

Inhibition of ongoing allergic reactions using a novel anti-IgE DARPin-Fc fusion protein

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Abstract

Background: Aggregation of the high-affinity IgE receptor (FcεRI) with the low-affinity IgG receptor (FcγRIIb) on basophils or mast cells has been shown to inhibit allergen-induced cell degranulation. Molecules cross-linking these two receptors might therefore be of interest for the treatment of allergic disorders. Here, we demonstrate the generation of a novel bispecific fusion protein efficiently aggregating FcεRI-bound IgE with FcγRIIb on the surface of basophils to prevent pro-inflammatory mediator release.

Methods: Alternative binding molecules recognizing receptor-bound human IgE were selected from DARPin (designed ankyrin repeat protein) libraries. One of the selected DARPins was linked to the Fc-part of a human IgG₁ antibody for binding to FcγRIIb.

Results: The resulting anti-IgE DARPin-Fc fusion protein was not anaphylactogenic and inhibited allergen-induced basophil activation in whole blood assays. Both binding moieties of the fusion protein, namely the anti-IgE DARPin as well as the IgG₁ Fc-part, were required to achieve this inhibitory effect. Most importantly, inhibition was faster and more efficient than with Omalizumab, a humanized anti-IgE antibody currently used for the treatment of severe asthma.

Conclusion: This novel anti-IgE DARPin-Fc fusion protein might represent a potential drug candidate for preventive or immediate treatment of allergic reactions.

Aggregation of the high-affinity IgE receptor (FcεRI) on human basophils and mast cells triggers degranulation of pro-inflammatory mediators inducing allergic responses (1). Allergens as well as anti-IgE antibodies are able to cross-link FcεRI via receptor-bound IgE (2). To prevent allergic reactions, numerous strategies interfering with this degranulation mechanism have been investigated (3).

The humanized anti-IgE antibody Omalizumab (Xolair®) is currently used to treat patients suffering from severe asthma. It recognizes free but not receptor-bound IgE. Omalizumab has been shown to decrease serum IgE levels and to reduce the amount of FcεRI on the surface of basophils and mast cells (4). However, cost-effectiveness of Omalizumab treatment is only achieved in a restricted group of patients because of required high-dose injections and long duration of the therapy (5).

An alternative and promising approach is the negative regulation via inhibitory receptors (6). Several studies have shown that cross-linking of FcεRI with the low-affinity IgG receptor (FcγRIIb) on mast cells and basophils inhibits allergen-induced cell degranulation (7–12). Current models suggest that this negative regulation is mediated by immu-

notyrosine-based inhibition motifs at the cytoplasmic domain of FcγRIIb (13–16). Molecules having the ability to aggregate FcεRI, and FcγRIIb might therefore be of interest for the development of novel drug candidates.

In this study, we describe the generation of a novel fusion protein featuring such binding characteristics. For this purpose, we used nonimmunoglobulin-like binding scaffolds termed DARPins (designed ankyrin repeat proteins). We isolated binders recognizing FcεRI-bound IgE from two different DARPin libraries. One of the selected DARPins was fused to the Fc-part of a human IgG₁. Binding characteristics of the resulting anti-IgE DARPin-Fc fusion protein (DE53-Fc) were assessed, and its capacity to inhibit allergen-induced basophil activation was tested in functional assays.

Methods

Selection of anti-IgE DARPins

The basic principle of DARPin selection is described elsewhere in detail (17). DARPin libraries were screened by

ribosome display to isolate specific binders for IgE (see Data S1) as previously published (18). Selected binders were transformed into *E. coli*. Randomly picked single colonies were cultured in 1 ml lysogeny broth (LB) medium containing 50 µg/ml carbenicillin, and DARPin expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) overnight at 25°C. The following day cells were harvested and lysed in 200 µl B-PER (ThermoFischer Scientific, Rockford, IL, USA) and taken up in 800 µl TBS₅₀₀ (50 mM Tris-HCl pH8.0, 500 mM NaCl). Crude DARPin extracts were screened by ELISA to identify binders specific for FcεRI-bound IgE (data not shown, see Data S1). This screening led to the identification of DARPin E53.

Generation and production of bivalent DARPin and DE53-Fc construct

DARPin E53 was rendered bivalent using pQI-bi-2_2. The first DARPin was cloned into the vector via *Bam*HI/*Hind*III (Fermentas, St. Leon-Rot, Germany) and the second via *Bgl*II/*Bsa*I (NE Biolabs, Ipswich, MA, USA) restriction sites.

The DNA sequence of DARPin E53 was joined to the sequence of a human IgG₁ Fc-part (Uniprot: P01857, amino acids 106–330) via a linker coding for (Gly₄-Ser)₃. The N-terminal sequence coding for the amino acids Met, Arg, Gly, Ser (MRGS) as well as the *Hind*III restriction at the 5' end of the original DARPin was removed. The resulting sequence was synthesized at GeneArt (Regensburg, Germany), where codons were optimized for expression in Chinese hamster ovary (CHO) cells (Fig. S1). The DNA fragment was ligated into a pcDNA3.1(–) based expression vector, which was linearized by *Pvu*I digestion (Roche, Basel, Switzerland) and transfected into CHO cells using polyethylenimine. Supernatants of CHO cell cultures were collected and purified over a protein G column (Protein G Sepharose™, GE Healthcare, Chalfont St. Giles, UK). The column was washed with 150 ml 0.02 M phosphate buffer (pH 6.8) and subsequently eluted with glycine HCl buffer (pH 3.0). DE53-Fc protein concentrations in the eluted fractions were determined by optical density measurement at 280 nm (UV mini Photospectrometer; Shimadzu, Kyoto, Japan). Fractions containing protein were pooled and dialyzed (GE Healthcare Mini Dialysis kit) against PBS. Expression was confirmed by nonreducing SDS–PAGE.

