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Combined *in vitro* and cell-based selection display method producing specific binders against IL-9 receptor in high yields

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We combined cell-free ribosome display and cell-based yeast display selection to build specific protein binders to the extracellular domain of the human interleukin 9 receptor alpha (IL-9Rα). The target, IL-9Rα, is the receptor involved in the signalling pathway of IL-9, a pro-inflammatory cytokine medically important for its involvement in respiratory diseases. The successive use of modified protocols of ribosome and yeast displays allowed us to combine their strengths—the virtually infinite selection power of ribosome display and the production of (mostly) properly folded and soluble proteins in yeast display. The described experimental protocol is optimized to produce binders highly specific to the target, including selectivity to common proteins such as BSA, and proteins potentially competing for the binder such as receptors of other cytokines. The binders were trained from DNA libraries of two protein scaffolds called 57aBi and 57bBi developed in our laboratory. We show that the described unconventional combination of ribosome and yeast displays is effective in developing selective small protein binders to the medically relevant molecular target.

Introduction

Small engineered nonantibody molecules derived from protein scaffolds are promising alternatives to antibodies as research tools or for medical applications [1]. The properties of small, stable protein molecules—protein scaffolds—can be modified to selectively bind specific targets by randomizing amino acids in carefully selected positions.

Protein scaffolds are used as alternatives and/or supplements to monoclonal antibodies for research [2], diagnostics [3] and treatment purposes [4] because they possess several advantages over the more frequently used monoclonal antibodies: they are smaller, nonglycosylated and easier to produce in prokaryotes. Of increasing importance seems to be that there is no need for animal immunization to produce them and that their production can be cheaper than the production of antibodies.

Some scaffolds have been successfully exploited to develop binders that are currently in advanced stages of clinical trials [5,6]. Treatment by protein binders derived from the Anticalin scaffold is promising in the areas of immuno-oncology, metabolic and respiratory

Abbreviations
APC, allophycocyanin; BiP, binding protein; *E. coli*, *Escherichia coli*; EB, elution buffer; FACS, fluorescent-activated cell sorting; gammaC, gamma chain; HM-1, Hansenula mrakii-1; HRP, horseradish peroxidase; HSA, human serum albumin; IL-10, interleukin 10; IL-2, interleukin 2; IL-9, interleukin 9; IL-9Rα, interleukin 9 receptor alpha; ILRG2, interleukin 2 receptor gamma; IPEI, polyethyleneimine; LB, lysogeny broth; MST, microscale thermophoresis; PE, phycoerythrin; PVP, polyvinylpyrrolidone; RiD, ribosome display; *S. cerevisiae*, *Saccharomyces cerevisiae*; S2 cells, Schneider 2 insect cells; scFvs, single-chain variable antibody fragments; SubP, subtractive panning; TMB, 3,3′,5,5′-tetramethylbenzidine; VEGF-A, vascular endothelial growth factor A; WT, wild-type; YPD, yeast extract-peptone-dextrose; YstD, yeast display.

diseases [7]. For instance, the Anticalin scaffold variant PRS050 was engineered into a variant effectively recognizing and preventing the activation of VEGF-A, a marker of solid tumours [8]. Despite that tens of scaffolds are in use, they are mostly used in basic research projects and only a few have undergone biopharmaceutical development up to the clinical stage [9]. We believe this is an indication that there is a need for enrichment of the portfolio of scaffolds to suit a wider range of applications and to improve their applicability.

The standard strategy to develop high-affinity binders based on a scaffold protein is to perform selection from combinatorial DNA libraries using one of the selection display methods. Widely used techniques for this purpose are the non-cell-based ribosome display and the cell-based yeast display; each has its strengths and limitations. Ribosome display is an *in vitro* method, providing the highest complexity. Its theoretical number of 10^{14} produced variants is practically limited only by the number of ribosomes used in the reaction [10]. However, this technique can lead to the selection of incompletely translated and poorly expressed variants. This is a lesser issue in yeast display, where the final binding variants need to be properly folded to be displayed on the surface of a single yeast cell [11]. On the other hand, the complexity of yeast display cannot compete with the complexity of ribosome display, its automatization is also complicated.

For our display selection experiments, we used two scaffold molecules developed in our laboratory [12]. The first scaffold called 57aBi has already been successfully trained to bind human IL-10 [12] and to function as interferon-lambda 1 surrogate [13]. The second scaffold called 57bBi is used here for the first time. 57aBi and 57bBi are trained to recognize the extracellular domain of the human IL-9 receptor α-chain (IL-9Rα). We decided to target the IL-9 signalling pathway because IL-9 is an important pro-inflammatory cytokine that signals via binding to its specific receptor IL-9Rα and common gamma chain cytokine receptor subunit which IL-9 shares with the other members of the IL-2 interleukin family [14]. Besides its normal function in defence against viruses, IL-9 is involved in triggering several respiratory diseases such as asthma [15]. Specific inhibition of IL-9Rα might therefore prove beneficial in treatment of these illnesses.

Successful development of a protein binder is typically evaluated by the affinity to its target molecule. However, high specificity of the binding is of the same if not of higher importance. While the problem of a lower affinity can be solved by increasing the concentration of the binder in the system, the binder's nonspecificity can cause severe side effects rendering the binder useless for any therapeutic purposes. This was the call for the development of our methodical platform where highly specific protein binders would be selected and trained. The platform we present here offers an unconventional combination of modified *in vitro* subtractive ribosome display and cell-based competitive yeast display selection techniques in one workflow to boost their advantages and minimize flaws. The pipeline is employed here for the development of protein binders produced in a soluble form and in high yields and specifically binding the medically relevant target, IL-9Rα.

Results and Discussion

Preparation of DNA libraries of the protein scaffolds

Our target for the development of protein binders is the extracellular domain of the human IL-9 receptor α-chain (IL-9Rα, Uniprot code Q01113, residues 41–270). To generate the binders, we used variants of two protein scaffolds called 57aBi and 57bBi, which were previously developed in our group [12]. In each scaffold protein, we identified 10 mutable residues (Fig. 1), for which we created degenerated DNA libraries. The usability of the 57aBi scaffold was shown previously by the successful selection of variants binding the human IL-10 at ~ 10 nM affinity [12]. 57aBi is based on PIH1D1 N-terminal domain Alpha-X beta2 integrin I domain, PDB ID 4PSF [16]. Performance of the other scaffold, 57bBi, is tested in this work; it is based on the crystal structure of alpha-X beta2 integrin domain, PDB ID 1N3Y [17].

The combinatorial DNA libraries for the ribosome display were synthesized using NNK codons technology. This partial randomization approach has been very successful in both academia and industry, as it introduces only one STOP codon, compared with the fully randomized NNN methodology that can introduce up to all three STOP codons to libraries. The NNK approach still has a better price-performance ratio compared with a potentially more powerful TRIM or Trimer technology that cannot generate STOP codons and lowers the risk of synonymous mutations [18].

