

DARPin as Bispecific Receptor Antagonists Analyzed for Immunoglobulin E Receptor Blockage

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The concept of multispecific antibodies is of high therapeutic interest but has failed to produce pharmaceutical products due to the poor biophysical properties of such molecules. Here, we propose an alternative and simple way to generate bispecific binding molecules using designed ankyrin repeat proteins (DARPin). For this purpose, monovalent DARPins with different epitope specificities were selected against the α chain of the high-affinity receptor for human immunoglobulin E (IgE) (Fc ϵ RI α). Two of the isolated binders interfering with IgE binding to the receptor were joined to each other or to themselves via a flexible protein linker. The resulting bivalent and bispecific DARPins were tested for their ability to prevent allergen-induced cell degranulation using rat basophilic leukemia cells stably transfected with human Fc ϵ RI α . The bispecific DARPin construct was the most potent one, efficiently blocking the IgE–Fc ϵ RI interaction and preventing the release of proinflammatory mediators. Noteworthy, the multivalent and multispecific DARPin construct did not show any alteration of the beneficial biophysical properties of the monovalent parental DARPins. Hence, bispecific DARPins may be used to generate receptor antagonists simultaneously targeting different epitopes on the same molecule. Moreover, they easily overcome the limiting immunoglobulin binding paradigm (one binding molecule=one epitope) and thereby represent an alternative to monoclonal antibodies in cases where the immunoglobulin scaffold is unsuitable.

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Introduction

Their ability to bind an appropriate antigen with high specificity and affinity turned antibodies into an indispensable tool for targeted disease therapy.¹ In certain cases, the application of multispecific

molecules simultaneously targeting distinct antigens might increase treatment efficiency. Therefore, attempts to generate bispecific antibodies or antibody fragments (Fab, diabodies) by hybridoma cell fusion, recombinant DNA expression or chemical conjugation have been undertaken.² Often the expression yield and quality of such bispecific binders were not sufficient.³ In this work, we propose a simple strategy to generate efficient and functional bispecific binders using designed ankyrin repeat proteins (DARPins).

DARPins were recently described as an alternative binding scaffold based on a consensus sequence derived from natural ankyrin repeat proteins. The molecular assembly of DARPins and the production of DARPin libraries have been described elsewhere.^{4,5} The use of ribosome display as a selection technique allows isolation of high-affinity binders against any given target protein from these DARPin

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Abbreviations used: α -HSA- α , human serum albumin N- and C-terminally fused to human Fc ϵ RI α ; DARPin, designed ankyrin repeat protein; Fc ϵ RI, high-affinity IgE receptor; Fc ϵ RI α , α chain of the high-affinity IgE receptor; IgE, immunoglobulin E; NIP-BSA, 4-hydroxy-5-iodo-3-nitrophenylacetyl joined to bovine serum albumin; PBS, phosphate-buffered saline; RBL-2H3-hu α , rat basophilic leukemia cells stably transfected with human Fc ϵ RI α ; RU, response units.

libraries.^{6,7} Due to their globular structure and the lack of cysteine residues, DARPins may be expressed in high quantities and in soluble form in *Escherichia coli*.⁸ DARPins with distinct epitope specificities may be genetically linked to each other by a simple [Gly₄-Ser]₄ linker in order to generate bispecific binding molecules.

To assess the functionality and efficiency of bispecific DARPins *in vitro*, we chose a model in which the application of antibodies has failed so far.⁹ Immunoglobulin E (IgE) interacts with its high-affinity receptor (FcεRI) via two binding sites located on the α subunit of the receptor (FcεRIα), resulting in a K_d of 1.4×10^{-10} M.^{10–12} Allergens cross-linking IgE on sensitized mast cells or basophils aggregate FcεRI, leading to cell degranulation.^{13,14} Released proinflammatory mediators finally induce allergy symptoms, such as coughing, wheezing, swelling, shortness of breath or even anaphylactic shock. Different anti-FcεRIα antibodies inhibiting IgE binding to the receptor have previously been isolated.^{15–17} However, due to the bivalency of antibodies, they cross-link FcεRIα on the cell surface and consequently trigger cell degranulation. Thus, monospecific anti-FcεRIα antibodies would not be useful as receptor antagonists preventing allergic reactions.

Garman *et al.* mapped the two IgE binding sites to the extracellular part of FcεRIα: one is located in the first Ig-like domain, and the other is on top of the receptor in the interface of the first and second Ig-like domains (Fig. 1a).¹⁰ It was previously suggested that an efficient FcεRIα antagonist must block both IgE binding sites on the receptor (Fig. 1b).¹⁸ Using the DARPin technology, we successfully selected binders against different epitopes on the receptor, allowing us to generate bispecific FcεRIα antagonists with improved potencies in inhibiting an allergic reaction.

Results

Selection of FcεRIα binders

A DNA library coding for DARPins with two randomized ankyrin repeat modules (N2C) and another coding for DARPins with three modules (N3C) were screened for FcεRIα binders using ribosome display. Four selection rounds were performed as described in **Materials and Methods**. Enrichment of FcεRIα binders was observed after the first three rounds on recombinant α-HSA-α (FcεRIα N- and C-terminally fused to human serum albumin). In each round, selection pressure was increased to favor isolation of DARPins with higher affinities. ELISA screening after the third and fourth selection rounds revealed that 29 of the 500 tested DARPins specifically recognized FcεRIα showing low reactivity to control proteins (Fig. 2).

