Poster 4552

Tumor-restricted immune-modulation by multispecific molecules from the DARPin® toolbox

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Background

During the last years, immune-modulating drugs have become an important cornerstone in the treatment of cancer patients. In particular, PD1/PDL-1 and CTLA-4 antagonists have revolutionized the field. However, only a limited number of patients benefit from these antagonistic molecules and more combination therapies are on the way to increase the number of patients benefiting from these novel therapies. Among the combinations, drugs that are T-cell or myeloid cell agonists belonging to the TNFR-superfamily show initial promising clinical results. However, systemic immune activation bears the risk of severe side effects that will not allow using these powerful drugs at an effective dose. We have developed a new class of DARPin® molecule that enable tumor-restricted immune cell activation of TNFR-superfamily agonists in the tumor only, thereby preventing systemic immune-activation.

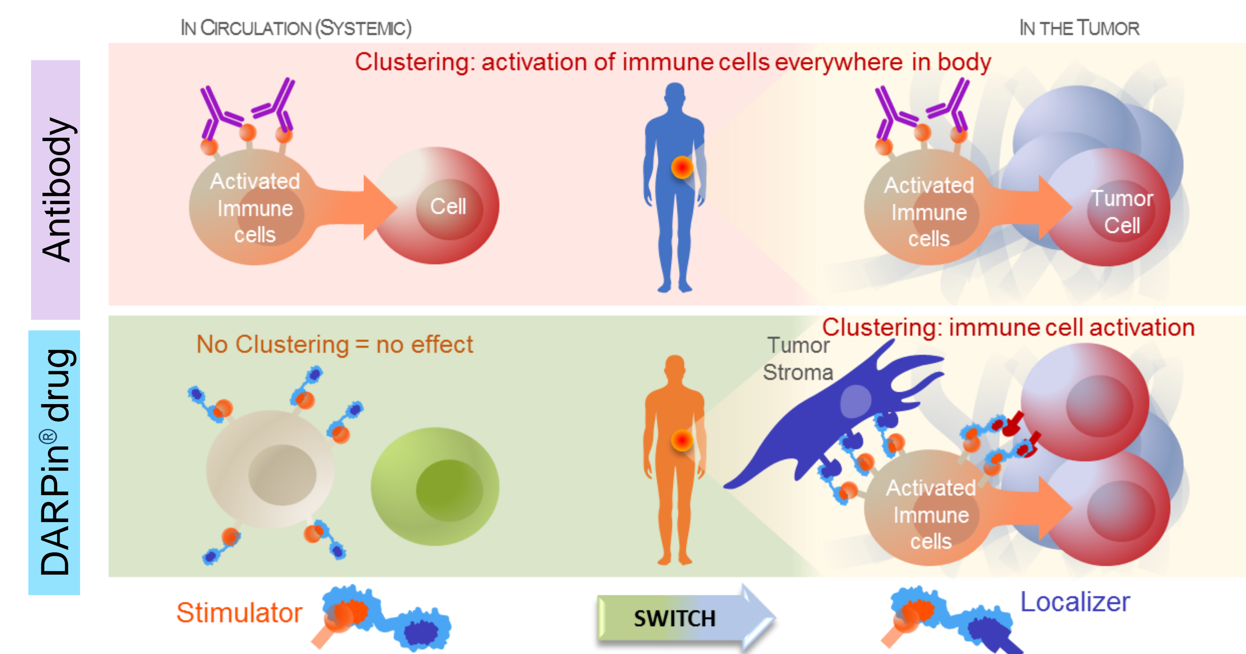


Figure 1. Cartoon illustrating localized immune stimulation concept

Methods

Surface-Plasmon-Resonance (SPR)

SPR measurements were performed using a ProteOn XPR36 instrument (BioRad). Briefly, the targets (ED-B, FAP, EGFR, HER2, 4-1BB, OX40) were immobilized on GLC chips, before injecting DARPin® molecules in a serial (1:3) dilution starting with 30 nM, with an association of 120 s and dissociation of 1800 s. To determine binding constants against CD40, an inversed set-up was applied by immobilizing DARPin® molecules on the chip surface. The signals were double referenced against the running buffer (PBS-Tween) treated control lanes.

Flow cytometry

Expression of FAP on CHO cells, EGFR on A431 cells and HER2 on BT474 cells was determined by flow-cytometry using target specific antibodies.