Overview of the workflow of binder selection

We used the combinatorial libraries of the 57aBi and 57bBi scaffolds to select binders against the

(A)

57aBi variants	Position																									
	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134
57aBi-WT	R	E	L	V	I	T	I	A	R	E	G	L	E	D	K	Y	N	L	Q	L	N	P	E	W	R	M
57aBi-A_01	R	W	L	V	I	R	I	A	S	K	G	L	E	W	K	Y	G	L	H	L	Y	P	W	W	L	M
57aBi-A_02	R	L	L	V	I	S	I	A	L	T	G	L	E	V	K	Y	R	L	F	L	H	P	G	W	I	M
57aBi-A_03	R	R	L	V	I	T	I	A	L	R	G	L	E	L	K	Y	P	L	C	L	R	P	A	W	H	M
57aBi-A_06	R	C	L	V	I	P	I	A	R	Q	G	L	E	Q	K	Y	C	L	P	L	R	P	F	W	T	M
57aBi-A_10	R	Q	L	V	I	L	I	A	W	S	G	L	E	M	K	Y	W	L	K	L	D	P	W	W	P	M
57aBi-A_11	R	C	L	V	I	S	I	A	Y	L	G	L	E	M	K	Y	F	L	R	L	T	P	G	W	C	M
57aBi-A_12	R	R	L	V	I	S	I	A	F	R	G	L	E	F	K	Y	S	L	L	L	S	P	S	W	S	M
57aBi-A_13	R	A	L	V	I	Y	I	A	R	A	G	L	E	G	K	Y	L	L	T	L	P	P	I	W	P	M
57aBi-A_14	R	S	L	V	I	D	I	A	F	S	G	L	E	W	K	Y	F	L	K	L	R	P	G	W	T	M
57aBi-A_15	R	F	L	V	I	G	I	A	S	L	G	L	E	Y	K	Y	P	L	A	L	R	P	L	W	D	M
57aBi-D_11	R	G	L	V	I	Y	I	A	A	P	G	L	E	S	K	Y	S	L	L	L	S	P	P	W	Y	M
57aBi-D_14	R	L	L	V	I	D	I	A	C	P	G	L	E	C	K	Y	S	L	L	L	V	P	L	W	N	M

(B)

57bBi variants	Position																	~					~										
	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34		44	45	46	47		65	66	67	68	69	70	71	72	73	74	75	76
57bBi-WT	S	R	N	F	A	T	M	M	N	F	V	R	A	V	I	S	Q		L	M	Q	S		S	N	P	L	S	L	L	A	S	V	H	Q
57bBi-A_01	M	M	N	F	E	T	M	M	S	F	V	R	C	V	I	S	D		L	M	Q	S		S	C	P	L	D	L	L	-	S	V	M	Q
57bBi-A_05	S	G	N	F	L	T	M	M	Q	F	V	R	G	V	I	S	-		L	I	Q	S		S	E	P	L	T	L	L	P	S	V	I	Q
57bBi-A_06	K	V	N	F	T	T	M	M	H	F	V	R	C	V	I	S	T		L	M	Q	S		S	R	P	L	Y	L	L	C	S	V	S	Q
57bBi-A_07	G	G	N	F	P	T	M	M	R	F	V	R	G	V	I	S	L		L	M	Q	S		S	I	P	L	G	L	L	P	S	V	A	Q
57bBi-A_08	-	H	N	F	T	T	M	M	A	F	V	R	G	V	I	S	R		L	M	Q	S		S	H	P	L	A	L	L	R	S	V	N	Q
57bBi-A_10	T	L	N	F	F	T	M	M	H	F	V	R	-	V	I	S	P		L	M	Q	S		S	G	P	L	Q	L	L	L	S	V	I	Q
57bBi-A_11	W	A	N	F	Q	T	M	M	D	F	V	R	W	V	I	S	L		L	M	L	S		S	P	P	L	L	L	L	V	S	V	P	Q
57bBi-A_13	G	L	N	F	S	T	M	M	Y	F	V	R	S	V	I	S	H		L	M	Q	S		S	C	P	L	S	L	L	P	S	V	S	Q
57bBi-A_15	S	V	N	F	V	T	M	M	E	F	V	R	L	V	I	S	W		L	M	Q	S		S	P	P	L	L	L	L	S	S	V	D	Q
57bBi-A_16	-	Q	N	F	L	T	M	M	L	F	V	R	R	V	I	S	-		L	M	Q	S		S	V	P	L	V	L	L	M	S	V	T	Q
57bBi-D_06	A	G	N	F	R	T	M	M	N	F	V	R	S	V	I	S	D		L	M	Q	S		S	S	P	L	A	L	L	N	S	V	S	Q
57bBi-D_14	A	Y	N	F	G	T	M	M	V	F	V	R	L	V	I	S	T		L	M	Q	S		S	I	P	L	A	L	L	P	S	V	A	Q

Fig. 1. Protein sequences of the combinatorial libraries of the (A) 57aBi and (B) 57bBi scaffolds. On top, the initial, 'wild-type' sequences are displayed with their 10 mutable sites highlighted in yellow. The following 10 sequences demonstrate sequence variability after the fifth round of the ribosome display (mutated residues in cyan, inadvertently mutated in black); the last two lines show sequences of the best binders after the 6th round of ribosome display and yeast display (the mutations in pink). The sequences are shown for the region with the 10 mutable residues corresponding to residues 138–164 of UniProt Q9NWS0 for 57aBi and residues 164–221 of UniProt P20702 for 57bBi. Position numbers refer to positions on the scaffold proteins 57aBi and 57bBi.

extracellular domain of IL-9Rα using directed evolution. For the initial steps of our workflow, we used the ribosome display technique, since it allowed us to screen the largest applicable library size amongst the display methods [10]. However, we modified the typical ribosome display workflow in three ways; (a) we utilized protein-free blocking reagents, (b) we introduced one to three subtractive pre-selection rounds (subtractive panning) and (c) after the fifth round of ribosome display, we performed one round of competitive yeast display to increase the specificity of binders and ensure good expression levels. The pipeline can be schematized as follows:

SubP – RiD1 – SubP – RiD2 – SubP – RiD3 – SubP – RiD4 – SubP – RiD5 – YstD – SubP – RiD6,

where SubP stands for subtractive panning, RiD for ribosome display and YstD for yeast display. The steps of the selection process are discussed below.

Ribosome display using protein-free blocking reagents

To increase binding specificity, we utilized protein-free blocking reagents in the ribosome display process. In the standard protocols, the target protein is immobilized on the surface of microplates and their surface is blocked by protein-based blockers, BSA protein [19,20], non-fat-skimmed milk [21] or gelatin [22,23]. These proteins can and apparently do serve as unwanted selection targets, lowering the specificity of the selected variants.

To increase the selective power of ribosome display we utilized protein-free blocking reagents. One of the first protein-free solutions used as a blocking agent in immunochemical studies was polyvinylpyrrolidone (PVP) [24]. Nowadays, there are several protein-free reagents available on the market that are suitable for use in both membrane- and plate-based binding assays; we used Pierce Protein-Free (PBS) Blocking Buffer (Thermo Scientific, Waltham, MA, USA).

Pre-selection by subtractive panning

To avoid the selection of nonspecific binders, we decided to include subtractive panning into our workflow. The method of subtractive panning has been introduced to ribosome display selection to increase the binding specificity of single-chain variable antibody fragments (scFvs) [25]. It was shown to be effective to avoid binding to the carrier protein [26], to develop specific antibodies recognizing HM-1 killer toxin [27] or to identify MKN-45, a poorly differentiated diffuse gastric adenocarcinoma cell line [28].