Specificity ELISA with DE53-Fc and bivalent DARPin E53

Antigens (monoclonal human IgE-Sus11, FcεRIα-bound IgE-Sus11, polyclonal human IgM and FcεRIα each at 50 nM) were immobilized on a 96-well Maxisorp plate (Nunc; Thermo Fisher, Waltham, MA, USA) by 4°C overnight incubation. The next day wells were blocked with PBS/0.15% casein for 1 h at room temperature and 1 h at 4°C. The plate was washed twice with PBS and a serial dilution of DE53-Fc (300, 100, 30 and 10 nM) and bivalent DARPin E53 (30, 10, 3 and 1 nM) was added for 1 h at 4°C. Binding of bivalent DARPin E53 was detected using a murine anti-His₆-tag antibody followed by a peroxidase labeled goat anti-mouse

antibody. DE53-Fc was detected in one step using a polyclonal horseradish peroxidase-labeled anti-human-IgG antibody. Detection antibodies were diluted 1 : 1000 in PBS/casein and 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 OD450 nm in a standard ELISA reader (BIO-TEK EL808; BioTek, Bad Friedrichshall, Germany).

Affinity measurement using surface plasmon resonance

Target binding analysis was performed on a Biacore X instrument (Biacore, Uppsala, Sweden). HBS-EP (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20) was used as running buffer (flow rate 10 µl/min). To determine binding parameters of the different IgE binders, 2500 response units of IgE-Sus11 was immobilized on a CM5 sensor chip. Samples were injected for 3 min at different concentrations (bivalent DARPin E53: 1.5–125 nM, DE53-Fc: 50–500 nM, Le27: 1.5–100 nM). The dissociation rate was measured for 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 kinetics.

Basophil activation test

Three individuals with elevated total IgE levels (Donor 1: 266 KU/l; Donor 2: 1357 KU/l; Donor 3: 12.5 KU/l) were included in this assay. Donors were previously screened by immuno solid phase allergen chip (ISAC; Phadia Multiplexing Diagnostics GmbH, Vienna, Austria) as well as immunoCAP® analysis.

The basophil activation test is based on the Flow2 CAST® of Bühlmann Laboratories AG (St. Gallen, Switzerland) that uses CD63 as a basophil activation marker. Several reports have previously demonstrated that CD63 correlates with histamine release (19–21).

Blood was drawn into an EDTA-containing S-Monovette® (Sarstedt, Nümbrecht, Germany). Briefly, 50 µl of blood was added to 100 µl stimulation buffer containing IL-3 (B-CCR-STB, Flow2 CAST® kit). Basophils were stimulated with 10 µl allergen for 25 min at 37°C and stained with an anti-CCR3 PE/anti-CD63 FITC antibody mix (B-CCR-SR, Flow2 CAST® kit). Red blood cells were lysed with 2 ml BD FACS™ Lysing Solution (BD Bioscience, San Jose, CA, USA) for 7 min. After centrifugation, cells were washed once with BD Cell Wash (BD Bioscience) and resuspended in 600 µl BD cell wash. Cells were kept at 4°C until analysis by FACS measurement (BD FACSCanto device, BD FACSDiva Software). Samples were measured in triplicates and individual groups compared by Student's *t*-test.

To assess whether different anti-IgE molecules trigger basophil activation, whole blood samples of donor 1 were incubated with different concentrations (0.01–100 nM final concentration) of monoclonal murine anti-IgE antibody

Le 27, bivalent DARPin E53 and DE53-Fc. IgG Fc-fragments alone and DARPin off7 were included as controls.

Additionally, DE53-Fc (100 nM) was preincubated for 10 min with a polyclonal anti-human IgG-Fc antibody (100 nM) to form complexes. Blood samples from donor 1 were either incubated with DE53-Fc alone or with the precomplexed DE53-Fc. Bivalent DARPin E53 (100 nM) was used as stimulation control, which was set to 100% activation.

To determine a suitable concentration for subsequent inhibition assays, an allergen titration with timothy grass extract (BAG-G6; Bühlmann Laboratories AG) was performed with donor 1. The concentration of 2 ng/ml was used for the following experiments. Whole blood samples in stimulation buffer were incubated for 1 h or 5 min with 10 µl Omalizumab (100 nM), IgG Fc-fragments (100 nM), different concentrations of DE53-Fc (5–100 nM) or buffer (PBS) previous to stimulation with allergen extract. Basophil activation with allergen extract alone was set to 100%.