Randomly chosen FcεRIα-specific binders were sequenced and aligned to test the diversity of

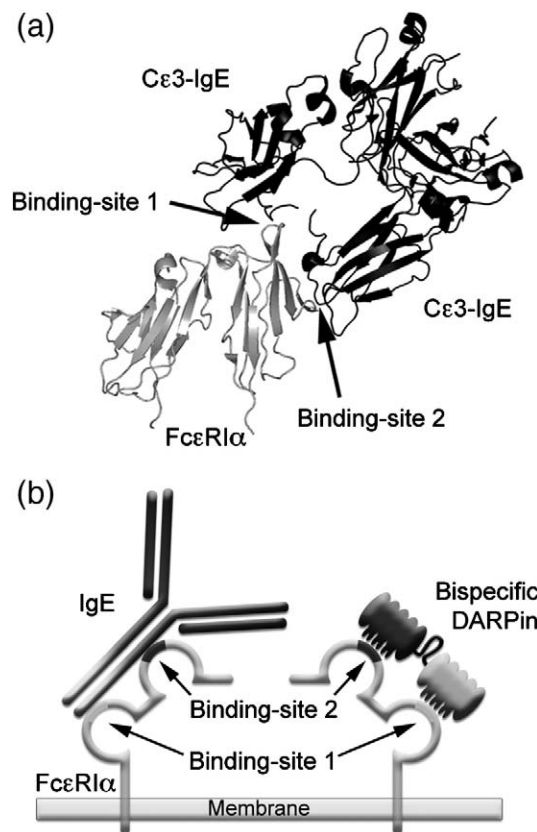


Fig. 1. Interaction of IgE and bispecific DARPin with FcεRIα. Panel (a) depicts the crystal structure of IgE-FcεRIα with the high-affinity IgE receptor (Protein Data Bank code 1f6a).¹⁰ Panel (b) shows a simplified model of the IgE-FcεRIα interaction, as well as the aim of the study, which lies in the generation of a bispecific DARPin simultaneously interfering with both IgE binding sites on the receptor.

selected DARPins. All sequences differed at the variable interaction residues, indicating that different epitope specificities were selected. Two DARPins, termed B-A4-85 and C-A3-30, were chosen for further characterization.

Epitope analysis and generation of bispecific anti-FcεRIα DARPins

Epitope specificities of the two DARPins were assessed by surface plasmon resonance on an α-HSA-α-coated chip. Both of them were previously rendered bivalent to increase the avidity of the binders and thus to warrant better chip saturation. In brief, we cloned the DARPins into an expression vector (pQI-bi-2_2) containing a region coding for a [Gly₄-Ser]₄ linker. Bivalent DARPins were expressed in the same way as monovalent DARPins in *E. coli*. The chip surface was saturated with bivalent DARPin C-A3-30 until the sensorgram reached its maximal response (Fig. 3). Subsequently, bivalent DARPin 85/85 was co-injected together with bivalent DARPin 30/30 three more times. Bivalent DARPin B-A4-85 was still able to bind even though

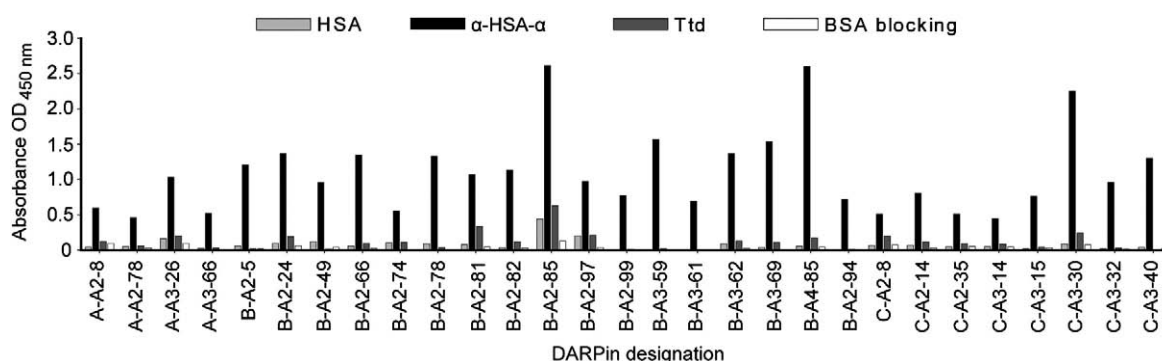


Fig. 2. Specificity screening of selected DARPins by ELISA. DARPins from three selections (A–C; see [Materials and Methods](#)) were tested for FcεRIα binding by ELISA. α-HSA-α (black gray bars) was used as target. Control antigens consisted of HSA (light gray bars), tetanus toxoid (dark gray bars) and PBS/0.5% BSA (white bars). Extracts from selection A were used undiluted, while those from panning B and panning C were diluted in PBS at 1:2 and 1:20, respectively. Binding was revealed using a specific monoclonal murine antibody against the N-terminal His₆ tag of DARPins and a horseradish-peroxidase-labeled goat anti-mouse antibody.

C-A3-30 epitopes were saturated. Hence, the two DARPins are able to recognize different sites on FcεRIα at the same time, which is a prerequisite for the generation of functional and bispecific DARPins. Monovalent DARPins B-A4-85 and C-A3-30 were then fused in two orientations (DARPins C-A3-30-linker-DARPins B-A4-85=30/85 or DARPins B-A4-85-linker-DARPins C-A3-30=85/30) using the same expression vector (pQI-bi-2_2) as previously described for the bivalent DARPins.

Affinity of monovalent, bivalent and bispecific DARPins

Kinetics of monovalent, bivalent and bispecific anti-FcεRIα DARPins were assessed by surface plasmon resonance on immobilized α-HSA-α ([Table 1](#)). The two monovalent DARPins recognized FcεRIα

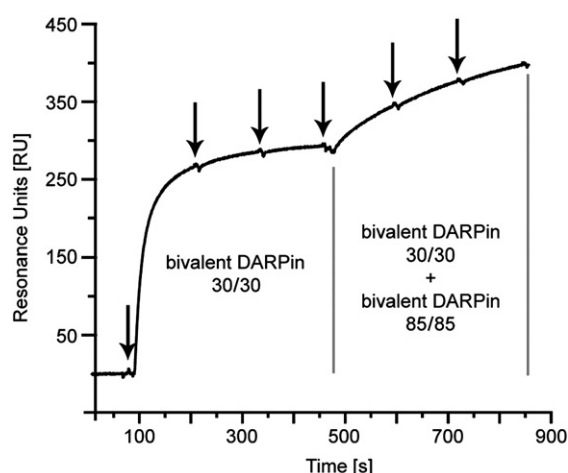


Fig. 3. Epitope specificities of anti-FcεRIα DARPins. Surface plasmon resonance was used to compare epitope specificities of two DARPins on FcεRIα. The sensor chip displaying recombinant FcεRIα was first saturated with three serial injections (arrows) of bivalent DARPins 30/30 (400 nM), each lasting 2 min. Subsequently, bivalent DARPins 30/30 (200 nM) and 85/85 (100 nM) were co-injected three more times.