Every ribosome display selection round of the 57aBi and 57bBi combinatorial libraries towards IL-9Rα was preceded by one-step subtractive panning against BSA. After the fifth round of selection, 16 variants of each scaffold were cloned into a vector for protein expression in *Escherichia coli* BL21-Gold (DE3) strain. The DNA of these clones was isolated and sent for sequence analysis. Even after five rounds of ribosome display, the sequences still showed high variability in the 10 mutated positions (Fig. 1). The 57aBi scaffold had mutations only in the randomized positions, while the 57bBi scaffold had two clones (A_05, A_11) with mutations outside of the randomized positions and five clones (A_01, A_05, A_8, A_10, A_16) with codon deletion without open reading frame shift.

The elegance of the subtractive ribosome display method is that only the nonbinding variants in the supernatant from the nonspecific target well are displaced into the neighbouring well on one microplate that contains the specific target. This setup makes this method suitable for further automatization, which is a big advantage of ribosome display compared with *in vivo* selection. Targets for pre-selection steps can be chosen independently in each round and they do not need to be labelled with fluorescent antibodies, which decreases the costs.

Pre-selection strategies in the 6th round of ribosome display

We evaluated the binding of the variants after the 5th round using ELISAs (Fig. 2A). Binding of some variants is relatively strong but binding to BSA remains also high. We therefore decided to test the power of the pre-selection process by running the 6th round of ribosome display in two different setups: (a) one round and (b) three rounds of subtractive panning. However, even these pre-selection steps did not resolve the issue of no or low expression and relatively high affinity to BSA. In addition, we detected only a small number of variants recognizing IL-9Rα. Some defective variants can be proteins shortened by the STOP codons present at the mutable sites of 57aBi or 57bBi, some can simply be insoluble. To eliminate clones with low expression and/or low solubility, we incorporated yeast display into the pipeline, as described in the next section.

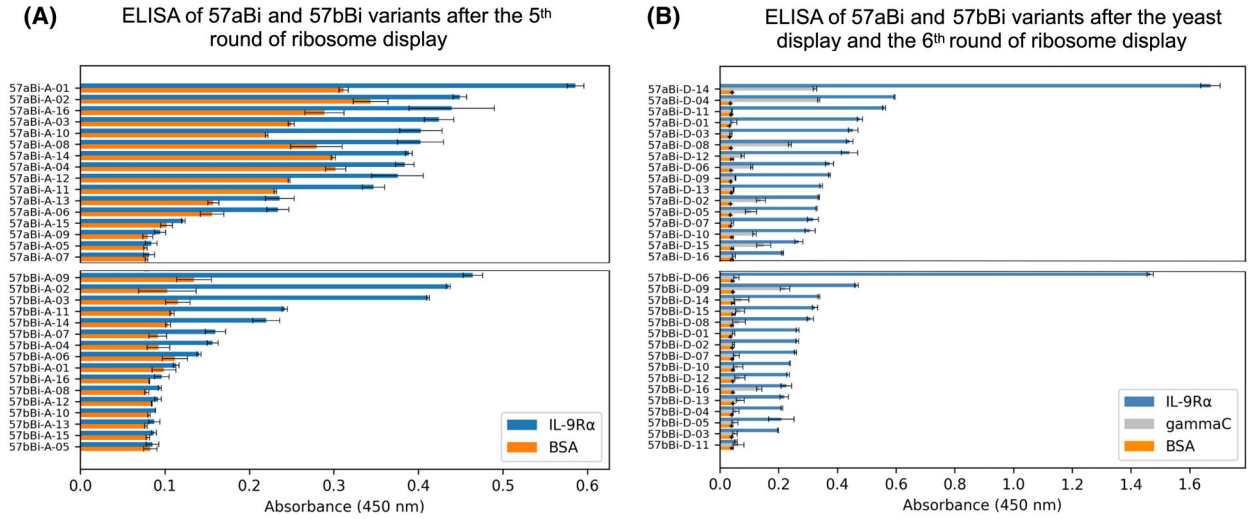

Fig. 2. ELISA binding assays of the 57aBi and 57bBi scaffold variants towards IL-9Rα, BSA and towards common gamma chain cytokine receptor subunit, labelled gammaC in the figure. Variants are ordered according to the strongest binding towards IL-9Rα. (A) Clones selected after 5th round of the ribosome display. (B) Clones selected after the additional yeast display and the 6th round of ribosome display with three-step subtractive pre-selection. Data were measured in triplicates; error bars are defined as standard deviations.