Furthermore, two additional donors (donors 2 and 3) were tested using a different allergen (6-gras-mix, BAG-GX1; Bühlmann Laboratories AG) at various concentrations (donor 2: 4.5, 10, 25 ng/ml; donor 3: 8, 25, 50 ng/ml). Blood samples in stimulation buffer were incubated for 1 h with or without DE53-Fc (100 nM) prior to allergen challenge.

In a next step, it was investigated whether the inhibitory effect of DE53-Fc may be abrogated by neutralization of either the DARPin or the Fc-binding moiety. Thus, DE53-Fc (5 nM) was preincubated for 10 min with a 100-fold excess of IgE-Sus11 (500 nM), anti-Fc antibody (500 nM) or with buffer (PBS). Blood samples from donor 1 were incubated for 1 h with the preincubated molecules prior to stimulation with allergen extract (timothy grass extract). Basophil activation with allergen extract alone was set to 100%.

Results

Fusion of IgE-specific DARPin to human IgG Fc-part

Binders specifically recognizing constant regions of IgE were selected from two different DARPins libraries using ribosome display (17, 18, 22). In the subsequent ELISA screening, DARPin E53 was identified. It showed reactivity against a non-FcεRIα epitope recognizing free as well as receptor-bound IgE (data not shown). To generate a fusion protein capable of cross-linking FcεRI-bound IgE with FcγRIIb, DARPin E53 was joined to a human IgG₁ Fc-part via a standard protein linker (Fig. 1 and Fig. S1). Affinity purified DE53-Fc from CHO cell supernatants was analyzed by non-reducing SDS-PAGE. DE53-Fc was expressed as homodimer with an apparent molecular weight of 100 kDa (data not shown). HPLC analysis revealed that the preparation contained 75% homodimeric DE53-Fc (see Fig. S2 and Data S1).

Binding characteristics of DE53-Fc

Binding characteristics of DE53-Fc and bivalent DARPin E53 were assessed by means of ELISA (Fig. 2). Both mole-

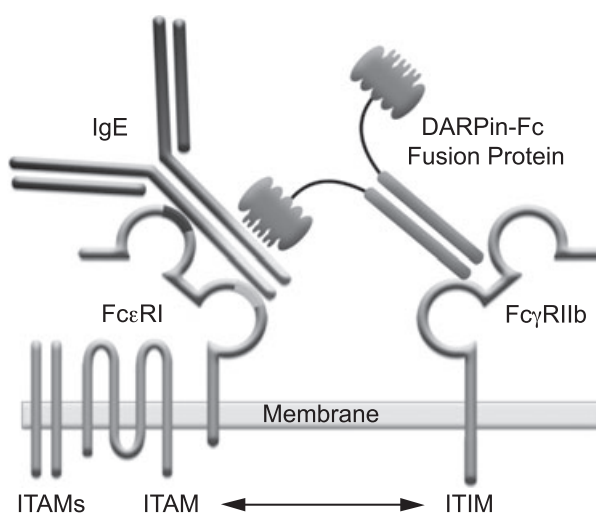


Figure 1 DARPin-Fc fusion protein induced FcεRI-FcγRIIb aggregation. Our hypothesis is that fusion of an anti-IgE DARPin to a human IgG Fc-part results in a bispecific molecule with the ability to simultaneously interact with FcεRI-bound IgE and FcγRIIb. Aggregation of these two receptors has been described to inhibit allergen-induced basophil and mast cell activation (8–12).

cules specifically recognized free as well as FcεRIα-IgE, whereas little background binding was detectable on control antigens. Compared to DE53-Fc concentrations, ten-times less bivalent DARPin E53 was required to reach the same signal intensity. Notably, DE53-Fc recognized free IgE to the same extend as receptor-bound IgE.

Binding kinetics of bivalent DARPin E53, DE53-Fc and monoclonal anti-IgE antibody Le27 was measured by surface plasmon resonance (Table 1). Le27 showed the highest affinity for IgE. This was mainly because of a small dissociation constant. The affinity of DE53-Fc and bivalent DARPin E53 differed by two orders of magnitude. Bivalent DARPin E53 featured a faster on-rate and a slower off-rate than DE53-Fc. Additionally, binding kinetics of DE53-Fc on FcεRIα-bound IgE was measured using a different SPR platform. Affinities for free as well as for receptor-bound IgE were in the same range (data not shown).

DE53-Fc is not anaphylactogenic

Most anti-IgE antibodies recognizing non-FcεRIα epitopes on IgE trigger basophil degranulation. To assess whether bivalent DARPin E53 and fusion protein DE53-Fc are anaphylactogenic, basophil activation was tested using whole blood samples from an allergic individual.