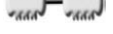
with affinities in the nanomolar range. Although an almost unaltered on-rate was observed for bivalent DARPins, they had a lower off-rate and thus an overall higher affinity compared with their monovalent analogs. Measured affinities of bivalent DARPins increased by at least 1 order of magnitude. However, for these binders, improved affinities are due to increased avidity.

Interestingly, the two bispecific DARPins showed different affinities for FcεRIα. While bispecific DARPins 85/30 bound the receptor with almost the same affinity as monovalent DARPins, its opposite, DARPins 30/85, recognized the receptor in the range of a bivalent DARPins. The orientation of the two monovalent DARPins in the bispecific construct played an important role for its binding characteristics. The bispecific binders recognized FcεRIα with different affinities depending on whether DARPins C-A3-30 or DARPins B-A4-85 is joined to the N-terminus or the C-terminus of the linker. Compared with the monovalent DARPins, bispecific DARPins 30/85 but not bispecific DARPins 85/30 showed improved affinity to FcεRIα. This indicates that only bispecific DARPins 30/85 was able to bind both epitopes on FcεRIα simultaneously. As observed for bivalent DARPins, the affinity improvement of bispecific DARPins 30/85 was mainly due to a lower off-rate. Affinities of monovalent DARPins were in the same range as those of different anti-FcεRIα autoantibodies, LTMα15, LTMα35 and CBMα8, previously described.^{9,15} Bivalent DARPins 30/30 and bispecific DARPins 30/85 even exceeded the affinity of IgE, the natural ligand of FcεRIα.

Inhibition of IgE–FcεRIα interaction

To assess whether anti-FcεRIα DARPins may inhibit IgE binding, we performed inhibition experiments by surface plasmon resonance using the same chip as for epitope analysis. The initial binding signal of IgE without competitor was compared with the remaining signal after prior chip saturation with the different DARPins. The maximal IgE

Table 1. Affinities of FcεRIα binding DARPins and antibodies

Binder type	Structure	Designation	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	K_d (M)
Monovalent DARPIn		C-A3-30	1.08×10^6	2.3×10^{-3}	2.12×10^{-9}
Monovalent DARPIn		B-A4-85	2.67×10^5	1.04×10^{-3}	3.9×10^{-9}
Bivalent DARPIn		30/30	1.98×10^5	9.4×10^{-8a}	4.75×10^{-13a}
Bivalent DARPIn		85/85	2.32×10^5	8.46×10^{-5}	3.65×10^{-10}
Bispecific DARPIn		30/85	9.43×10^5	6.55×10^{-5}	6.71×10^{-11}
Bispecific DARPIn		85/30	7.59×10^4	3.31×10^{-4}	4.36×10^{-9}
Human antibody		LTMα15 ^b	—	—	7×10^{-9}
Human antibody		LTMα35 ^b	—	—	1×10^{-8}
Human antibody		CBMα8 ^c	5.32×10^4	3.54×10^{-4}	6.66×10^{-9}
Murine antibody		15/1 ^c	2.54×10^3	8.27×10^{-7}	3.25×10^{-10}
Human antibody		IgE-Sus11 ^c	2.48×10^5	3.58×10^{-5}	1.44×10^{-10}

^a Values are approximations as they were out of detection range of the measurement apparatus.

^b Published values.^{9,15,19,20}

^c Own measurements.

response [1096 RU (response units)] was reduced to 171 RU (86% inhibition) using bivalent DARPIn 85/85 as antagonist and to 156 RU (85% inhibition) using bivalent DARPIn 30/30 as antagonist (Fig. 4). Both bivalent DARPins were able to block IgE binding to FcεRIα to a certain extent even though they recognized different epitopes on FcεRIα. IgE showed accelerated dissociation from the receptor when binding was competed with bivalent DARPIn 85/85. Next, we investigated whether a combined application of both bivalent DARPins would result in an improved inhibition. Indeed, simultaneous application of both bivalent DARPins resulted in an additive inhibitory effect (to 29 RU; 97% inhibition) and almost completely inhibited the IgE–FcεRIα interaction. As expected, both bispecific DARPins

30/85 and 85/30 showed the same effect as simultaneously added bivalent DARPins (to 33 RU; 97% inhibition), suggesting that both epitopes recognized by DARPins B-A4-85 and C-A3-30 have to be blocked to reach efficient inhibition.

From the affinity measurements, there was evidence that bispecific DARPIn 30/85 but not DARPIn 85/30 is able to simultaneously block both epitopes on one receptor molecule. In this experiment, the chip surface was completely presaturated with corresponding bispecific DARPins or the bivalent DARPIn mixture prior to adding IgE. Therefore, inhibition was dependent on whether both epitopes were blocked, not on the binding affinity or stoichiometry of the individual DARPins.

Inhibition of mediator release from rat basophilic leukemia cells stably transfected with human FcεRIα

Next, we studied whether bivalent and bispecific DARPins are also able to inhibit IgE-mediated degranulation *in vitro*. Rat basophilic leukemia cells stably transfected with human FcεRIα (RBL-2H3-huα cells) were incubated with NIP-BSA (4-hydroxy-5-iodo-3-nitrophenylacetyl joined to bovine serum albumin)-specific IgE in the presence of different DARPins or the commercially available anti-IgE antibody Omalizumab as a control (Fig. 5). NIP-BSA was used to trigger degranulation of the cells. Both bivalent DARPins only inhibited degranulation at the highest concentration. A stronger and additive effect was observed by using the bivalent DARPins in combination. The efficacy of bispecific DARPIn 85/30 was similar to that of simultaneously applied bivalent DARPins. Best inhibition was reached with bispecific DARPIn 30/85, however. It showed an effect similar to Omalizumab. Again, there was evidence that bispecific DARPIn 30/85 but not DARPIn 85/30 was able to simultaneously bind both epitopes

