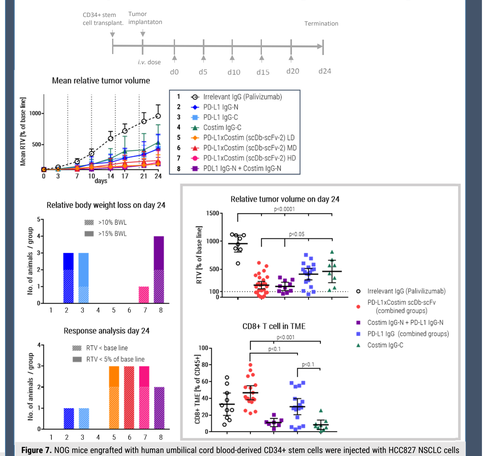


Figure 7. NOG mice engrafted with human umbilical cord blood-derived CD34+ stem cells were injected with HCC827 NSCLC cells and tumors were allowed to grow to an average of 80 to 100 mm³ prior to randomization into treatment groups (n=10 each). Mice were treated with Palivizumab (0.1 mg), proprietary anti-PD-L1 IgG-N (0.1 mg), competitor analogue PD-L1 IgG-C (0.1mg), competitor analogue Costim IgG-C (0.1 mg), PD-L1xCostim scDb-scFv2 at 3 different dose levels (0.02 mg, 0.1 mg and 0.5 mg) or PD-L1 IgG-N + Costim IgG-N combination (0.1 mg each) on day 0, 5, 10, 15 and 20 (dotted vertical lines). Tumor growth and body weight was recorded twice weekly. Tumors were harvested at the end of the study and assessed for infiltration of human T cells by flow cytometry (mean (95% CI) One way ANOVA Bonferroni).**PD-L1xCostim scDb-scFv-2 therapy resulted in stronger reduction of tumor growth than therapy with PD-L1 IgGs or Costim IgGs. PD-L1xCostim scDb-scFv-2 therapy led to higher response rates (30% vs 20%) and was generally better tolerated than combination therapy with PD-L1 IgG-N and Costim IgG-N. This correlated with higher frequency of cytotoxic T cells (CD8+), and CD8+GrB+ double positive T cells in the tumor (not shown).**

Conclusions

- The PD-L1xCostim scDb-scFv induces Costim signaling exclusively in the proximity of PD-L1 expressing cells and thereby directs its activity to PD-L1 expressing tumor tissue.
- The requirement for a secondary signal through T cell receptor (TCR) further limits stimulation of T cells by the PD-L1xCostim scDb-scFv to anti-tumor T cells.
- PD-L1xCostim scDb-scFv therapy in humanized mouse models of NSCLC resulted in stronger reduction of tumor growth than therapy with PD-L1 IgG or Costim IgG.
- PD-L1xCostim scDb-scFv therapy led to higher response rates (30% vs 20%) and was generally better tolerated than combination therapy with PD-L1 IgG and Costim IgG. This correlated with a higher frequency of CD8+ and CD8+/GrB+ T cells in TME (not shown).
- In humanized NOG mice, the PD-L1xCostim scDb-scFv showed dose-dependent exposure in serum with a terminal half-life of approximately 40 hours.