Stimulation with the murine monoclonal anti-IgE antibody Le27 resulted in a dose-response curve with an optimal triggering concentration of 1 nM (Fig. 3A). As it has previously been described, activation declined at supraoptimal concentrations (2). Triggering with bivalent DARPin E53 activated basophils at significantly lower concentrations (Le27 EC₅₀: ~0.4 nM; bivalent DARPin E53 EC₅₀:

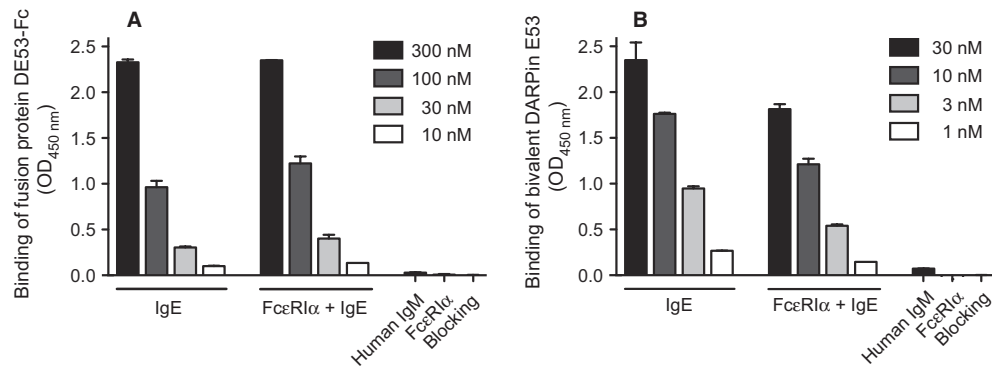


Figure 2 Bivalent DARPin E53 and DE53-Fc recognize free as well as receptor-bound IgE. ELISA to assess IgE binding specificity. A serial dilution of fusion protein DE53-Fc (300, 100, 30, 10 nM) (A) and bivalent DARPin E53 (30, 10, 3, 1 nM) (B) on monoclonal

human IgE-Sus11 and FcεRIα-bound IgE-Sus11 is shown. Only the highest concentration was tested on polyclonal human IgM, FcεRIα and casein blocking. Bars represent mean values ($n = 2$).

Table 1 Kinetics of different anti-IgE molecules

	Target	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	Affinity (M)
Anti-IgE antibody Le 27	IgE	8.61×10^4	1.83×10^{-6}	2.13×10^{-11}
Bivalent DARPin E53	IgE	4.60×10^5	8.21×10^{-4}	1.78×10^{-9}
DE53-Fc Fusion Protein	IgE	1.17×10^4	6.87×10^{-3}	5.90×10^{-7}

~ 0.06 nM). In contrast to Le27, supraoptimal concentrations did not result in a decrease in activation. No bell-shaped curve was observed, and activation reached a plateau of

almost 100% activation. Interestingly, stimulation with DE53-Fc did not induce basophil activation at all. IgG Fc-fragments alone as well as a control DARPin had no effect on basophil activation.

To assess whether potential immunogenicity of DE53-Fc might induce anaphylactogenicity, the construct was preincubated for 10 min with polyclonal anti-Fc-part antibodies to form aggregates or with buffer as control. Samples were tested in the same whole blood assay (Fig. 3B). The response using anaphylactogenic bivalent DARPin E53 was set to 100%. Again, no stimulation was observed for DE53-Fc alone. Interestingly, antibody-aggregated DE53-Fc showed no significant increase in activation. Anti-Fc-part antibodies alone, which were used as negative control, had no effect.

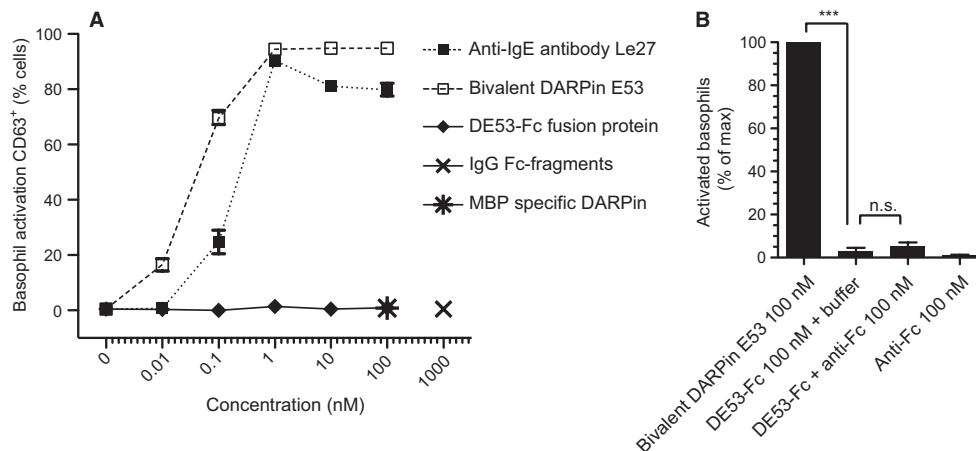


Figure 3 Anaphylactogenicity of different anti-IgE molecules. Basophil activation test with whole blood samples of an allergic individual to assess anaphylactogenicity of anti-IgE molecules. (A) Different concentrations of bivalent DARPin E53 (open squares), DE53-Fc (closed diamonds) or monoclonal antibody Le27 (closed squares) were assessed. IgG Fc-fragments (cross) and a maltose

binding protein-specific DARPin off 7 (asterix) served as controls. Each point represents mean values ($n = 3$). (B) Bivalent DARPin E53, DE53-Fc, anti-Fc antibody aggregated DE53-Fc, and polyclonal anti-Fc antibodies alone were added to the blood samples. Activation with bivalent DARPin E53 was set to 100%. Bars represent mean values ($n = 3$, $*P < 0.001$, n. s. = not significant).

