Published in final edited form as:

J Allergy Clin Immunol. 2014 June ; 133(6): 1709–1719.e8. doi:10.1016/j.jaci.2014.02.005.

Accelerated dissociation of IgE:FcεRI complexes by disruptive inhibitors actively desensitizes allergic effector cells

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Abstract

Background—The remarkably stable interaction of immunoglobulin E (IgE) with its high-affinity receptor FcεRI on basophils and mast cells is critical for the induction of allergic hypersensitivity reactions. Due to the exceptionally slow dissociation rate of IgE:FcεRI complexes such allergic effector cells permanently display allergen-specific IgE on their surface and immediately respond to allergen challenge by releasing inflammatory mediators. We have recently described a novel macromolecular inhibitor that actively promotes the dissociation of IgE from FcεRI through a molecular mechanism termed facilitated dissociation.

Objective—Here, we assessed the therapeutic potential of this non-immunoglobulin based IgE inhibitor DARPin E2_79 as well as a novel engineered biparatopic DARPin bi53_79 and directly compared them to the established anti-IgE antibody omalizumab. Methods: IgE:FcεRI complex dissociation was analyzed *in vitro* using recombinant proteins in ELISA and surface plasmon resonance, *ex vivo* using human primary basophils with flow cytometry and *in vivo* using human FcεRI transgenic mice in a functional passive cutaneous anaphylaxis test.

Results—We show that E2_79 mediated removal of IgE from primary human basophils fully abrogates IgE-dependent cell activation and release of pro-inflammatory mediators *ex vivo*. Furthermore, we report that omalizumab also accelerates the dissociation of IgE from FcεRI albeit

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Disclosure of potential conflict of interest: P.F. is founder and shareholder of Molecular Partners AG, which controls the DARPin technology.

All other authors declare no competing financial interests.

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much less efficiently than E2_79. Using the biparatopic IgE targeting approach we further improved the disruptive potency of E2_79 by ~100 fold and show that disruptive IgE inhibitors efficiently prevent passive cutaneous anaphylaxis in mice expressing the human FcεRI alpha chain.

Conclusion—Our findings highlight the potential of such novel IgE inhibitors as important diagnostic and therapeutic tools to managing allergic diseases.

Keywords

Allergy; Basophils; Mast Cells; IgE; FcεRIα; DARPins. Inhibitors

Introduction

The interaction of IgE antibodies with its high-affinity receptor FcεRI, mainly expressed on the surface of tissue-resident mast cells and blood-borne basophils, is a critical step in most allergic reactions. IgE binding to the α-chain of FcεRI is mediated through the Fc region of IgE that is comprised of three domains, Cε2-Cε3-Cε4. While the Cε3 domains directly contact receptor, the Cε4 domains form the heavy chain dimerization interface ¹. Interestingly, the Cε2 domains are not necessary for FcεRI binding, but slow down both on and off rates for complex formation ². Since the binding affinity of IgE to FcεRI is remarkably high (<1 nM), IgE and FcεRI form stable complexes on mast cells and basophils prior to allergen binding, priming these allergic effector cells to respond. Allergen induced cross-linking of receptor-bound IgE stimulates cell degranulation and release of pre-stored as well as *de novo* synthesized pro-inflammatory mediators promoting classic allergic disease symptoms ^{3–5}.

The central importance of IgE-receptor binding in allergic diseases has drawn considerable attention on this interaction as a therapeutic target. Small oligonucleotide aptamers ^{6,7}, phage-display selected peptides ^{8–10}, anti-IgE antibodies ^{11,12}, anti-FcεRI antibodies ^{13–15}, as well as designed ankyrin repeat proteins (DARPins) ^{16–18} have been identified as high-affinity inhibitors of IgE-receptor binding. However, only the anti-IgE antibody omalizumab (trade name Xolair®) is currently available for the treatment of moderate-to-severe persistent asthma. The binding epitope of omalizumab has been mapped to the Cε3 domain of IgE overlapping with the FcεRI binding-site ¹⁹. Therefore, it is widely accepted that omalizumab neutralizes free IgE but does not interfere with receptor-bound IgE ^{12,19–21}. Since IgE stabilizes the receptor on the cell surface and thereby prevents its internalization ^{22,23} the amount of soluble IgE present in serum directly correlates with FcεRI levels on basophil granulocytes *in vivo* ²⁴. Indeed various clinical studies have observed a decrease in the amount of FcεRI on basophils of omalizumab treated patients ^{23,25–27}.

Using recombinant proteins we recently reported that a novel IgE inhibitor, namely DARPin E2_79, not only prevents binding of free IgE to FcεRI but additionally actively disrupts pre-formed IgE:FcεRI complexes *in vitro* through a facilitated dissociation mechanism ²⁸. Here, we describe that the disruptive anti-IgE inhibitor E2_79 has the ability to interfere with IgE:receptor complexes on the surface of human allergic effector cells *ex vivo* as well as *in vivo*. Furthermore, we demonstrate that a fusion of two anti-IgE DARPins exhibits

significantly increased efficiency in disrupting IgE:Fcε RI complexes. Unexpectedly, we found that high concentrations of omalizumab also promote IgE:receptor complex dissociation and that omalizumab directly binds to IgE-Fc:receptor complexes lacking the IgE Cε2 domain. We establish that *in vitro* biochemical binding studies and the efficiencies of receptor complex dissociation correlate well with the ability of these inhibitors to strip IgE from the surface of human blood basophils and to block IgE-dependent responses in a humanized mouse model of passive cutaneous anaphylaxis. Overall, these studies reveal an additional mode of action for the therapeutic anti-IgE antibody omalizumab and demonstrate that DARPin-based disruptive anti-IgE inhibitors offer an attractive therapeutic approach for the treatment of allergic disease.

