Poster 690

Application of the DARPin® technology for specific targeting of tumor-associated MHC class I:peptide complexes

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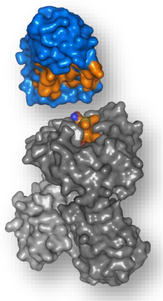
Introduction

Major histocompatibility complex (MHC) class I molecules with peptides derived from tumor-specific or tumor-associated antigens represent a significant subset of targets which can be recognized by tumor-specific cytotoxic T-lymphocytes which play a primary role in immunological control of malignant transformation. Development of biologics or cell therapeutics which target MHC class I:peptide (pMHC) complexes for recognition and elimination of tumor cells is hindered by low affinity, cross-reactivity or challenging biochemical properties of antibody- and T-cell receptor-based binders. We hypothesized that DARPin® proteins may be particularly effective in solving this problem due to structural characteristics of their antigen binding surface and excellent biophysical properties. Panels of binders highly specific for a given pMHC complex were isolated from DARPin® libraries through several rounds of selection and counter selection on the relevant or irrelevant but structurally similar pMHC complexes using ribosome display. DARPin® binders were successfully isolated against pMHC complexes composed of different MHC class I alleles with various peptides derived from either tumor associated antigens or non-self viral proteins.

Here we present characterization of a selected panel of DARPin® binders specific to HLA-A2 molecule in association with SLLMWITQC (SLL-peptide), a peptide derived from NY-ESO-1, was used to create bi-specific T-cell engagers containing another moiety binding to the the CD3 complex, thus allowing highly sensitive analysis of pMHC specificity and potential cross-reactivity as well as evaluation of T-cell activation and cytotoxicity. Using a number of cellular assays, including peptide pulsing of TAP-deficient T2 cells, we confirmed high specificity of selected DARPin® proteins to the HLA-A2:SLL complex. This was manifested as effective and specific activation of T-cells in the presence of relevant DARPin® construct and HLA-A2$^+$ cells pulsed with the SLL-peptide or HLA-A2$^+$/NY-ESO-1$^+$ tumor cells. Furthermore, HLA-A2$^+$/NY-ESO-1$^+$ cells but not HLA-A2$^+$/NY-ESO-1$^-$ cells were effectively killed in the presence of HLA-A2:SLL-specific T-cell engagers.

Alanine scanning mutagenesis and X-scan analysis demonstrated that interactions with several peptide residues located across the entire peptide sequence are critical for binding of selected DARPin® proteins to the pMHC complex. These data suggest that peptide residues exposed outside of the MHC peptide binding grove create the focal point of MHC:peptide:DARPin® interactions.

DARPins® characteristics for pMHC binding



- DARPin® proteins are small binding moieties (11-15kDa; 300-500bp genes) whose binding surface may still fully cover the entire peptide region of a pMHC. Individual DARPin® domains may be easily assembled into multi-specific, single polypeptide molecules
- Structural fit between pMHC molecules and the DARPin® scaffold may result from:
  - Rigidity of DARPin® binding surface (relative to TCRs and antibodies) proven to mediate highly specific interactions with various protein targets
  - Predicted congruency of the interacting surfaces between the pMHC and DARPin® domain (see *in **Fig. 1***)
- With our highest quality DARPin® libraries in combination with well-established selection and screening technologies, we can physically select from 10^{12} variants to find the best solutions which perfectly matches the target peptide region. We generated well-behaved, highly specific and highly pure pMHC binding DARPin® T-cell engagers containing an additional CD3-binding DARPin® domain within 3 months from initiation of selection to the final production batch
- We successfully selected specific DARPin® binders against multiple pMHC targets represented by several self or non-self peptides restricted by different HLA class I alleles (HLA-A*02 or HLA-A*01)

In this poster, we present data on selection, production and characterization of two distinct SLL:HLA-A0201 binding DARPin® candidates in a T-cell engager format: NY_1xCD3 and NY_2xCD3.