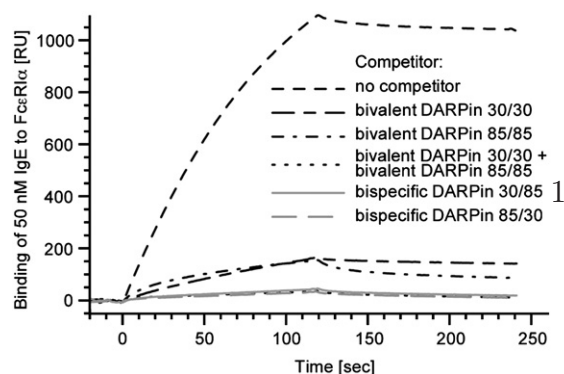


Fig. 4. Inhibition of IgE–FcεRIα interaction. The ability of bivalent and bispecific DARPins to inhibit the IgE–FcεRIα interaction was investigated by surface plasmon resonance. The response of 50 nM IgE–Sus11 either before or after saturation of an α-HSA-α-coated chip with bivalent DARPIn, two bivalent DARPins in combination or with bispecific DARPins was measured. The initial response without saturation (1096 RU) with DARPins was defined as 0% binding inhibition.

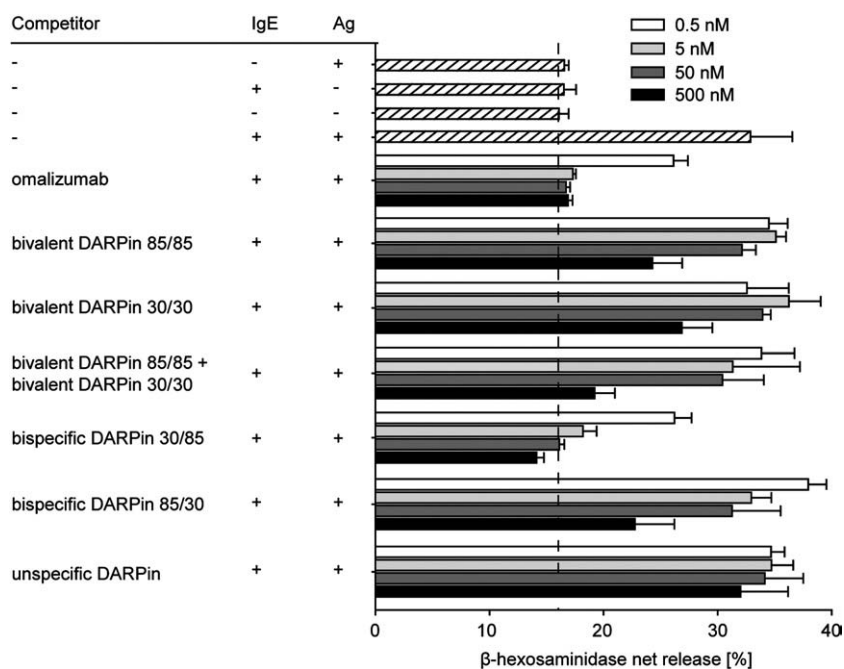


Fig. 5. Inhibition of mediator release from RBL-2H3-hu α cells. RBL-2H3-hu α cells were incubated with Omalizumab (Xolair®) or selected anti-Fc ϵ RI α DARPins. IgE-JW8 (IgE) was added overnight. On the next day, cells were triggered with NIP-BSA (Ag). β -hexosaminidase in the supernatant and stored in the cells was analyzed after receptor cross-link. Increasing concentrations of anti-Fc ϵ RI α binders were used in the experiment to compete with IgE for Fc ϵ RI α (white bar, 0.5 nM; light gray bar, 5 nM; gray bar, 50 nM; black bar, 500 nM). Hatched bars represent maximal net release without addition of competitor and spontaneous net release without sensitization and triggering of cells. For negative control, an unspecific DARPIn was used. Degranulation is expressed as percentage of net release of β -hexosaminidase.

on Fc ϵ RI α . Moreover, this experiment proved that neither bivalent nor bispecific anti-Fc ϵ RI α DARPins are anaphylactogenic.

Aggregation analysis of Fc ϵ RI α with bivalent and bispecific DARPins

To analyze complex formation, we performed HPLC size exclusion with α -HSA- α alone or that

together with the bivalent and bispecific DARPins. The chromatograms show that the two bivalent DARPins and bispecific DARPIn 85/30 form aggregates with Fc ϵ RI α (Fig. 6). In contrast, almost no aggregation was observed using bispecific DARPIn 30/85, confirming our hypothesis that only one of the two bispecific DARPins is capable of simultaneously binding both epitopes on the same receptor molecule.