Reporter cell assays

Activation of human CD40, OX40 and 4-1BB was measured in reporter cell assays. CD40 activation was determined using HEK293 cells transfected with both CD40 and a NF-κB/luciferase reporter gene. Similarly, 4-1BB activation was determined using HT1080 cells transfected with human 4-1BB and a NF-κB/luciferase reporter gene. OX40 activation was determined using HT1080 cells transfected with human OX40. Activation of OX40 was measured by ELISA determination of IL8 secretion upon receptor activation.

Primary cell assays

B-cells, CD4⁺ and CD8⁺ T-cells were purified from buffy-coats using standard procedures. Activation of 4-1BB on CD8⁺ T-cells was determined by co-coating of anti-CD3 antibody (OKT3) and neutravidin on plastic. The respective biotinylated targeted was bound on neutravidin. The respective DARPin® molecule was added together with CD8⁺ T-cells. 48h later IFNγ secretion in culture supernatants was analyzed by ELISA (PeproTech). OX40 activation was determined in a similar manner except that CD4⁺ T-cells were used. CD40 activation was determined on B-cells. Primary B-cells were co-cultured with the respective cell line expressing the tumor or stromal target. After 48h incubation with the respective DARPin® molecule, B-cells were analyzed by FACS for the upregulation of activation marker CD86.

Immuno-histochemistry

ED-B (domain contained in oncofetal fibronectin isoforms) was detected in OTC-embedded MC38 tumors stained with an ED-B specific primary and a respective secondary antibody. Fluorescent signals from individual fluorophores was acquired using an Olympus IX-73 Microscope.

The DARPin® Toolbox

DARPin® molecules are small engineered proteins, derived from natural ankyrin repeat proteins, that are selected to bind to specific targets with high affinity. Individual DARPin® molecules can be linked together genetically in order to create multi-specific drug molecules. The cartoon below shows the construction of the multi-specific DARPin® molecules.

Schematic of a bi-specific DARPin® molecule

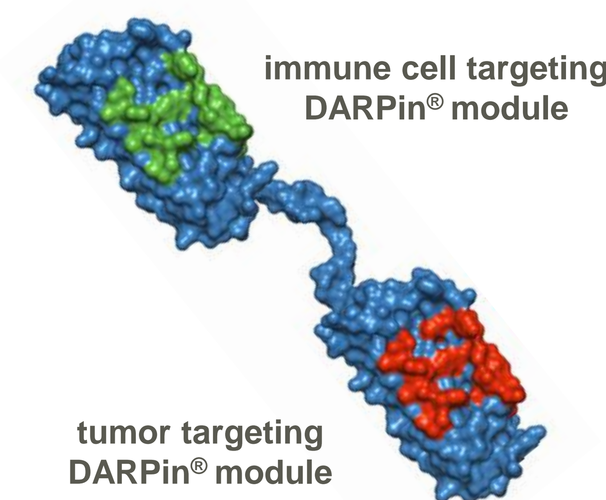


Figure 2: Schematic representation of a DARPin® molecule

The DARPin® toolbox

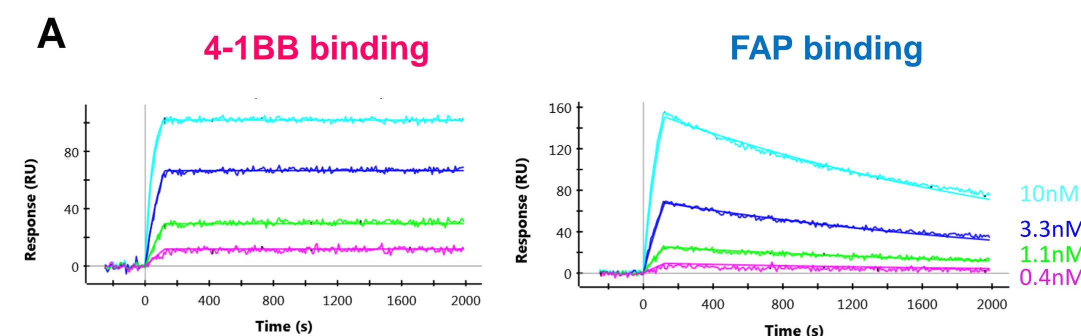
Table: Matrix of individually generated bispecific DARPin® molecules binding to one tumor localizer (e.g. ED-B) and one TNFR (e.g. CD40)

Module	CD40 (myeloid cell)	4-1BB (CD8 T-cell)	OX40 (CD4 T-cell)
ED-B (matrix)	✓	✓	✓
FAP (stroma cell)	✓	✓	✓
EGFR (tumor cell)	✓	✓	✓
HER2 (tumor cell)	✓	✓	✓