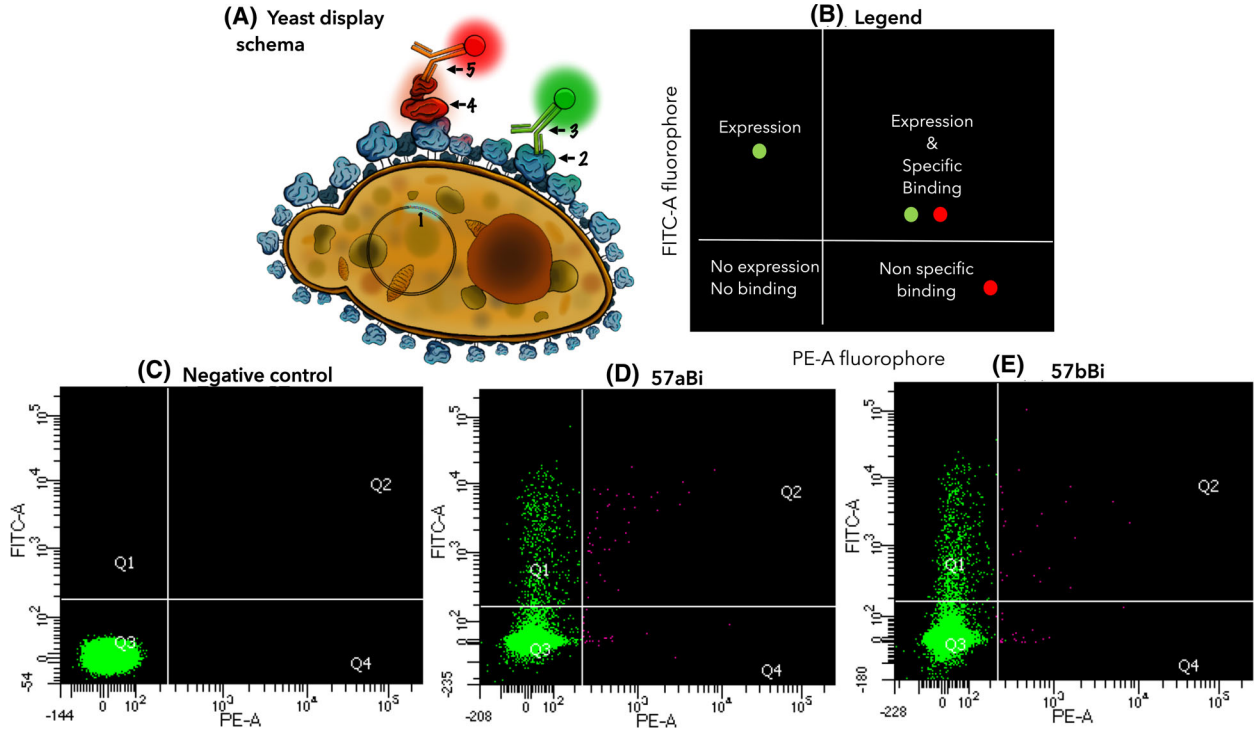

Fig. 3. Yeast display of 57aBi and 57bBi scaffolds trained to bind IL-9Rα protein. (A) Scheme of competitive yeast display selection: 1: vector with cloned combinatorial library, 2: protein binder variant displayed on the yeast surface, 3: anti-c-myc APC conjugated antibody used to detect fully translated scaffold variant, 4: IL-9Rα, 5: anti-IL-9Rα PE-conjugated antibody used to detect bound target IL-9Rα. (B) The panel Legend shows the strategy of the detection of expression of the variants on the yeast cell surface and binding to IL-9Rα subunit with fluorescent antibodies. (C–E) Flow cytometry assisted yeast display selection of the combinatorial libraries of the 57aBi and 57bBi scaffolds performed after the 5th round of ribosome display. Variants of the 57aBi and 57bBi combinatorial libraries presented on the yeast cell surface were incubated with 100 nM IL-9Rα and were competing for binding for 1 h. Cells with fluorescence positivity on FITC22-A and PE-A (double positives) in quadrant Q2, which exhibit both good expression level and binding affinity to IL-9Rα, were sorted, and their plasmid DNA was isolated.

Competitive yeast display improved expression and solubility

To alleviate the low production and solubility of binders after the 5th round of ribosome display, we decided to implement a yeast display step into the selection workflow. Firstly, only properly folded and soluble protein scaffold variants could be secreted and exposed on the yeast surface. Secondly, only fully translated scaffold variants retained the C-terminal c-myc tag, which was detected by a fluorescent-labelled antibody. Expressed and soluble binders presented on the yeast surface then competed for IL-9Rα (used at 100 nM concentration). Bound IL-9Rα was detected by another antibody with a conjugated fluorophore (Fig. 3A).

Following the competitive yeast display, the resulting DNA library underwent another 3-step subtractive pre-selection against BSA and the 6th round of ribosome display selection against IL-9Rα. The harvested variants were tested for binding towards IL-9Rα, BSA and the shared receptor for IL-9, the common gamma chain cytokine receptor subunit (UniProt P31785 ILRG2_HUMAN, residues 23–255). The results of the ELISA showed that the additional selection by yeast display improved dramatically the selectivity of binding to IL-9Rα compared with BSA and common gamma chain cytokine receptor subunit (Fig. 2B). While the affinity to BSA is still detectable after 5th round of ribosome display (Fig. 2A), it is at the background level after the yeast display and 6th round of ribosome display. In addition, binding to the common gamma chain cytokine receptor subunit is also undetectable for most variants.

Switching between two display techniques significantly improved the binding specificity of selected binders to the target and decreasing it to unwanted molecules, in our case BSA and common gamma chain. It can be explained by the different environments in

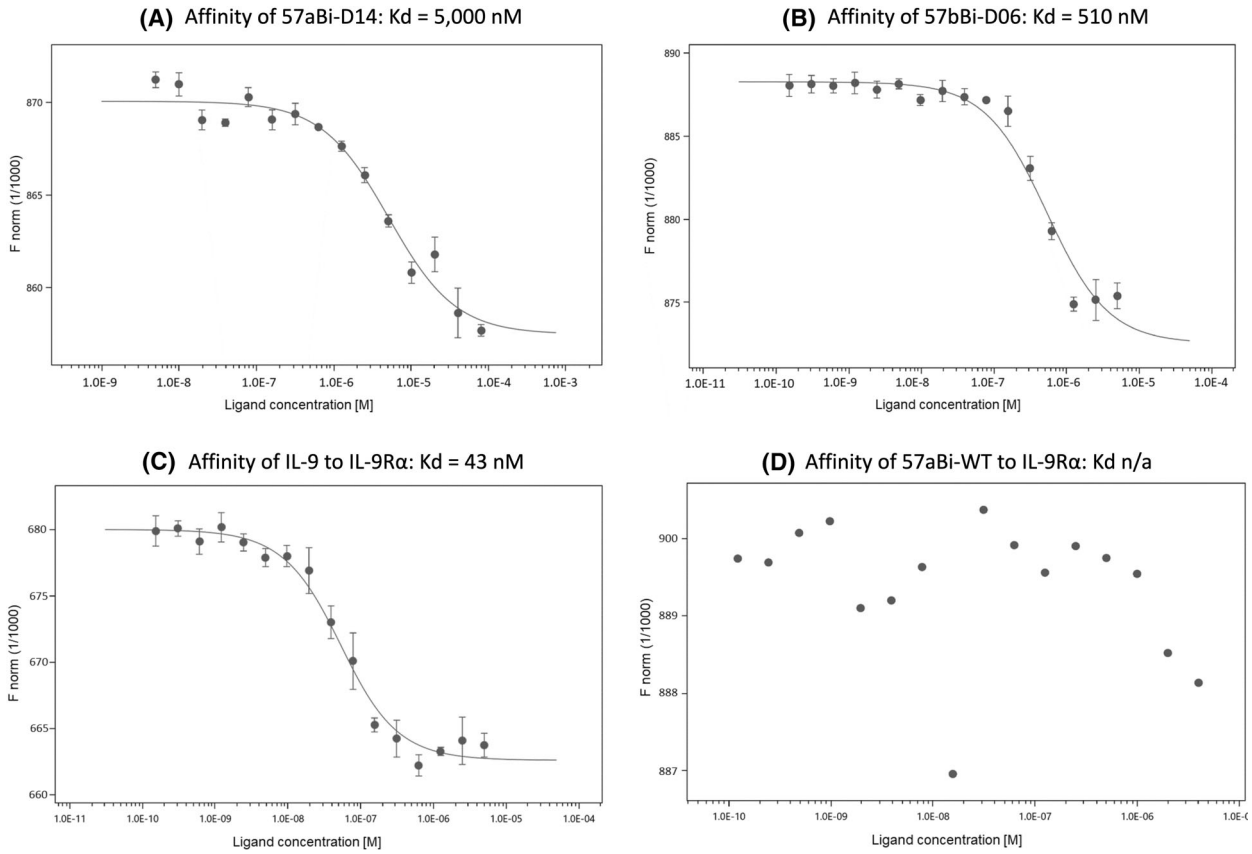

Fig. 4. Affinity measurement between human IL-9Rα and its ligands measured by MST. The curves show the level of binding between IL-9Rα and (A) 57aBi-D14 variant, (B) 57bBi-D06 variant, (C) IL-9 (natural ligand, positive control) and (D) 57aBi-WT (negative control). Data were measured in triplicates; error bars are defined as standard deviation.

Table 1. Results of the affinity measurement between human IL-9Rα and its ligands measured by MST.