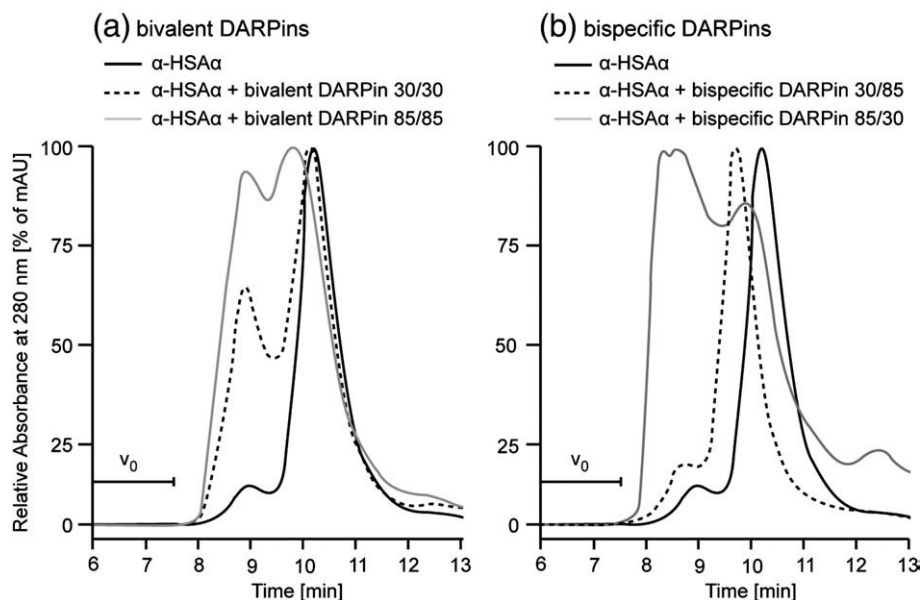


Fig. 6. Aggregation analysis of Fc ϵ RI α by HPLC. It was assessed using HPLC whether bivalent or bispecific anti-Fc ϵ RI α DARPins may aggregate the receptor. Five runs were performed on a TSK G3000SWXL size-exclusion column. α -HSA- α was applied either alone (15 μ M) or after prior incubation with an excess of bivalent or bispecific DARPins (1:4 ratio; 3.75 to 11.25 μ M). Panel (a) depicts an overlay of the chromatograms of α -HSA- α plus bivalent DARPins (30/30, dashed line; 85/85, light gray line) with the chromatogram of α -HSA- α alone (black line). Panel (b) shows the overlay of the same α -HSA- α chromatogram (black line) with those of α -HSA- α plus bispecific DARPins (30/85, dashed line; 85/30, light gray line). The void volume (V_0) of the column is 5.3 ml (7.6 min at a flow rate of 0.7 μ l/min). Aggregates of more than 500 kDa are not separated on this column and thus appear in the aggregate peak (between 7.6 and 9.5 min at a flow rate of 0.7 μ l/min).

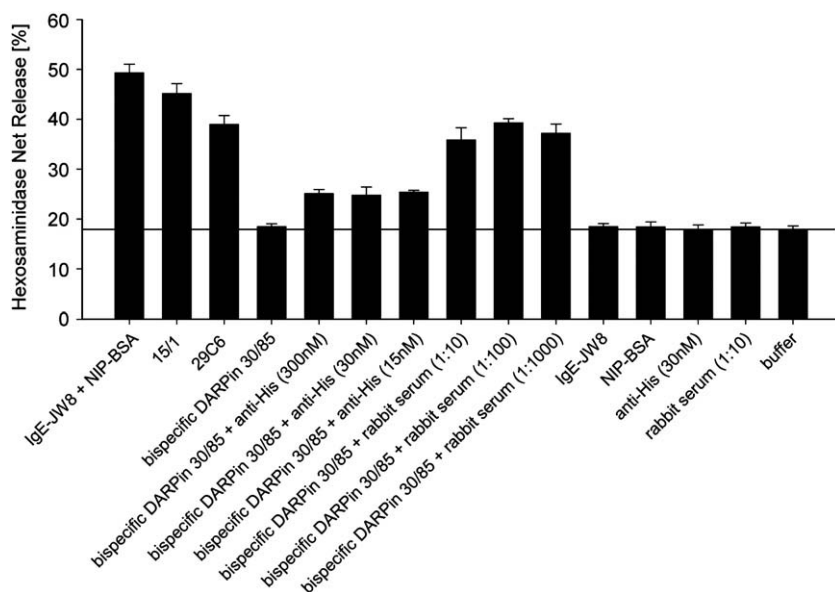


Fig. 7. Anaphylactogenicity assessment. RBL-2H3-hu α cells were incubated with bispecific DARPIn 30/85 alone or with bispecific DARPIn 30/85 subsequently cross-linked by anti-DARPIn antibodies to assess their anaphylactogenic potential. Different concentrations of anti-DARPIn antibodies were used. IgE-JW8 and anti-Fc ϵ RI α antibodies 15/1 and 29C6 were used as controls for degranulation. Results are expressed as percentage of net release of β -hexosaminidase. The horizontal line represents the amount of spontaneous released hexosaminidase (from buffer control).

Anaphylactogenicity of bispecific DARPIn 30/85

Previously described antagonistic anti-Fc ϵ RI α antibodies triggered cell degranulation of basophils or mast cells by cross-linking the receptor.^{9,15,17} In contrast, we have shown that anti-Fc ϵ RI α DARPins do not induce receptor aggregation on the cell surface. Nevertheless, we investigated whether degranulation may be induced by cross-linking receptor-bound DARPIn with anti-DARPIn antibodies. Bispecific DARPIn 30/85 was applied either alone or in combination with different anti-DARPIn antibodies (Fig. 7). Since the DARPIn was expressed with an N-terminal His₆ tag, it could be cross-linked by a murine monoclonal anti-His₆-tag antibody. Moreover, a rabbit serum containing polyclonal anti-DARPIn IgG was applied together with bispecific DARPIn 30/85 to induce mediator release. Two anaphylactogenic anti-Fc ϵ RI α antibodies (15/1 and 29C6) and IgE-JW8 cross-linked by NIP-BSA were included as positive controls. We confirmed our observation that the application of bispecific DARPIn 30/85 alone does not trigger mediator release. However, addition of anti-DARPIn antibodies clearly resulted in receptor aggregation followed by degranulation.

Discussion

Antibodies inhibiting the binding of IgE to Fc ϵ RI α have been described, but they never reached clinical phase due to risk of anaphylactogenicity. The two-armed structure of antibodies induces Fc ϵ RI α cross-link on the cell surface, resulting in the release of proinflammatory mediators.^{9,15–17} Using DARPins as an alternative binding molecule scaffold enabled us to circumvent these structural limitations of immunoglobulins.

DARPins have several advantages over antibodies: They are efficiently expressed in *E. coli* as

they do not contain cysteine residues, and they are superior to antibodies in terms of production yields and stability.⁸ Furthermore, selecting DARPins against target proteins is a rapid process fully carried out *in vitro* without the need for immunization.²¹ As we have shown, the use of DARPins to generate functional bispecific binding molecules is a straightforward strategy. In contrast to the cumbersome procedures to produce bispecific antibodies or antibody fragments, DARPins may simply be combined, similar to building blocks, via a suitable protein linker. Depending on whether the monovalent binders within the bispecific construct were selected against the same or different proteins, bispecific DARPins may be constructed recognizing two distinct epitopes either on the same target or on two targets.