DE53-Fc inhibits allergen-induced basophil activation

Allergen-specific IgG as well as antibody-based fusion proteins capable of cross-linking Fc ϵ RI with Fc γ RIIb have been shown to inhibit allergen-induced basophil activation (8–12). As DE53-Fc was generated to simultaneously target these two receptors, we tested its inhibitory potential in a functional basophil activation assay.

Incubation of the blood from donor 1 samples with DE53-Fc for 60 min prior to allergen stimulation resulted in a concentration dependent inhibition of basophil activation (IC_{50} : ~20 nM). Maximal inhibition of 75% was observed using 100 nM of DE53-Fc (Fig. 4A). Higher concentrations did not further increase the effect (data not shown). Omalizumab as well as IgG Fc-fragments showed no inhibition. Background level of activated cells without allergen-triggering was approximately 1%.

The same experiment was performed with shorter preincubation time (5 min). Overall, the effect was less prominent but still concentration dependent (Fig. 4B). Again, maximal inhibition of ~25% basophil activation was achieved using 100 nM DE53-Fc.

To investigate whether the inhibitory effect of DE53-Fc is donor dependent, we tested blood samples of two additional individuals (donor 2 and 3). At the same time, we assessed the influence of different allergen concentrations on DE53-Fc-mediated inhibition (Fig. 4C). Samples of both donors showed a clear reduction in basophil activation when preincubated with 100 nM DE53-Fc. Except for the highest allergen concentration with donor 2, all inhibitions were significant for both donors.

Both binding moieties are required for inhibition

Using the same basophil activation test, it was investigated whether competition of the anti-IgE DARPin or the Fc-part of the fusion molecule might abrogate the observed inhibitory effect. For this purpose, DE53-Fc was preincubated with an excess of free IgE or polyclonal anti-Fc antibody. Mixes were added to the whole blood samples for 1 h prior to allergen challenge. The control containing no inhibitory molecules was set to 100%. As already shown in Fig. 4A, 5 nM of DE53-Fc significantly inhibited basophil activation. Notably,

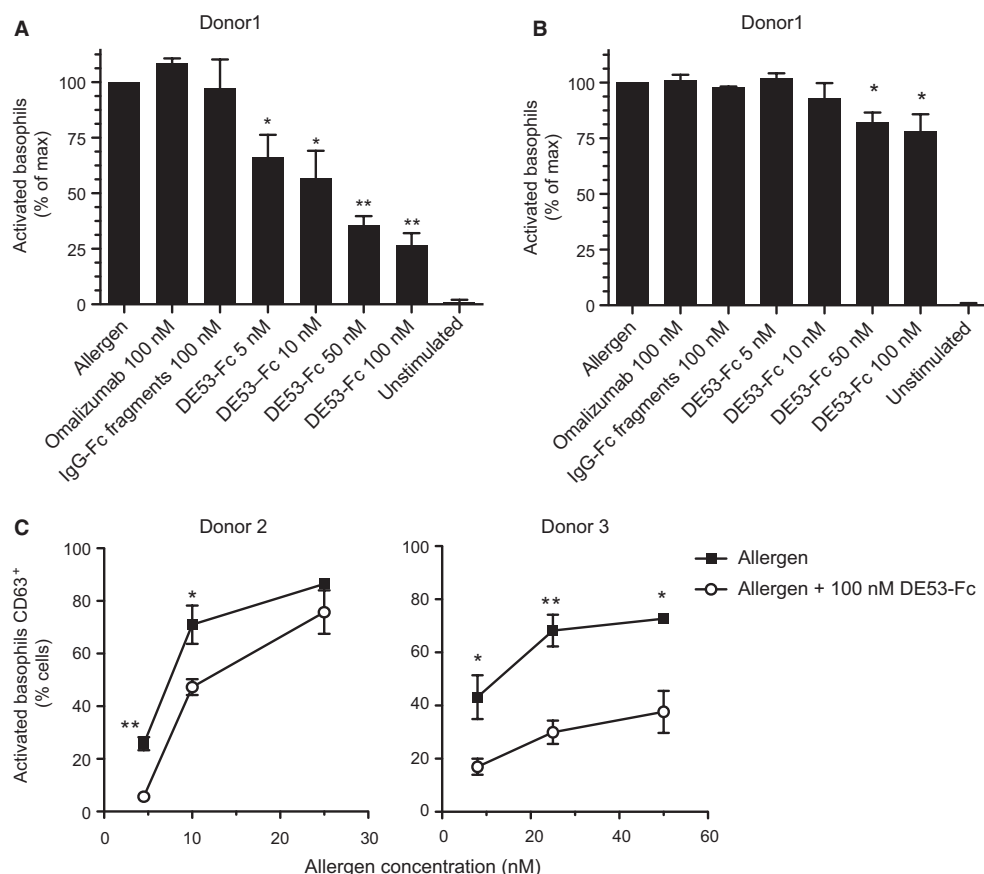


Figure 4 Inhibition of allergen-induced basophil activation. Basophil activation test using whole blood samples of three different donors. (A) Prior to allergen challenge (timothy grass extract) blood samples of donor 1 were incubated with Omalizumab (100 nM), IgG Fc-fragments (100 nM) or different concentrations of DE53-Fc (5, 10, 50, 100 nM). Activation with allergen alone was set to 100% (B) The

same test as in (A) was repeated with shorter incubation time (5 instead 60 min) prior to allergen stimulation. (C) Samples of two additional individuals (donor 2 and 3) were stimulated with different concentrations of allergen mix (6-gras-mix) after preincubation with DE53-Fc (100 nM, black squares) or PBS (open circles). Bars and points represent mean values ($n = 3$, * $P < 0.05$, ** $P < 0.01$).