	Affinity (nM)	Amplitude	No. of replicates
57aBi-WT	Not measurable	–	3
IL-9	43	17	3
57aBi-D14	5000	13	3
57bBi-D06	510	16	3

each technique, the nonspecific variants occurring in one of them lose their binding partners in the other selection technique and are discarded during the selection process. A few rounds of ribosome display should be used in the initial steps of the selection to take advantage of the ability of ribosome display to deal with the high complexity of libraries. After the complexity is reduced, yeast display eliminates the selection of incompletely and/or poorly expressed variants. The final round of ribosome display ensures that binders can be produced by a prokaryotic expression system (albeit in the cell-free system).

Affinity measurement of two best binder candidates

Affinities of the IL9-Rα binders were measured for two best binders, one derived from the 57aBi scaffold and one from the 57bBi scaffold by Microscale thermophoresis (MST) as dissociation constants K_d (Fig. 4, Table 1) in triplicates. The affinity measured for the best 57aBi variant 57aBi-D14 was 5 μM, and the best 57bBi variant 57bBi-D06 had a higher affinity, 510 nM. Untrained 57aBi-WT used as a negative control had unmeasurably low affinity to IL9-Rα. The affinity of the natural ligand of IL9-Rα, IL-9, was 43 nM. The IL-9 affinity to IL-9Rα has been previously reported in the literature as sub-nanomolar [29]. This high affinity has been estimated for the mouse IL-9/IL9-Rα system by MST-unrelated method directly in the cell culture of murine T-cell population.

Comparing the MST affinities of the binders and IL-9 indicates a need for further affinity maturation of binders. The difference between the measured affinities

of the binders and IL-9 suggests that further use of secondary mutagenesis tools such as such as error-prone PCR, and rational computer-driven design has a good chance to further increase binder affinities. Increasing binder's affinity would be the first step towards their use for diagnostic or therapeutic purposes that would need to be followed by proving their power to disrupt the IL-9 signalling pathway. However, these steps are beyond the scope of this methodological work.

Conclusions

In this work, we present a proof-of-concept study combining two display selection methods into one workflow. We demonstrated that the combination of *in vitro* ribosome and cell-base yeast display methods presents a powerful tool for the selection of highly expressed and specific protein binders. We used this platform to select binders against the specific IL-9 receptor, IL-9Rα, an important member of the IL-9 signalling pathway that is involved in regulating inflammation and response to viruses.

We used the ribosome display technique in the initial selection steps, as it enabled us to cover the high-complexity libraries. After several rounds of ribosome display, as the complexity of the combinatorial library was reduced, we introduced yeast display. This enabled us to select only well-folded and fully translated protein variants with a high expression level in the soluble form. Importantly, the implementation of yeast display into the workflow increased the specificity of the selected variants to IL-9Rα and eliminated nonspecific binding to BSA and to the common gamma chain cytokine receptor subunit.

During the selection process in the ribosome display, we applied subtractive panning against a nonspecific BSA target. This, together with the usage of non-protein-blocking reagents instead of protein-based solutions helped to increase the specificity of binders to the target protein, IL-9Rα.

For the creation of combinatorial libraries of protein variants, we used two protein scaffolds, 57aBi and 57bBi, previously designed in our laboratory. The experiments presented here show that the newly developed pipeline combining *in vitro*, and cell-based selection display methods are effective in producing of soluble specific binders.

Materials and methods

Construction of DNA library of scaffolds

The 57aBi and 57bBi scaffolds were designed and 10 amino acid positions for randomization in each of them were selected in previous studies (Fig. 5) [12]. The combinatorial libraries of both 57aBi and 57bBi scaffolds were synthesized by the GENEWIZ company (Leipzig, Germany) using NNK codons technology. The DNA construct contained an open reading frame coding the protein scaffold sequence, elements for *in vitro* transcription and translation (T7 promoter, 5′ stem-loop and ribosome-binding site) and restriction sites on both termini. In addition, the libraries consisted of N-terminal Strep-tag II peptide for further purification and C-terminal c-myc detection tag followed by TolA-spacer. For further selection by ribosome display technique, there was an absence of STOP codon for the creation of a stable complex of mRNA/ribosome/protein. The ordered DNA libraries were amplified by PCR using T7b and TolAk primers (Table 2).

Cloning of recombinant proteins

Gene for human IL-9Rα extracellular domain (Uniprot Q01113 residues 41–270) was ordered in the form of DNA string (Thermo Fisher, Waltham, MA, USA) with optimized codons for their expression in the eukaryotic system. The DNA was cloned into a pMTH vector [12] to obtain a final construct of a human IL-9Rα extracellular subunit with N-terminal BiP signal peptide and C-terminal His-tag purification tag (UniProt code Q01113 residues 41–270) containing C-terminal 6xHis purification tag and cloning sites in bold.

RSSVTGEGQG PRSRTFTCLT NNILRIDCHW SAPE LGQGSS PWLLFTSNQA PGGTHKCILR GSECTVV LPP EAVLVPSDNF TITFHHCMSG REQVSLVDPE YLPRRHVKLD PPSDLQSNIS SGHCILTWSI SPALEP MTTL LSYELAFKKQ EEAWEQAQHR DHIVGVTWLI LEAFELDPGF IHEARLRVQM ATLEDDVVEE ERY TGQWSEW SQPVCFQAPQ RQGPLIPPWG WP**LE HHHHHH**

The DNA coding human common gamma chain cytokine receptor subunit (UniProt code P31785 residues 23–255) was kindly provided by J. Spangler (Johns Hopkins University, Baltimore, MD). The gene was cloned into gWiz vector (Genlantis, San Diego, CA, USA).

Expression and purification of proteins

The recombinant human IL-9Rα extracellular domain was produced similarly as described elsewhere [30]. Briefly, the Schneider 2 insect (S2) cells were transfected and further cultivated in an Insect-XPRESS Protein-free Insect Cell Medium (Lonza, Bazel, Switzerland). Protein expression was induced by the addition of $CuSO_4$ and secreted IL-9Rα protein was purified via Ni-NTA affinity chromatography followed by size exclusion chromatography using Superdex 200 Increase 10/300 GL column (Figs 6 and 7A). The column was equilibrated into the buffer containing 50 mM Tris pH 8.0 and 300 mM NaCl. Human IL-9