Here, we aimed to block two distinct epitopes overlapping with the IgE binding sites on Fc ϵ RI α . The extracellular part of the receptor, including both Ig-like domains where the IgE binding sites are located, was used as selection target (Fig. 1a).¹⁰ We showed that DARPins C-A3-30 and B-A4-85 simultaneously recognize different epitopes on Fc ϵ RI α (Fig. 3) and that they both interfere with IgE binding to the receptor (Fig. 4). Thus, DARPins C-A3-30 and B-A4-85 represented an optimal pair of binders to be fused together in order to generate bispecific Fc ϵ RI α antagonists. The linker did not seem to influence binding of the individual DARPins in the construct as bivalent and bispecific constructs were still able to recognize Fc ϵ RI α . We showed by plasmon surface resonance measurements that linking of two DARPins resulted in enhanced affinity values (Table 1). For bivalent binders, this is due to increased avidity. In contrast, bispecific DARPins are able to simultaneously recognize distinct epitopes, thereby interacting with a bigger binding surface.

The orientation of the two monovalent DARPins in the bispecific construct plays an important role for its binding characteristics. This was previously

observed for recombinant human bispecific diabodies²² and holds true for our bispecific anti-FcεRIα binders 30/85 and 85/30. They recognize the receptor with different affinities depending on whether DARPin C-A3-30 or DARPin B-A4-85 is joined to the N-terminus or the C-terminus of the linker. Compared with the monovalent DARPins, bispecific DARPin 30/85 but not bispecific DARPin 85/30 showed improved affinity to FcεRIα (Table 1). This might indicate that only bispecific DARPin 30/85 is able to bind both epitopes on FcεRIα simultaneously (Fig. 1b). We assume that bispecific DARPin 85/30 recognizes only one epitope on a single receptor molecule for stoichiometric reasons.

As we have shown, targeting of only one epitope on FcεRIα is not enough to completely inhibit IgE binding. Complete inhibition was only achieved by using either bispecific DARPins or combined application of both bivalent DARPins (Fig. 4). Therefore, we conclude that both epitopes, recognized by DARPins C-A3-30 and B-A4-85, have to be blocked to prevent IgE binding to FcεRIα. The epitopes of DARPins C-A3-30 and B-A4-85 have not been exactly mapped yet. Our data suggest that they differ from each other (Fig. 3) and that they might at least partially overlap with the IgE binding sites, as both DARPins inhibited the IgE–FcεRIα interaction (Fig. 4).

In functional cell assays, administration of the DARPins before sensitization (Fig. 5) or incubation of sensitized cells with DARPins overnight (data not shown) resulted in a concentration-dependent inhibition of proinflammatory mediator release. The applied DARPins efficiently blocked free accessible FcεRIα and competed with IgE for its binding sites. The dissociation rate of receptor-bound IgE, which was described to be in the order of weeks,²³ could be significantly reduced by the application of antagonistic DARPins. Due to their high affinities, we speculate that they might even displace receptor-bound IgE.

Different results support our hypothesis that bispecific DARPin 30/85 simultaneously recognizes two epitopes on one FcεRIα and thus forms a 1:1 complex with the receptor. Best evidence was delivered by aggregation analysis using HPLC size exclusion (Fig. 6) where bispecific DARPin 30/85 did not aggregate FcεRIα. Moreover, it did not trigger RBL-2H3-huα cell degranulation and is thus not anaphylactogenic (Fig. 7).

The administration of therapeutic antibodies often elicits anti-antibody responses—even in the case of humanized antibodies. Repetitive low-dose injections of the humanized murine antibody Humira led to the development of an anti-Humira response in 5% of treated patients.²⁴ Therefore, even though DARPins are designed from naturally occurring ankyrin repeat proteins and are very close to human ones, they may also induce an anti-DARPin response in some individuals. In the case of anti-FcεRIα DARPins, this might be problematic, as such antibodies could induce anaphylactic reactions. Thus, the potential immunogenicity of anti-FcεRIα DARPins is currently a limiting factor for *in vivo* application. As

of today, there is no peer-reviewed publication on the *in vivo* application of DARPins available. However, there are promising preclinical data concerning the *in vivo* use of DARPins against vascular endothelial growth factor and tumor markers (P. A., personal communication).

In summary, we have shown the selection of monovalent DARPins against different epitopes of FcεRIα. We were able to generate a functionally active, bispecific anti-FcεRIα DARPin using two of those selected binders. The possibility of producing bispecific binding molecules might represent a novel approach in the development of new therapeutics even though this is not the case for anti-FcεRIα DARPins. Such molecules not only may be important in the prevention of high-affinity protein–protein interactions, as shown here, but also might have a major impact on the development of new drugs in other fields where the antibody scaffold is limiting or superior affinity is required.

Materials and Methods

Recombinant receptor proteins and antibodies

Two recombinant FcεRIα constructs were used as target proteins. α-HSA-α was a kind gift from Dr. H. Kocher (Novartis Pharma, Basel, Switzerland). It consists of HSA N- and C-terminally fused to the extracellular part of FcεRIα. The second construct, designated α-huIgG₁(Fc), is a fusion protein of the extracellular part of FcεRIα and the Fc part of a human IgG₁. It was provided by Dr. M. Fux (Glenmark, La Chaux-de-Fonds, Switzerland). Both constructs were expressed in CHO (Chinese hamster ovary) cells.

The monoclonal mouse anti-human FcεRIα antibody 15/1 (batch SDS653)²⁵ was obtained from Dr. F. Kricek (Novartis). The murine monoclonal antibody 29C6 was provided by Hoffmann–La Roche (Nutley, NJ).¹⁷

IgE-Sus11 is a monoclonal human IgE produced by a hybridoma cell line¹⁹ in our laboratory. IgE-JW8, produced by a chimeric hybridoma cell line,²⁶ specifically binds to NIP. The monoclonal humanized anti-IgE antibody Omalizumab (Xolair®, Novartis, Genentech)²⁷ was included in several experiments as control and reference.