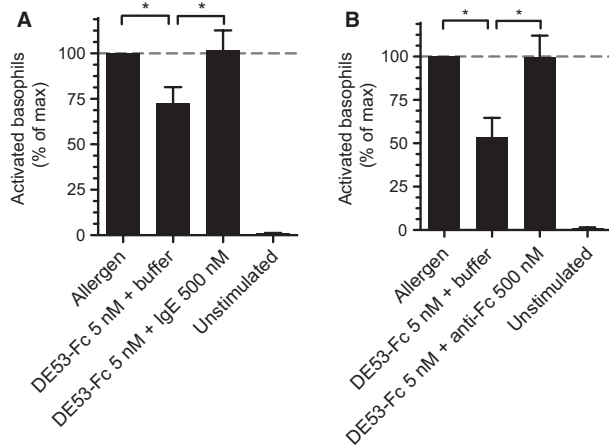


Figure 5 Reversibility of DE53-Fc mediated inhibition. Basophil activation test using whole blood samples of an allergic individual. Fusion protein DE53-Fc was preincubated with an excess of soluble IgE (A), anti-Fc antibodies (B) or with buffer and added to blood samples. Inhibitory capacity was assessed after allergen challenge (timothy grass extract). Unstimulated samples are included as background control. Activation with allergen alone was set to 100%. Bars represent mean values ($n = 3$, $*P < 0.05$).

the inhibitory effect differed between individual experiments. This might be because of common variations of basophil releasability (23). Most important, inhibition was completely abrogated by competing either of the two binding moieties of DE53-Fc with an excess of free IgE (Fig. 5A) or polyclonal anti-Fc antibodies (Fig. 5B).

Discussion

Before monoclonal anti-IgE antibodies were developed, it was generally agreed that triggering of basophils with anti-IgE sera or polyclonal anti-IgE antibodies results in a bell-shaped dose-response curve (2, 14). Interestingly, supraoptimal concentrations of anti-IgE antibodies decrease basophil mediator release. In our whole blood basophil activation test, a similar tendency although not as prominent as with polyclonal anti-IgE antibodies was observed using monoclonal murine anti-human IgE antibody Le27 (Fig. 3A). However, the mechanism underlying the bell-shaped activation curve is still poorly understood.

So far, two reasonable explanations have been suggested to account for this characteristic shape of the activation curve. Initially, it was speculated that an excess of polyvalent allergen or anti-IgE antibodies leads to the formation of non-stimulatory monovalent IgE/allergen or IgE/anti-IgE complexes that no longer cross-link FcεRI. More recently, it has been described that co-aggregation of FcεRI and FcγRIIb inhibits basophil or mast cell degranulation (15, 24, 25). Thus, anti-IgE antibodies might induce a negative feedback loop by simultaneously binding to FcεRI-bound IgE via the Fab fragment and to FcγRIIb via the Fc-part. Here, we provided evidence supporting this latter suggestion. Namely, in contrast to Le27, the dose-response curve of bivalent

DARPin E53 lacking an IgG Fc-part did not show the characteristic bell shape (Fig. 3A). Moreover, significantly lower concentrations of bivalent DARPin E53 were required to induce activation, indicating that already at low concentrations, the Fc-part of Le27 might act inhibitory.

Whether bivalent anti-IgE molecules induce basophil or mast cell degranulation not only seems to depend on the presence or absence of an Fc-part. The affinity for IgE influencing the ratio of aggregated FcεRI-FcεRI (activation signal) vs FcεRI-FcγRIIb (inhibitory signal) might play an important role as well (26). We showed that Le27 binds to IgE with high affinity ($K_D \sim 10^{-11}$ M). In contrast, the IgG Fc-part of Le27 has a low-affinity for FcγRIIb ($K_D \sim 10^{-6}$ M) (27). Thus, activation signals are likely to dominate inhibitory signals. As described earlier, Le27 most likely starts to form nonstimulatory monovalent complexes with receptor-bound IgE beyond a certain concentration, rebalancing signals and leading to a bell-shaped stimulation curve. Our fusion protein DE53-Fc has a lower affinity ($K_D \sim 10^{-7}$ M) for IgE. Thus, co-aggregation of FcεRI as well as cross-linking of FcεRI with FcγRIIb might be more balanced. This would explain why DE53-Fc is not anaphylactogenic compared to the anti-IgE antibody Le27 despite the fact that both molecules are bivalent. Extensive future studies on the consequences of affinity improvement or weakening of DE53-Fc will be needed. At the moment, we can only speculate whether affinity maturation of the anti-IgE DARPin or abrogation of Fc binding would affect the triggering capacity of DE53-Fc.