Figure 1: Schematic illustration

DARPin® (blue with binding surface, orange) docking to the peptide region (orange) of HLA-A02 (grey)

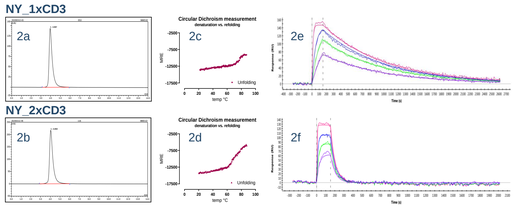


Figure 2: Biophysical characterization of NY_1xCD3 (upper panel) and NY_2xCD3 (lower panel)

2a/b) HPLC-SEC traces for both candidates demonstrate monodispersity without any signs of aggregation or oligomerization

2c/d) protein unfolding in Circular Dichroism proves very high temperature stability up to 70° C (NY_1xCD3) respectively 60° C (NY_2xCD3)

2e/f) SPR multi-concentration measurement reveals different binding kinetics despite comparable KD values.

NY_1xCD3: Ka: 7.97E+05 1/Ms; Kd: 1.17E-03 1/s; KD: 1.46E-09 M

NY_2xCD3: Ka: 2.55E+06 1/Ms; Kd: 1.90E-02 1/s; KD: 7.45E-09 M

DARPins® mediate specific T-Cell Activation

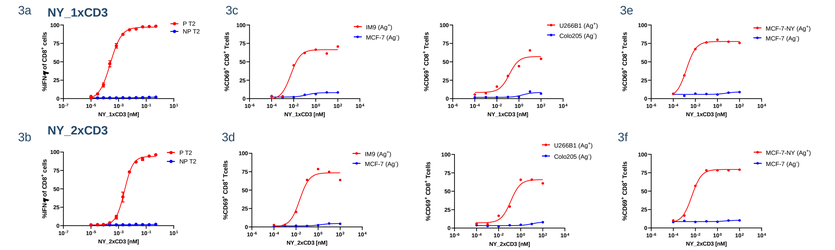


Figure 3: DARPin® mediated T cell activation. (a-b) *Pulsed (PT2) or unpulsed TAP-deficient T2 cells (NP T2) were incubated with effector CD8$^+$ T cells for 4 hours in the presence or absence of NY_1xCD3 (**b**) or NY_2xCD3 (**b**). Intracellular IFN-γ levels obtained on CD8$^+$ T cells are shown. (**c-f**) HLA-A2$^+$/NY-ESO-1$^+$ tumor cells lines (IM9, U266B1, **c** and **d**) or HLA-A2$^+$/ NY-ESO-1$^-$ tumor cell lines (MCF-7, Colo205, **c-f**) or the transfected MCF-7-NY-ESO-1 cell line (MCF-7-NY, **e** and **f**) were incubated with PBMCs for 48 hours in the presence or absence of NY_1xCD3 (**c** and **e**) or NY_2xCD3 (**d** and **f**). Depicted are CD69 levels obtained on CD8$^+$ T cells. Ag: antigen/NY-ESO-1*

Results

NY_1xCD3 and NY_2xCD3 DARPin® candidates efficiently mediate highly specific T cell activation in a dose dependent manner in the presence of target cells which present either endogenously or exogenously formed SLL:HLA-A0201 complex at the cell membrane.