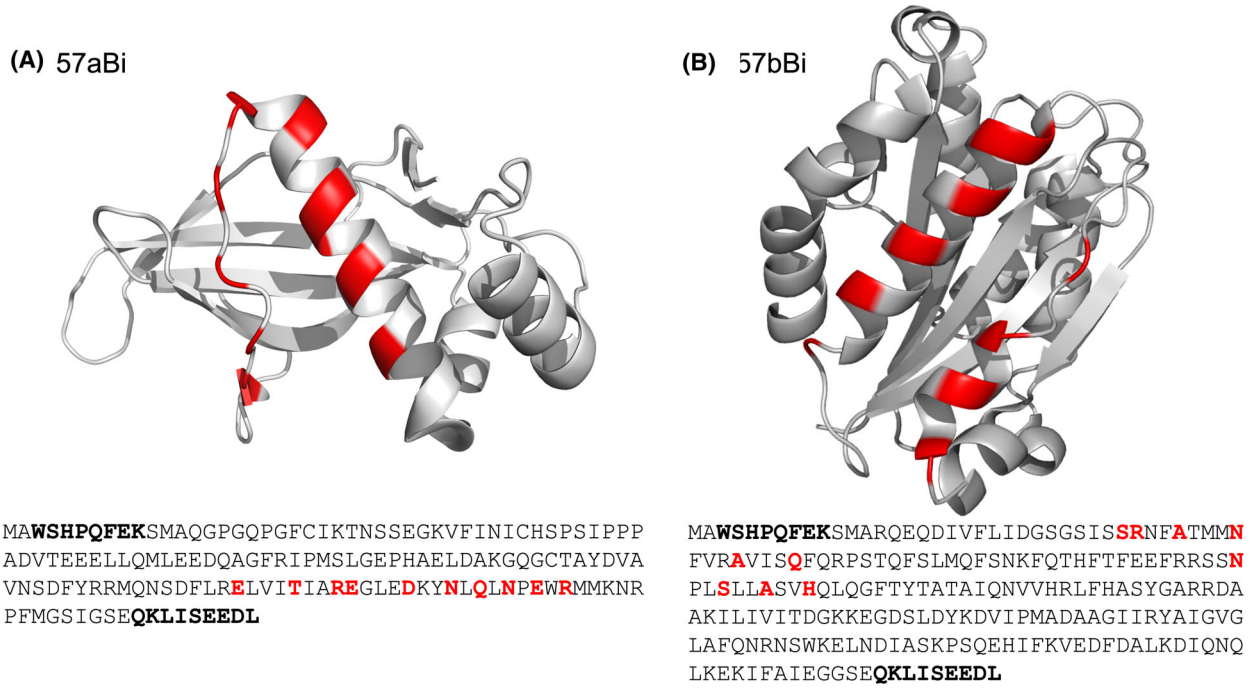

Fig. 5. Structures of (A) 57aBi scaffold and (B) 57bBi scaffold and their amino acid sequences. Ten red-labelled positions of each scaffold were chosen for further randomization. Scaffolds contain Strep-tag II purification tag on N-terminus and c-myc detection tag on their C-terminus (in bold). Molecular cartoons were drawn by program PYMOL (DeLANO Scientific LLC, San Carlos, CA, USA).

Table 2. List of used DNA primers. Specific sets of 57aBi_for/rev and 57bBi_for/rev were used for the amplification of scaffolds open reading frame sequence. Primers T7b and TolAk served for the amplification of ordered DNA libraries including ribosome display elements.

Primer name	Sequence
57aBi_for	AAGTCCATGGCACAGGGA
57aBi_rev	GTTCGGATCCGATGGAGCC CATGAATG
57bBi_for	AAGTCCATGGCAAGACAGGAG
57bBi_rev	GTTCGGATCCACCCTCAATG
T7b	ATACGAAATTAATACGACTCA CTATAGGGAGACCACAACGG
TolAk	CCGCACACCAGTAAGGTGTGCG GTTTCAGTTGCCGCTTTCTTTCT

(UniProt P15248 residues 19–144) used as a positive control in the microscale thermophoresis affinity essay was produced as a recombinant protein in the S2 insect cells and purified in the same way as IL-9Rα.

The expression of the human common gamma chain protein (CD132) was done in suspension-adapted HEK293T cells kindly provided by R. A. Aricescu [31]. The adaptation for suspension cultivation is described elsewhere [32]. The cells were transfected in high density. Briefly, 800 × 10^6 HEK293T cells were centrifuged (90 ***g***, 5 min) and resuspended in 34 mL of ExCELL293 medium. The cell suspension was then transferred to a 1 L square-bottom flask. To that, 800 µg of plasmid DNA dissolved in 6 mL of PBS for cell cultures was sterile-filtered and mixed. Linear 25 kDa polyethyleneimine (lPEI) in a ratio of 1 : 3 DNA : lPEI (w/w) was added directly to the culture. The cell suspension was incubated on a shaker for 90 min at 37 °C, 135 r.p.m. The culture was topped with fresh medium to 400 mL and valproic acid [33] was added to a final 2 mM concentration. The culture was harvested 5 days post-transfection. The supernatant was filtered (0.22 µm filter), diluted with PBS (1 : 1 volume ratio) and loaded to a 5 mL INDIGO Ni-Agarose column. Impurities were washed off with PBS and pre-eluted with 25 mM Imidazole in PBS. Protein was eluted with 250 mM Imidazole in PBS, concentrated to 250 µL and loaded to Superdex 200 10/300 GL column equilibrated with 10 mM HEPES buffer pH 7.5 and the appropriate fractions were collected.

WTs of 57aBi and 57bBi scaffolds and two best binding variants derived from each scaffold were purified in two steps, firstly by affinity chromatography using Streptactin XT resin (Iba, Göttingen, Germany) followed by size exclusion chromatography using Superdex 200 Increase 10/300 GL column (Fig. 7B). The column was equilibrated into the buffer containing 50 mM Tris pH 8.0 and 300 mM NaCl. Purified proteins were then analysed by SDS/PAGE to demonstrate their solubility and expression level in *E. coli*.

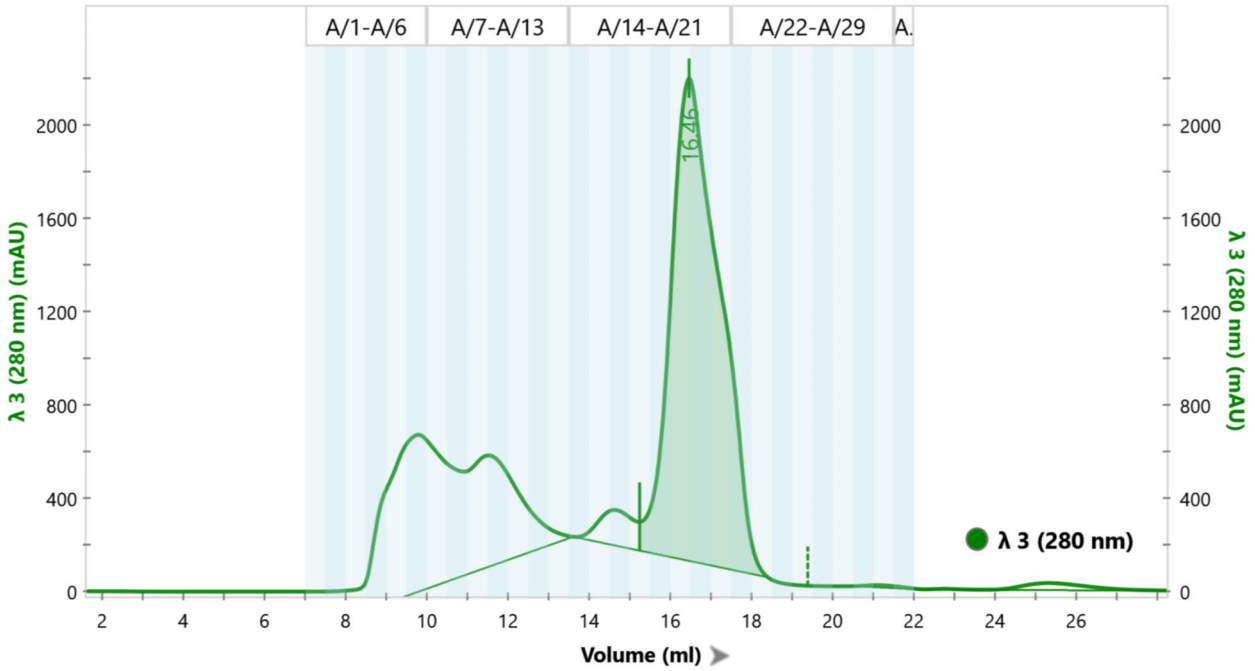

Fig. 6. Size exclusion chromatography of IL-9Rα extracellular domain followed after Ni-NTA affinity chromatography. Elution fractions 19–21 were collected for further assays and were analysed via SDS/PAGE.