Target binding

The DARPin® Toolbox molecules bind their respective target

- All proteins were purified from *E.coli* with yields >50 mg/L and displayed as a single monomeric peak in size exclusion chromatography runs.
- Strong binding against 4-1BB and OX40 was observed with no significant dissociation during the monitored time (no fitting possible)
- Determined binding constants varied for each target by a factor of 5 only (between all tested formats) → No major effect of formatting on the binding constants



B

Affinity against TNFR (K_d measured in nM) (columns); Affinity against tumor localizer (K_d measured in nM) (rows)

Localizer	CD40: vs localizer	CD40: vs TNFR	4-1BB: vs localizer	4-1BB: vs TNFR	OX40: vs localizer	OX40: vs TNFR
ED-B	0.04	0.63**	0.06	<0.02*	0.08	<0.02*
FAP	0.4	1.0**	0.5	<0.02*	0.4	<0.02*
EGFR	0.03	0.75**	0.05	<0.02*	0.14	<0.02*
HER2	0.02	0.89**	<0.02*	<0.02*	0.06	<0.02*

* accurate fit not possible due to slow off-rate
** inaccurate fit

Figure 3: Affinities of bispecific DARPin® toolbox molecules determined by SPR.
(A) Illustrative SPR traces showing high affinity binding (4-1BB binding: fitting of K_d not feasible due to slow off-rate) and intermediate affinity binding (FAP binding). For most constructs, the target was immobilized on the chip surface and the DARPin® protein was applied as analyte at different concentrations (10, 3.3., 1.1 and 0.4 nM). (B) Affinities for toolbox DARPin® molecules. *Horizontally* (→): K_d (nM) determined for binding to the tumor targets (ED-B, FAP, EGFR and HER2). *Vertically* (↓): K_d (nM) determined for binding to the TNFR-superfamily members (CD40, 4-1BB and OX40).

Targeting dependent receptor activation

Activation of TNFR-superfamily agonists expressed in reporter cell lines depends on binding to and clustering on tumor target expressing cell lines or the presence of recombinant ED-B