DARPins® induce specific T cell cytotoxicity

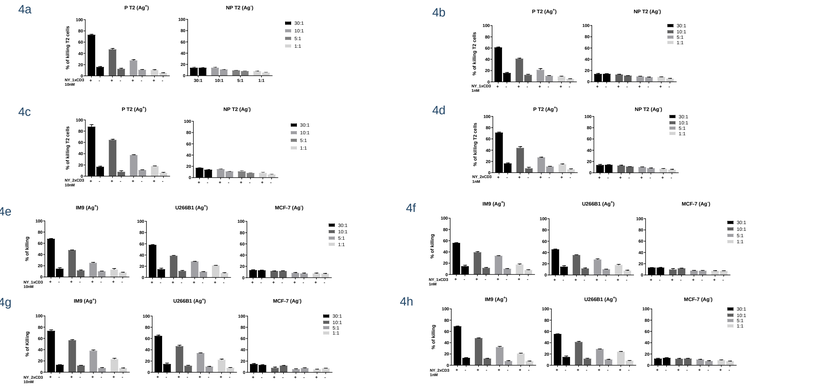


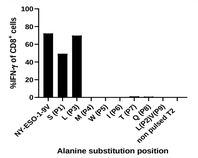
Figure 4: DARPin® mediated T cell cytotoxicity. *Pulsed (PT2) or unpulsed TAP-deficient T2 cells (NP T2) (**a-d**) or HLA-A2$^+$/ NY-ESO-1$^+$ tumor cells lines (IM9, U266B1,**e-h**) or HLA-A2$^+$/ NY-ESO-1$^-$ tumor cell lines (MCF-7,**e-h**) were incubated with effector CD8$^+$ T cells for 4 hours in the presence or absence of 10 nM or 1nM of NY_1xCD3 (**4a**, **4b**, **4e** and **4f**) or 10 nM or 1nM of NY_2xCD3 (**4c**, **4d**, **4g** and **4h**). The percentage of specific lysis of the T2 cells or tumor cell lines obtained by the chromium release assay is plotted for different effector to target ratios (E:T). Ag: antigen/NY-ESO-1*

Results

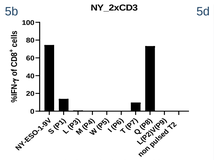
NY_1xCD3 and NY_2xCD3 mediate T cell cytotoxicity against peptide-pulsed T2 cells or tumor cell lines presenting the SLL:HLA-A0201 complex.

Specificity Assessment (Alanine/X-Scan)

5a



5b



5c

NY_1xCD3

Position	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
P1	S	89.7	94.3	0.5	27.4	72.5	103.1	43.0	69.0	13.3	30.1	79.3	77.9	9.3	69.2	76.3	100.0	107.5	113.8	114.4	111.7
P2	L	53.0	81.8	2.5	0.3	12.1	0.2	0.3	101.3	6.5	100.0	105.4	0.1	0.4	59.9	0.4	0.9	15.6	90.6	0.3	0.3
P3	L	96.1	52.9	96.7	3.5	70.4	39.0	70.0	98.7	2.8	100.0	97.0	93.9	44.2	95.9	0.1	37.7	86.8	75.4	102.6	4.6
P4	M	0.3	1.3	0.1	19.0	34.3	0.4	0.0	95.7	3.0	82.9	100.0	1.1	0.3	13.8	11.2	0.1	98.3	29.3	24.5	2.2
P5	W	0.1	0.2	0.1	0.2	16.4	0.1	0.1	0.3	0.3	0.4	1.0	0.4	0.1	0.1	0.0	0.1	0.2	0.0	100.0	0.2
P6	I	0.1	1.3	0.0	0.0	84.1	0.1	0.3	100.0	0.6	45.4	54.7	0.0	1.0	0.1	0.0	0.4	17.4	113.8	0.2	6.7
P7	T	5.5	32.1	0.1	24.8	114.6	0.3	13.0	95.3	1.0	93.3	102.4	14.1	1.6	78.1	82.3	75.2	100.0	74.8	7.3	52.7
P8	Q	23.1	0.2	0.0	17.8	0.1	20.2	0.7	0.2	0.6	3.4	0.9	0.1	0.1	100.0	0.1	12.6	57.1	3.7	0.3	0.2
P9	V	89.4	39.7	0.4	0.7	0.3	11.2	0.7	94.6	1.0	21.0	1.0	1.0	1.1	0.4	0.7	5.3	42.3	100.0	0.8	0.4