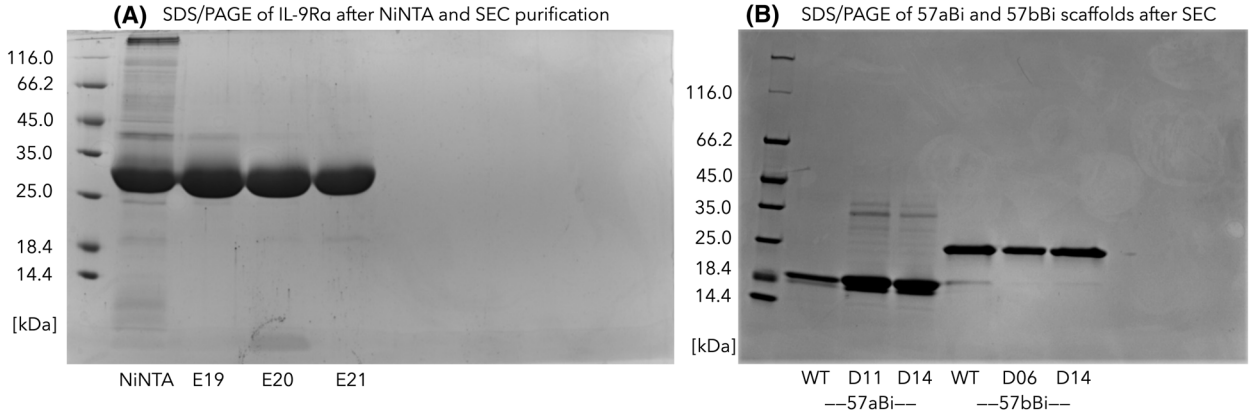

Fig. 7. (A) SDS/PAGE of IL-9Rα extracellular domain purified by Ni-NTA affinity chromatography and size exclusion chromatography. (B) SDS/PAGE of 57aBi and 57bBi WTs and two best binders of both scaffolds: 57aBi-D11, 57aBi-D14; 57bBi-D06, 57bBi-D14 after two-step purification (Streptactin XT purification and size exclusion chromatography).

Preparation of cell lysates for ELISA testing

Libraries after the fifth and sixth ribosome display selection were cloned into pETsm vector (a modified pET-26b (+) vector containing N-terminal Strep-tag II peptide and C-terminal c-myc tag with Stop codon) [12]. Chemically competent *E. coli* BL21-Gold (DE3) strain was transformed with this vector according to the standard protocol for the transformation of chemocompetent cells.

Protein expression was evaluated on 16 random clones of both 57aBi and 57bBi variants. Cells were cultivated in LB medium with kanamycin antibiotic in the deep 24-well plate. After the cultivation, cells were harvested by centrifugation (1100 ***g***, 60 min). Cell pellets were resuspended and lysed in B-PER Bacterial Protein Extraction Reagent (Thermo Fisher) by shaking at 300 r.p.m. for 45 min. Lysates were clarified by centrifugation (1100 ***g***, 60 min). Resulted supernatants containing binding variants were

analysed for binding to IL-9 receptor subunits and BSA by ELISA.

Construction of mRNA-ribosome-scaffold complex

Constructs coding combinatorial libraries of 57aBi and 57bBi scaffolds designed for ribosome display were transcribed and translated in one step at 30 °C for 6 h using RTS 100 *E. coli* HY commercial kit (Biotechrabbit, Berlin, Germany). Because of the absence of STOP codon, a stable mRNA-ribosome-scaffold complex was formed. Complexes were held on ice until processed in the subtractive pre-selection in the ribosome display.

Subtractive pre-selection in ribosome display

Subtractive pre-selection was done before every round of ribosome display selection. Stable complexes of mRNA-ribosome-scaffold were supplemented by 0.5% BSA and 200 mg·mL^{-1} of heparin in PBS buffer and incubated for either 1 × 60 min or 3 × 30 min at 4 °C with 3% BSA immobilized on the plastic surface of Nunc Polysorp plate (Invitrogen, Waltham, MA, USA).

Ribosome display selection

Supernatants from the pre-selection wells contained the pre-selected mRNA-ribosome-scaffold complexes and then were transferred into the selection wells coated with IL-9Rα diluted in Bicarbonate coating buffer pH 9.6. Concentrations of coated target protein, concentrations of used Pluronic F-127 detergent and number of washing steps during the selection process varied in the individual rounds of the display method (Table 3). Complexes of mRNA-ribosome-scaffold were incubated with IL-9Rα for 60 min at 4 °C. The well was washed with TBS pH 7.4 buffer containing Pluronic F-127 detergent. The library complexes were incubated with an Elution buffer (EB) composed of 50 mM Tris-acetate, 150 mM NaCl, 50 mM EDTA, final pH 7.5. EB contained 1 mg·mL^{-1} of *Saccharomyces cerevisiae* RNA and 200 mg·mL^{-1} of heparin. EDTA causes disruption of the mRNA-ribosome-scaffold complex, and mRNA is then isolated using High Pure RNA isolation Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The purified mRNA was reverse transcribed using GoScript Reverse Transcription System (Promega, Madison, WI, USA) using reverse primers 57aBi_rev and 57bBi_rev (Table 2) according to the manufacturer's protocol. The reverse transcribed cDNA was amplified by PCR using commercial Q5 polymerase (New England Biolabs, Ipswich, MA, USA) with 57aBi_for and 57aBi_rev primers for 57aBi library, and 57bBi_for and 57bBi_rev primers for 57bBi library. The amplified DNA was cloned into the pRDVsm vector [12] with NcoI and BamHI restriction enzymes and ligated with T4 ligase (New England Biolabs). Libraries cloned in pRDVsm vector were used for further selection and pre-selection processes. In the final rounds of ribosome display selections, DNA libraries were cloned into the pETsm vector and transformed into *E. coli* BL21-Gold (DE3) chemocompetent cells (Agilent, Santa Clara, CA, USA) for the analysis of sequences of the selected protein binders and for the evaluation of their binding properties by ELISAs.

Table 3. Concentrations of coated target molecules (IL-9Rα), concentrations of Pluronic F-127 detergent in washing buffer and number of washing steps of specific round during ribosome display selection.