Vectors, bacterial strains, libraries and cell lines

The ribosome display vectors pRDV (GenBank accession no. AY327136) and pQI-bi-2_2, which was used for the generation of bivalent and bispecific DARPins, were obtained from Molecular Partners AG (Schlieren, Switzerland). As previously described, pRDV contains all DNA regions required for ribosome display.⁴ The vector pQI-bi-2_2 allows the fusion of two proteins via a [Gly₄-Ser]₄ linker located between a BamHI/HindIII cloning site and a BglIII/BsaI cloning site. The protein expression vector pQE30 (Qiagen, Hilden, Germany), containing a His₆-tag sequence, was used to produce DARPins with an N-terminal His₆ tag in *E. coli* XL-1 Blue (Stratagene, San Diego, CA).

Two DARPin DNA libraries, one coding for DARPins containing two randomized ankyrin repeat modules (N2C) and the other coding for DARPins containing three modules (N3C), were received from Molecular

Partners AG. The structure of DARPins and the generation of the libraries have been published.^{8,28}

RBL-2H3-hu α cells were used for degranulation assays.²⁹

In vitro selection

The selection of DARPins using ribosome display was performed as previously described.³⁰ To select Fc ϵ RI α -specific binders, we performed three panning rounds on α -HSA- α with two DARPin libraries (N2C and N3C) in parallel (selection A). HSA was used to preabsorb non-Fc ϵ RI α -specific DARPins prior to each selection. α -HSA- α (40 nM) and HSA (72 nM) were immobilized in 100 μ l of phosphate-buffered saline (PBS; pH 7.4) overnight at 4 °C directly on a Maxisorp plate (Nunc, Roskilde, Denmark). Translated DARPin libraries were added to the target proteins. Selection pressure was increased from round to round by extending the wash duration prior to the recovery of mRNA. Reverse-transcription PCR cycle numbers were decreased from 45 to 35 to 28 throughout selection round 1 to selection round 3. All other PCRs were performed with 30 cycles.

An additional fourth round on two Fc ϵ RI α constructs was carried out after the third selection round in parallel (selections B and C). Starting with the same DNA pool, one panning was performed on α -HSA- α with two prepannings on HSA. In the other panning, α -hulG₁ (55 nM) was used as target antigen. Three prepannings were performed, one on HSA (72 nM) and two on a human IgG₁ (33 nM). All other steps were performed as described for selection rounds 1 to 3.

ELISA

Randomly picked *E. coli* single colonies of the third and fourth selection rounds were cultured in 1 ml of LB medium containing 50 μ g/ml of carbenicillin. At an optical density at 600 nm of 0.6, DARPin expression was induced with 1 mM isopropyl- β -D-thiogalactopyranoside overnight at 25 °C. On the following day, cells were harvested and lysed in 200 μ l of B-PER (Pierce, MA). To increase the sample volume, we added 800 μ l of TBS500 (50 mM Tris-HCl, pH 8.0, and 500 mM NaCl) to each lysate. After an additional centrifugation step, the supernatants (crude extracts) were used to characterize individual DARPins.

In order to analyze the potential of selected DARPins to specifically recognize Fc ϵ RI α , we immobilized three proteins overnight at 4 °C on a Maxisorp plate (Nunc). Wells were coated with HSA (72 nM), α -HSA- α (40 nM) and tetanus toxoid (33 nM) in 100 μ l of PBS. The plate was washed twice with PBS and blocked with 200 μ l of PBS/0.5% BSA for 1 h at 25 °C and 1 h at 4 °C. For each tested extract, an uncoated, blocked well served as additional control for unspecific binding. The plate was washed twice with PBS after blocking, and crude extracts were added for 1 h at 4 °C (extracts were undiluted in the third round, extracts from α -hulG₁ panning were diluted 1:2 in PBS/BSA in the fourth round and extracts from α -HSA- α panning were diluted 1:20 in PBS/BSA in the fourth round). The plate was then washed four times with PBS containing 0.01% Tween-20 and four times with PBS. DARPins were detected via their N-terminal His₆ tag with a murine anti-His IgG (Amersham Bioscience, Freiburg, Germany), followed by a horseradish-peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, USA). Detection antibodies were diluted 1:1000 in PBS/BSA and subsequently incubated for 1 h at 4 °C. TMB (3,3',5,5'-tetramethylbenzidine) was used as substrate to

develop the ELISA. The reaction was stopped after 5 min with 1 M sulfuric acid, and the absorbance was measured at an optical density at 450 nm in a standard ELISA reader (BIO-TEK EL808, Bio8Tek, Bad Friedrichshall, Germany).

Production, purification and sequencing

DARPins were produced in large expression culture volumes (up to 250 ml). Plasmid DNA was produced from 10 ml of the culture. DARPins were sequenced at Microsynth (Balgach, Switzerland) using the oligonucleotide pQE30rev (5'-GTT CTG AGG TCA TTA CTG G-5'). The remaining 240-ml culture was purified over a metal-affinity chromatography column according to the manufacturer's manual (TALON™ Metal Affinity Resins User Manual, Clontech, CA). Purified DARPins were used in further characterization experiments, such as surface plasmon resonance measurements and *in vitro* cell assays.

Production of bivalent and bispecific DARPins

Two selected monovalent DARPins were cloned into pQI-bi-2_2. The first DARPin fragment was inserted into the vector via the BamHI/HindIII (Roche, Basel, Switzerland) restriction site. The second fragment was cloned via the BglIII/BsaI (NE Biolabs, MA) restriction site into pQI-bi-2_2. Bispecific DARPins were produced as DARPin1-linker-DARPin2 and DARPin2-linker-DARPin1 constructs to test whether the orientation plays a role for binding characteristics. Expression and purification of bivalent and bispecific DARPins were performed as described above.

Epitope mapping using surface plasmon resonance

Binding epitopes of DARPins were analyzed by surface plasmon resonance. α -HSA- α was immobilized on a CM5 sensor chip as described for affinity measurement. The surface was first saturated with three serial injections of C-A3-30 (400 nM), each lasting 2 min, until all epitopes were covered (sensorgram reached threshold value) to find out whether DARPins C-A3-30 and B-A4-85 recognize different epitopes on Fc ϵ RI α . DARPin B-A4-85 (100 nM) was then co-injected three times with C-A3-30 (200 nM) for 2 min.