Therapies based on the neutralization of IgE have been described to prevent allergic reactions. However, conventional anti-IgE treatments are long-term therapies, not suitable for emergency treatment. As we have shown, DE53-Fc inhibits allergen-induced basophil degranulation with high efficacy in different donors (Fig. 4). Already 5 min after administration, significant reduction in basophil activation was observed. In comparison, the humanized anti-IgE Omalizumab, which is clinically used to treat patients with severe asthma, did not inhibit allergen-induced basophil activation in these assays. The preincubation time might have been too short to see a beneficial effect of Omalizumab. These results clearly underline the advantage of a noncompetitive inhibition strategy directly targeting receptor-bound IgE to prevent allergen-induced immediate hypersensitivity reactions.

The group of Zhu et al. previously described the generation of a similar fusion protein (Fcγ-Fcε: GE2) to inhibit IgE-mediated hypersensitivity reactions (12). However, in contrast to DE53-Fc, this molecule had to compete with IgE on sensitized basophils for FcεRI binding, decreasing its inhibitory capacity. Furthermore, it has been shown that GE2 was immunogenic *in vivo* (9). Induced antibodies against the IgE Fc-part of the fusion protein cross-linked native IgE on sensitized basophils and mast cells and led to anaphylaxis-like reactions in non-human primates. Assuming that DE53-Fc would be immunogenic as well, induced anti-DARPin antibodies would not be able to directly cross-link native IgE. Moreover, we showed that a polyclonal antibody preparation against the Fc-part of DE53-Fc did not increase basophil activation either (Fig. 3B). Of course, to further assess potential immunogenic-

ity of DE53-Fc and to evaluate the risk of anti-DE53-Fc antibodies in more detail, *in vivo* studies would be required.

In summary, we successfully generated a novel molecule consisting of an IgE-specific DARPIn linked to the effector portion of a human IgG. The resulting fusion protein, DE53-Fc, recognizes FcεRI-bound IgE without being anaphylactogenic. We assume that nonanaphylactogenicity is because of inhibitory signaling via FcγRIIb as it was previously described for similar molecules (8–12). Most important, DE53-Fc inhibited allergen-induced basophil activation in samples of different donors. Our results suggest that novel drug candidates based on this approach might be efficient as preventive as well as immediate treatment of allergic diseases.

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Conflict of interest

The University Institute of Immunology at the University of Bern has a research collaboration agreement with Molecular

Partners AG (MPAG), who owns the intellectual property on the DARPIn technology used in this study. M.J.B. and P.A. are employed by MPAG. A.E., P.B., M.V., and B.M.S. declare no competing financial interests.

Authorship

Contribution: A.E. performed the research, analyzed and interpreted the data and wrote the manuscript. P.B. performed the research. M.J.B. performed the research. P.A. provided DARPIn libraries, selection technology and knowledge. B.M.S. and M.V. supervised all studies and revised the manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article available at: www.wileyonlinelibrary.com

Data S1. Materials and methods.

Figure S1. Amino acid sequence of fusion protein DE53-Fc.

Figure S2. Analytical HPLC of fusion protein DE53-Fc.

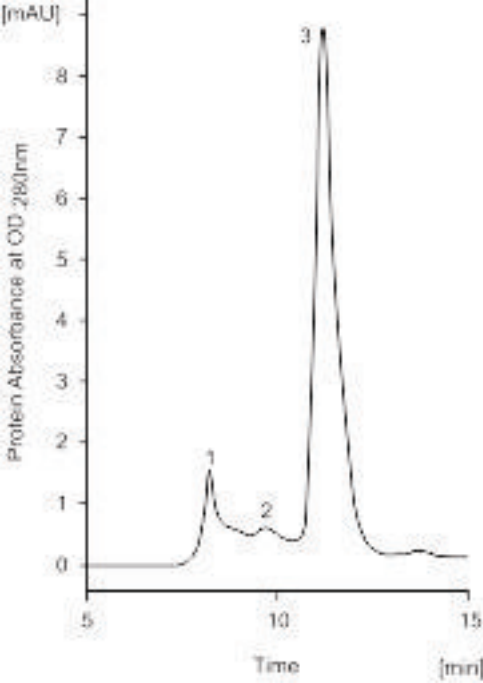
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DE53- Fc HHHHHHGS DLGKKLL EAARAGQDDEVRI LMANGADVNAI DHNGTTPLHLAAYAGH
LEI VEVLLKHGADV NARDLRGFTPLHLAAI DGHLEI VEVLLKYGADV NADDDYGT
TPLHLAAQYGHMEI VEVLLKYGADV NAQDKFGKTAFDI SIDNGNEDLAEI LQKAA
Linker *Hinge IgG1(Fc)*
GGGSGGGSGGGSGGGSTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI SRTPEVTCV
VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK
EYKCKVSNKALPAPI EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGF
YPSDI AVEWESNGQPENNYKTT PPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSV
MHEALHNHYTQKSLSLSPGK* *



Supplementary Materials

Inhibition of Ongoing Allergic Reactions Using a Novel Anti-IgE DARPin-Fc Fusion Protein