Selection round	Target concentration (μg·mL^{-1})	Pluronic F-127 concentration (%)	Number of washing steps
1	25	0.10	5
2	25	0.15	10
3	15	0.20	10
4	15	0.20	10
5	10	0.20	15
6	10	0.20	15

Construction of DNA libraries for yeast display selection and yeast cell transformation

DNA libraries after the fifth round of ribosome display selection were cleaved with NdeI and BamHI restriction enzymes and cloned into the pETcon(−) plasmid (Plasmid #41522; Addgene, Watertown, MA, USA) using T4 ligase (New England Biolabs).

Saccharomyces cerevisiae EBY 100 competent cells (Invitrogen) were cultivated in YPD medium (10 g·L^{-1} Yeast nitrogen base, 20 g·L^{-1} Peptone, 20 g·L^{-1} Glucose) at 30 °C and until OD600 reached 1.6. Cells were collected by centrifugation (800 ***g***, 3 min). Pellets were washed twice with 50 mL of ice-cold water and once with 50 mL of ice-cold electroporation buffer containing 1 M Sorbitol and 1 mM $CaCl_2$.

Pellets were resuspended in 20 mL of 0.1 M LiAc with 10 mM DTT and shaken in a culture flask for 30 min. Cells were again collected by centrifugation (800 ***g***, 3 min), washed once with 50 mL of ice-cold electroporation buffer and resuspended in 200 μL of the same buffer. The cells were kept on ice until electroporation.

Cells were gently mixed with plasmid-containing combinatorial libraries and transferred to a pre-chilled BioRad GenePulser cuvettes (BioRad, Hercules, CA, USA). Cuvettes were kept on ice for 5 min. Then, cells were transformed with prepared plasmid by electroporation at 2.5 kV and 25 μF.

Electroporated cells were transferred into 8 mL of 1 M Sorbitol/YPD media in a ratio 1 : 1. Cells were cultivated at 30 °C for 1 h, then collected by centrifugation (800 ***g***, 3 min) and resuspended in 50 mL of SDCAA media (6.7 $g \cdot L^{-1}$ Yeast nitrogen base, 5 $g \cdot L^{-1}$ Bacto casamino acids, 5.4 $g \cdot L^{-1}$ Na_2HPO_4, 8.56 $g \cdot L^{-1}$ NaH_2PO_4, 20 $g \cdot L^{-1}$ Glucose) and grew overnight.

Yeast display selection

The electroporated yeast cells (1 mL) were added to a mixture of 1 mL of SDCAA media and 9 mL SG-CAA media (6.7 $g \cdot L^{-1}$ Yeast nitrogen base, 5 $g \cdot L^{-1}$ Bacto casamino acids, 5.4 $g \cdot L^{-1}$ Na_2HPO_4, 8.56 $g \cdot L^{-1}$ NaH_2PO_4, 20 $g \cdot L^{-1}$ Galactose). Cells were cultivated overnight at 20 °C. When the yeast cells consumed all the glucose in the media mixture, they started to metabolize galactose. The protein scaffold variants were under the galactose promoter, therefore they were expressed and further displayed on the yeast cell surface.

Yeast cells presenting our protein binders on their surface were harvested by centrifugation (800 ***g***, 5 min) and resuspended in 1 mL of PBS buffer supplemented with 0.1% BSA (PBS-B). IL-9Rα in a final concentration of 100 nM was added and the reaction was incubated at 4 °C for 2 h.

Cells were harvested by centrifugation (800 ***g***, 5 min), washed with PBS and resuspended in 50 µL of PBS-B buffer together with 1 µL of chicken anti-c-myc tag antibody (BioLegend, San Diego, CA, USA). Reaction was held on ice for 1 h. After the incubation, cells were washed again and resuspended in 50 µL of PBS-B buffer together with 1 µL of anti-IL-9Rα antibody conjugated with PE and 1 µL of anti-chicken secondary antibody conjugated with APC (both BioLegend). The reaction was incubated on ice for 1 h. The cells were washed twice and resuspended in 1 mL of PBS-B buffer. Samples were analysed using BD FACSAria flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Variants with detected double fluorescence signals (Fig. 3C–E) were sorted into a fresh SDCAA medium.

Yeast plasmid isolation

Sorted yeast cells with presented protein scaffold variants binding IL-9Rα subunit were cultivated and recovered in SDCAA medium overnight at 30 °C. Cells were collected by centrifugation (800 ***g***, 5 min) and plasmid DNA was isolated using the Zymoprep kit (Zymo Research, Irwine, CA, USA) according to the attached manufacturer's protocol. Isolated DNA was used for the transformation of chemocompetent *E. coli* TOP10 strain. Cells were plated on LB agar plates with 100 $\mu g \cdot L^{-1}$ kanamycin antibiotic and colonies were grown overnight at 37 °C. Plasmids from single colonies were isolated using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the attached manufacturer's protocol. DNA was then cleaved by NcoI and BamHI restriction enzymes and cloned into the pRDVsm vector for the sixth round of selection by ribosome display.

ELISA

Nunc Polysorp 96-well microplate from Invitrogen was covered with 10 $\mu g \cdot mL^{-1}$ of IL-9Rα or common gamma chain cytokine receptor subunit diluted in Bicarbonate coating buffer pH 9.6. The plate was then blocked with 3% BSA diluted in PBS-P buffer (PBS buffer + 0.1% Pluronic F127). One control plate was also covered with 3% BSA for detection of the negative background level of binding. Plates were washed three times with PBS-P buffer.

Each plate well was filled with a cell lysate prepared from a different single colony expressing protein scaffold variant. Binding reaction was running for 1 h and plates were washed three times. The C-terminal c-myc detection tag of the binders was detected via anti-c-myc antibody conjugated with horseradish peroxidase (HRP) (Abcam, Cambridge, UK). Reaction was incubated for 1 h and plates were washed three times to remove the unbounded antibody. Then, a specific TMB-2 one-step substrate (Thermo Scientific) for the peroxidase was added, and the reaction was stopped with 2 M H_2SO_4. Absorbance at 450 nm was measured.

Microscale thermophoresis

According to ELISA results, the affinity of 57-aBi-D14 and 57-bBi-D6 variants were estimated by Microscale thermophoresis (MST) using the Monolith NT.115 instrument (NanoTemper Technologies, Munich, Germany); as a negative control was used 57aBi-WT, as the positive control S2-cell expressed IL-9. IL-9Rα was labelled via c-terminal His-tag with RED-tris-NTA labeling kit according to the attached kit protocol. Protein binders were diluted in buffer 50 mM TRIS; pH = 8, 300 mM NaCl and 0.1% Pluronic F-127. IL-9Rα was then titrated by protein binders, and the mix was loaded into NT.115 Standard treated capillaries and MST was measured using Medium MST power and 60% of LED power. MST measurements were done in MO. Control program and data were analysed in MO. Affinity Analysis v2.2.4 software (both NanoTemper Technologies).

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

PM, MH and BS conceived the project; MH, PM, LB and JT planned experiments; MH, DB, ŠH, LK and JT performed experiments and analysed data; MH, JT, LB, PM and BS wrote the paper.

Peer review

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Data availability statement

The data that support the findings of this study are openly available in the Zenodo server at https://doi.org/10.5281/zenodo.752555.

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