Affinity measurement using surface plasmon resonance

All measurements were performed on a Biacore X instrument (Biacore, Uppsala, Sweden). HBS-EP (10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20) was used as running buffer (flow rate, 10 μ l/min). To determine the binding parameters of the different anti-Fc ϵ RI α molecules, we immobilized 2500 RU of α -HSA- α on flow cell 2 of a CM5 sensor chip. Flow cell 1 remained uncoated and served as control for unspecific binding to the sensor chip surface. Samples were injected for 3 min at different concentrations (400 pM–100 nM). The dissociation rate was measured for another 3 min at constant buffer flow. For each sample, an additional buffer control was measured and subtracted from the sample sensorgram. BIAevaluation software (Biacore) was used to determine the kinetic parameters.

Inhibition of IgE-Fc ϵ RI α interaction

Using surface plasmon resonance, we tested whether the generated divalent DARPins antagonize binding of

IgE to immobilized Fc ϵ RI α . The CM5 sensor chip was prepared in the same manner as described above for the affinity measurement (2500 RU of α -HSA- α). In a first measurement, the response of 50 nM IgE-Sus11 with an injection time of 2 min and a flow rate of 10 μ l/min was assessed. The chip was then saturated with bivalent DARPIn 30/30 (100 nM) or 85/85 (100 nM), bispecific DARPIn 30/85 (400 nM) or 85/30 (400 nM) or the bivalent DARPins in combination (200 nM). The response of 50 nM IgE co-injected with the respective DARPIn for 2 min was remeasured. The unspecific buffer response was subtracted from each sensorgram.

Inhibition of IgE-mediated cellular degranulation

In a 96-well flat-bottom tissue culture plate (Falcon™, BD Bioscience, Heidelberg, Germany), 5×10^4 RBL-2H3-hu α cells were seeded per well in 100 μ l of phenol-red-free RPMI 1640 medium (Biochrome AG, Berlin, Germany) containing 10% fetal calf serum. Cells were incubated for 3 h at 37 °C in a 5% CO₂ incubator, permitting the cells to adhere. DARPins and Omalizumab were added in different concentrations (0.5, 5, 50 and 500 nM). Again, monovalent DARPins were not included in this experiment as their affinity was too low to compete with IgE. The unspecific control DARPIn was a bispecific binder selected against Omalizumab (not published). After 1 h of incubation, IgE-JW8 was added (final concentration, 30 nM). Cells were incubated overnight at 37 °C in 5% CO₂. On the next day, they were triggered with NIP-BSA (20 nM) for 45 min at 37 °C. Supernatants were collected after 1 h of incubation at 37 °C. Cells were lysed with 100 μ l of 0.2% Triton X100 in H₂O after triggering to quantify the remaining amount of β -hexosaminidase (store).

Supernatants and stores were incubated for 1 h at 37 °C with a β -hexosaminidase substrate, *p*-nitrophenyl *N*-acetyl- β -D-glucosaminidase (Sigma, Buchs, Switzerland). The reaction was stopped with 0.1 M carbonate buffer, pH 10, and the absorption was measured in a standard ELISA reader at an optical density at 405 nm. The percentage of net release was calculated by dividing the amount of released β -hexosaminidase by the net amount present in a cell.

Analytical HPLC

α -HSA- α alone or together with bivalent or bispecific DARPins was analyzed on a TSK G3000SWXL HPLC column (Tosoh Biosep GmbH, Stuttgart, Germany) using an HP 1050 HPLC system with an HP 1100 wavelength detector (Agilent Technologies, Wilmington, DE). The running buffer was EP (0.05 M Na₂HPO₄, 0.02 M NaH₂PO₄ and 0.4 M NaCl). Runs were performed at a flow rate of 0.7 μ l/min at 25 °C. The void volume (*V*₀) of the column is 5.3 ml. The following protein amounts were applied: 15 μ M α -HSA- α alone and α -HSA- α with DARPIn in a 1:3 ratio (3.75 μ M receptor/11.25 μ M DARPIn). Absorbance was measured at 280 nm. The maximal signal of the chromatograms was set as 100% relative response.

Anaphylactogenicity

Anaphylactogenicity of bispecific DARPIn 30/85 was assessed using RBL-2H3-hu α cells. The amount of released β -hexosaminidase was measured after incubation with DARPins alone or by cross-linking Fc ϵ RI α -bound DARPins with a monoclonal mouse anti-histidine anti-

body (Amersham Bioscience) or a polyclonal rabbit anti-DARPIn serum from an immunization experiment using DARPins with adjuvants (in house preparation; data not shown). Briefly, cells were prepared as described for the degranulation assay. Thereafter, 10 μ l of different anti-Fc ϵ RI α molecules was added to the cells to a final concentration of 30 nM. Cells were incubated overnight at 37 °C in the 5% CO₂ incubator. On the next day, cross-linking antibodies were added in different concentrations (300, 30 or 15 nM anti-His antibody; 1/10, 1/100 or 1/1000 of anti-DARPIn serum). As described for the degranulation assay, supernatants and stores were quantified. IgE-JW8 (30 nM) cross-linked by NIP-BSA (1.5 nM) and the two anaphylactic anti-Fc ϵ RI α antibodies 15/1 (30 nM) and 29C6 (30 nM) served as controls.

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Supplementary data

Supplementary data associated with this article can be found, in the online version at [doi:10.1016/j.jmb.2009.08.014](https://doi.org/10.1016/j.jmb.2009.08.014)

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Supplementary Material

Sequence alignment of different anti-FcεRIα DARPins

The amino acid sequence alignment of selected anti-FcεRIα DARPins with the N4C consensus sequence is shown in single-letter code. Variable residues being mainly responsible for target binding are termed x and z in the consensus sequence (first line) and are indicated with shaded boxes throughout all sequences. Single letters outside the shaded boxes depict framework mutations. Matching amino acids are represented by dots. Tildes stand for missing residues. Black arrows illustrate the beginning of N- and C-terminal cappings as well as of internal repeats. The His₆-tag (MRGSHHHHHHGS) at the N-terminus was removed from the sequences.