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MATERIALS AND METHODS

Antibodies and recombinant proteins

The human monoclonal IgE-Sus11 as well as the murine monoclonal anti-IgE Le27 are produced by a hybridoma cell line in our laboratory (1, 2). The myelomas IgE-Zavazal and IgE-PS were kind gifts from Dr. V. Zavázal (Pilsen, Czech republic) and Drs. T. and K. Ishizaka (La Jolla, CA, USA). The monoclonal humanized anti-IgE antibody Omalizumab (Xolair®, Novartis, Genentech) was obtained commercially. Polyclonal anti-human IgG-Fc antibody was purchased from Nordic (Tillburg, The Netherlands). Polyclonal human IgM was bought from The Binding Site (Birmingham, UK). IgG Fc- fragments were produced by papain digestion of intravenous immunoglobulin (IVIg) using the ImmunoPure® Fab Kit (Pierce). The IVIg preparation was a kind gift from Dr. Sylvia Miescher from CSL Behring (Bern, Switzerland). Human serum albumin N- and C-terminally flanked with the extracellular part of high affinity IgE receptor alpha chain (FcεRIα) was a kind gift of Dr. H. Kocher (Novartis Pharma, Basel, Switzerland) (3). The maltose binding protein specific DARPIn used as control in several experiments was received from Molecular Partners AG (Schlieren, Switzerland) (4).

Vectors, bacterial strains, libraries and cell lines

The ribosome display vector pRDV (GenBank accession no. AY327136) as well as pQI-bi-2_2, which was used for the generation of bivalent and bispecific DARPins, were obtained from Molecular Partners AG (Schlieren, Switzerland) (5). For DARPIn expression pQE30 (Qiagen, Hilden, Germany) containing a His₆-tag sequence was used. We received a pcDNA3.1(-) based vector (Invitrogen, Carlsbad, USA) containing an IgG₁ Fc-part (Uniprot: P01857, amino acids 106-330) and its sequence from Dr. M. Fux (Glenmark, La Chaux-de-Fonds, Switzerland). Selection of binders was performed using two DARPIn DNA libraries

received from Molecular Partners AG. Structure of DARPins and the generation of DARPins libraries are published elsewhere (6, 7). Expression of the DARPins-Fc fusion protein was performed in Chinese hamster ovary (CHO) cells (8). All DARPins were produced in *E.coli* XL-1 blue (Stratagene, San Diego, USA) and purified on IMAC columns (TALON™, Clontech, CA, USA).

Selection of anti-IgE binders from DARPins libraries

The selection of anti-IgE binders was previously published (9). Briefly, four panning rounds on three different IgEs (1st round: IgE-Sus11, 2nd round: IgE-Zavazal, 3rd round IgE-PS and 4th round: IgE-Sus11) with two DARPins libraries (N2C and N3C) were performed.

PBS/0.5% BSA blocking was used to preabsorb non-specific DARPins prior to each selection. IgEs (5 µg/ml) were immobilized in 100 µl of phosphate-buffered saline (PBS; pH 7.4) overnight at 4 °C directly on a 96-well Maxisorp plate (Nunc, Thermo Fisher, Waltham, USA). Translated DARPins 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. Starting with 45 cycles reverse-transcription PCR cycle numbers were decreased from round to round.

Screening ELISA to identify DARPins specific for receptor-bound IgE

Antigens (monoclonal human IgE-Sus11, FcεRIα- bound IgE-Sus11 or FcεRIα alone each at 5µg/ml) were immobilized on a 96-well Maxisorp plate (Nunc) overnight at 4°C. The next day wells were blocked with PBS/0.5% BSA for 1h at RT and 1h at 4°C. The plates were twice rinsed with PBS. Crude DARPins extracts from *E.coli* were added at a 1:10 dilution for 1h at 4°C. The plates were again rinsed extensively with PBS containing 0.01% Tween-20 and with PBS. Bound DARPins were detected using a anti-His₆-tag antibody (R&D Systems,

Minneapolis, USA) followed by a peroxidase labeled goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, USA). Detection antibodies were diluted 1:1000 in PBS/0.5% BSA and 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 OD_{450nm} in a standard ELISA reader (BIO-TEK EL808, BioTek, Bad Friedrichshall, Germany).

Analytical HPLC

DE53-Fc was analyzed on a TSK G3000SWXL HPLC column (Tosoh Biosep GmbH, Stuttgart, GER) using the HP 1050 HPLC-system with a HP 1100 wavelength detector (Agilent Technologies, Wilmington, DE, USA). The running buffer was EP (0.05 M Na₂HPO₄, 0.02 M NaH₂PO₄, 0.4 M NaCl). The run was performed at a constant flow rate of 0.7 ml/min at 25 °C. The void volume (V₀) of the column is 5.3 ml. The concentration of DE53-Fc was 5 µM. Absorbance was measured at 280 nm.

FIGURE LEGENDS

Figure 1E. Amino acid sequence of fusion protein DE53-Fc

Anti-IgE DARPin E53 was fused via a (Gly₄-Ser)₃ linker to the Fc part of a human IgG1 (Uniprot: P01857, amino acids 106-330). Asteriks indicate stop codons.

Figure 2E. Amino acid sequence of fusion protein DE53-Fc

Anti-IgE fusion protein DE53-Fc was applied to a HPLC size exclusion column (TSK G3000SWXL) to check the presence of natural aggregates in protein preparation. The void volume (v_0) of the column is 5.3 ml (7.6 min at a flow of 0.7 ml/min). According to molecular weight standards peak 1 corresponds to aggregates (11.5%), peak 2 to dimers (8.6%) and peak 3 to monomers (74.6%).

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