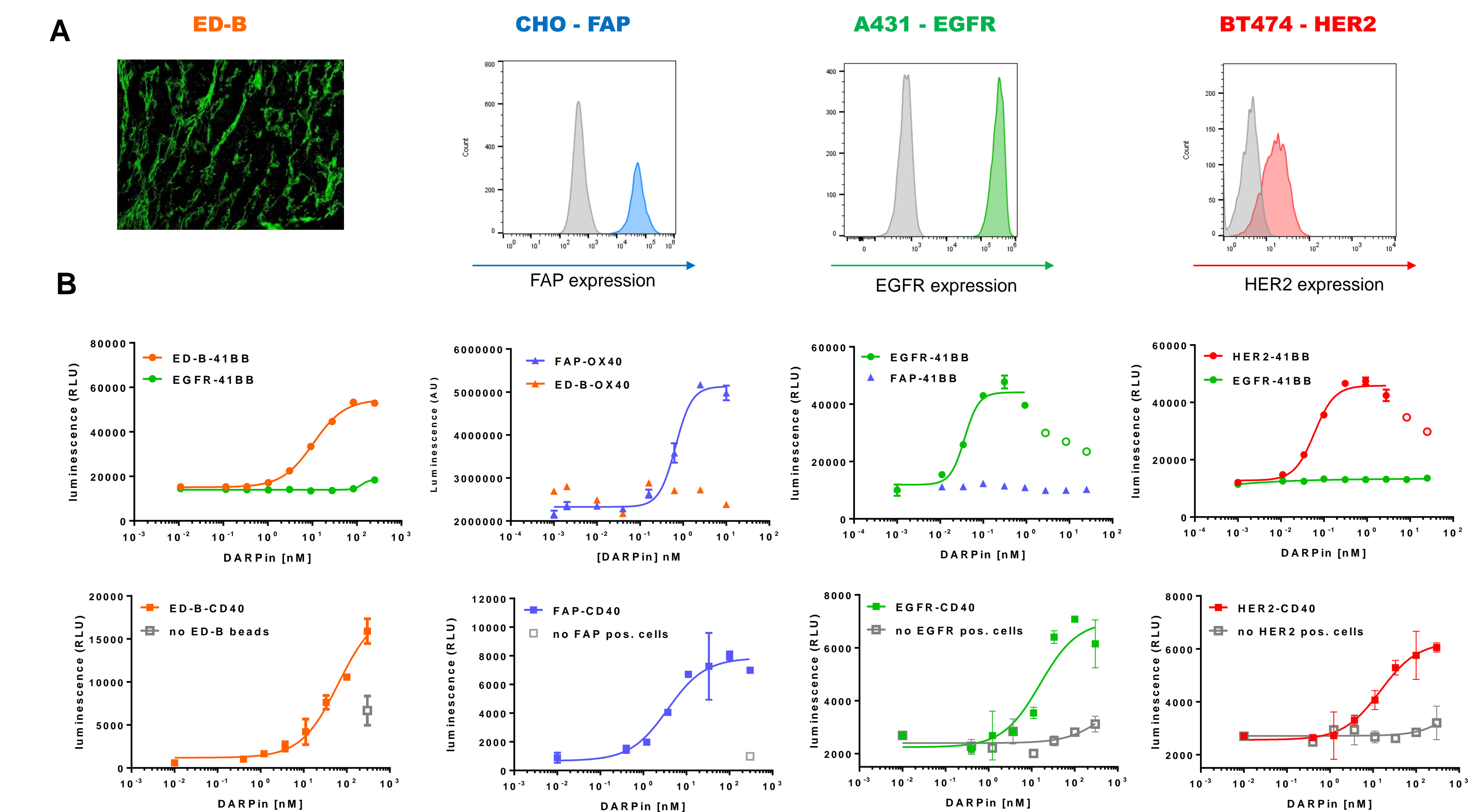


Figure 4. Activation of TNFR-specific reporter cell lines in the presence and absence of EGFR, HER2 or FAP expressing cell lines or recombinant ED-B. (A) ED-B expression in the tumor stroma is shown by IHC and target expression on cell lines by flow cytometry. (B) A titration of DARPin® molecules on reporter cell lines with tumor-target expressing cell lines or recombinant ED-B. The DARPin® molecule tested is depicted in the individual graphs. All combinations were tested and showed activation, representative data are shown.

Activation of TNFR-superfamily members in primary T-cells or B-cells is mediated by binding to and clustering on the tumor or tumor stromal target

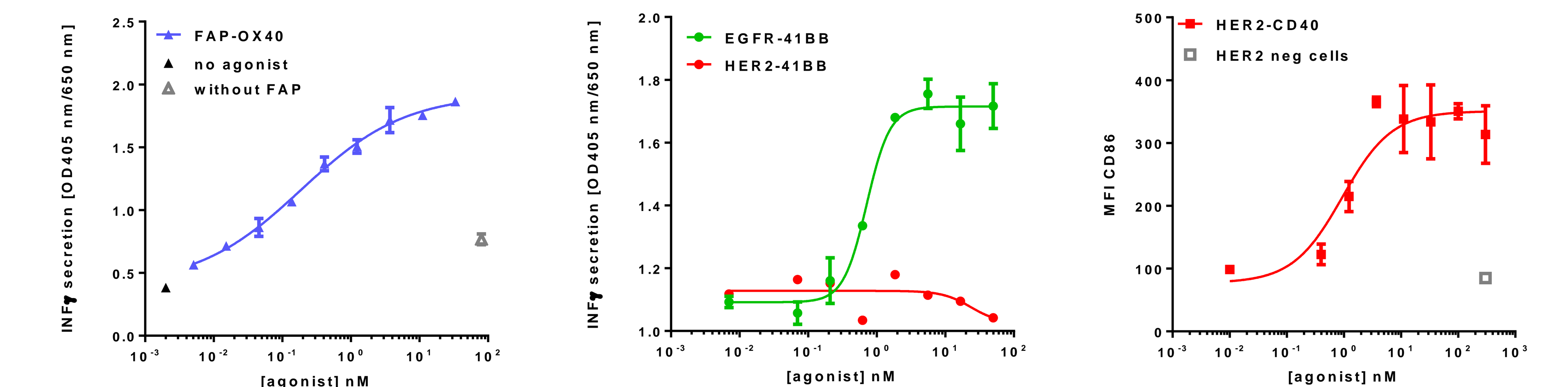


Figure 5. Activation of OX40 in primary CD4+ T-cells in the presence of FAP, 4-1BB activation in primary CD8⁺ T-cells in the presence of EGFR expressing cells and CD40 activation in B-cells in the presence of HER2 expressing cells. A titration of DARPin® molecules is shown. T-cell activation was detected by measuring IFNγ secretion by ELISA and B-cell activation by monitoring CD86 upregulation by flow-cytometry. All combinations were tested, representative data are shown.

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