5d

NY_2xCD3

Position	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
P1	S	78.1	73.6	27.7	29.5	68.3	2.0	57.2	69.6	0.6	13.1	81.1	109.9	64.9	97.9	35.2	100.0	110.7	108.4	106.8	93.2
P2	L	0.4	0.8	0.2	0.3	39.4	0.2	0.5	105.1	0.1	100.0	52.5	0.1	2.2	0.3	0.6	0.3	1.8	79.9	57.2	0.2
P3	L	0.3	0.1	0.4	0.2	0.6	0.3	0.1	110.2	0.0	100.0	83.4	0.1	2.5	48.6	0.5	0.6	0.2	71.3	20.2	0.1
P4	M	0.4	0.5	0.2	0.3	2.0	0.1	0.4	65.5	0.3	16.8	100.0	0.3	1.1	1.0	0.5	0.5	0.6	0.5	0.5	0.4
P5	W	0.3	0.1	0.4	0.3	13.7	0.6	0.4	1.3	1.5	1.0	2.1	0.7	0.3	0.3	0.3	0.3	0.5	100.0	0.7	
P6	I	2.1	4.8	0.1	0.2	74.6	1.6	0.8	100.0	1.3	102.3	75.0	1.2	107.4	0.1	0.6	4.8	0.2	98.5	36.6	0.0
P7	T	57.7	93.2	0.3	11.4	106.5	107.8	0.4	96.0	0.3	108.5	124.0	76.2	15.1	108.5	102.2	92.0	100.0	100.8	19.6	9.4
P8	Q	122.6	95.6	120.6	122.2	43.9	126.9	124.2	13.4	123.8	98.1	107.1	100.8	113.3	100.0	51.9	113.0	120.7	104.6	108.9	99.6
P9	V	104.1	86.1	16.8	0.4	76.4	56.3	1.9	104.0	1.8	95.1	24.5	27.4	4.6	10.3	0.4	56.8	56.2	100.0	0.9	0.1

Figure 5: Analysis of DARPin® interactions with the SLL:HLA-A0201 complex.

5a/b) *Results of Alanine-scan. Each amino acid of the target peptide sequence was sequentially replaced by alanine in the indicated position. Anchoring positions P2/P9 were substituted together. T2 cells were pulsed with each of the mutated peptides and incubated with effector CD8$^+$ T cells for 4 hours in the presence of NY_1xCD3 or NY_2xCD3 DARPin® candidate concentrations allowing EC90 levels for the wildtype peptide. Intracellular IFN-γ levels obtained on CD8$^+$ T cells are shown.*

5c/d) *Results of X-scan analysis. Each amino acid of the target peptide sequence was replaced by any amino acid in position Px. T2 cells were pulsed with each of the mutated peptides and incubated with effector CD8$^+$ T cells for 4 hours in the presence of NY_1xCD3 (**5c**) or NY_2xCD3 (**5d**) DARPin® candidate concentrations allowing EC90 levels for the wildtype peptide. Intracellular IFN-γ levels obtained on CD8$^+$ T cells are shown as a read-out. Each of the experiments were performed in two independent replicates. Values were averaged and normalized to 100% for the according wild-type residue (dark blue fields) in each position. All values above 30%, indicating no significant loss of T-cell activation, are marked in bold font and light blue color.*

Results

Our alanine scanning mutagenesis and X-scan analysis indicate that the two selected DARPin® lead candidates interact with multiple residues of the SLL-peptide presented in the peptide binding groove of HLA-A0201. While NY_1xCD3 tolerates a larger range of amino acid substitutions at the N-terminus of the SLL-peptide consistent with stronger interactions with the C-terminus, binding of the second candidate NY_2xCD3 appears to more oriented towards the N-terminus of the SSL-peptide.

Search for potential cross-reactive peptides using the Expasy Prosite database (https://prosite.expasy.org/scanprosite/) identified 43 unique human peptide sequences for NY_1xCD3 and 68 unique human peptide sequences for NY_2xCD3 that is comparable to values previously reported for natural T-cell receptors. Potential cross-reactivity to these peptide hits will be analyzed in further specificity characterizations.

Discussion / Conclusions

Our results demonstrate that the technology combined with our in-house-developed T-cell engagement platform allows effective selection and characterization of drug candidates which target tumor-specific/tumor-associated MHC class I:peptide complexes.

DARPin® domains with specificity comparable to that of natural or affinity matured T-cell receptors, as assessed by the low number of potentially cross-reactive peptides identified by X-scan and in vitro cellular assays, can be selected against HLA alleles and associated peptides.

Thus, the DARPin® technology may represent a versatile alternative to other biologics in the field of specific tumor targeting through MHC:peptide complexes. The outstanding biophysical properties, capacity for multi-specific formatting and level of specificity exhibited by pMHC-specific DARPin® proteins prompts investigations on their broader application for alternative treatment modalities (drug conjugates, radio therapy, CAR-T-cells) and pMHC-specific diagnostics.