Methods

See the Methods section in this article's Online Repository at www.jacionline.org for details about materials, inhibition ELISAs, BIAcore binding assays, cell isolation, basophil de- and resensitization, receptor timecourse assay, basophil activation test as well as passive cutaneous anaphylaxis.

Human samples and animals

Human primary basophils were isolated from whole blood of allergic and healthy donors with approval from the local ethics committee. Informed consent was obtained from all donors in accordance with the Helsinki Declaration. Mice transgenic for human FcεRIα and that have the murine FcεRIα knocked out were obtained from Dr. J.-P. Kinet. All animal experimentation was approved from the local committee.

Statistics

Statistical analysis was carried out with Prism 5.0 for Macintosh. All data are shown as mean ± s.d. Comparisons between different treatments were analyzed by One-way ANOVA with Bonferroni post-hoc tests. In all tests, P-values of less than 0.05 were considered statistically significant.

Results

Omalizumab accelerates IgE dissociation in vitro

In order to assess the therapeutic potential of different IgE inhibitors we compared the previously described disruptive anti-IgE DARPin E2_79 to the commercial anti-IgE antibody omalizumab in different assays. In all these experiments the non-inhibitory anti-IgE DARPin E3_58 served as a control. Using surface plasmon resonance (SPR) we first measured the kinetics of each binder on immobilized IgE (Fig E1 and Table E1). Omalizumab showed approximately 10-fold higher affinity for IgE ($K_D \sim 0.3$ nM) than E2_79 ($K_D \sim 5$ nM). The affinity of control DARPin E3_58 was almost 100-times lower ($K_D \sim 19$ M) compared to omalizumab. In order to assess the disruptive potential of the different anti-IgE binders we performed a competition ELISA in which we pre-complexed IgE with FcεRI prior to a 30 minutes incubation with increasing concentrations of DARPin E2_79, DARPin E3_58 and omalizumab (Fig 1, A). As expected we did not observe any effect on

IgE dissociation using E3_58 whereas IgE was removed from its receptor in a dose-dependent manner by E2_79. Surprisingly, we also observed complex dissociation with high concentrations ($>1\mu\text{M}$) of omalizumab, albeit to a significantly lesser extent than with E2_79. At the same time, we analyzed supernatants for the amount of dissociated IgE in a separate ELISA (Fig 1, B). Accordingly, we found the highest concentrations of removed IgE in E2_79 treated IgE:Fc ϵ RI complexes, whereas we did not detect changing levels of IgE in solution for DARPin E3_58, confirming that it does not have the ability to remove or compete IgE from Fc ϵ RI. Importantly, and consistent with the removal data, we also observed detached IgE in the supernatant of omalizumab treated IgE:receptor complexes.

To obtain more direct evidence for accelerated complex dissociation with omalizumab, we probed the interactions of the anti-IgE ligands with pre-formed IgE:Fc ϵ RI complexes by surface plasmon resonance (SPR). Fc ϵ RI protein was immobilized on the chip surface and loaded with full length IgE prior to injection of the different anti-IgE molecules, which was followed by buffer flow. We confirmed dose-dependent accelerated dissociation of pre-formed IgE:Fc ϵ RI complexes by E2_79 (Fig 1, C), whereas E3_58 did not remove IgE but formed ternary complexes with IgE:Fc ϵ RI (Fig 1, D). High concentrations ($>3\mu\text{M}$) omalizumab also showed dissociation of IgE (Fig 1, E), although not as efficiently as E2_79, which is consistent with the observations by ELISA. We also included soluble Fc ϵ RI (sFc ϵ RI) as a control for direct steric competition and we did not observe any accelerated dissociation (Fig 1, F). At lower concentrations (6–200 nM) omalizumab showed no direct binding to receptor-bound full length IgE (Fig 2, A). However, when we used IgE-Fc $_{3-4}$ consisting of the C ϵ 3-C ϵ 4 domains instead of full length IgE, we observed remarkable binding of omalizumab (Fig 2, B) suggesting that the lack of C ϵ 2 domains exposes at least one of the two C ϵ 3 domains in the IgE-Fc subunits, which have been described as omalizumab binding sites (Fig 2, C and D; PDB ID: 1F6A) ^{1,19,29}.

E2_79 and omalizumab remove IgE from cells *ex vivo*

In order to investigate whether the observed accelerated dissociation of IgE from recombinant receptor might have relevance for IgE bound to Fc ϵ RI-expressing cells we isolated primary human blood basophils. Increasing concentrations of E2_79, E3_58 and omalizumab were applied to the cells for different durations and the remaining IgE was quantified by flow cytometry (Fig 3, A–B and Fig E2). IgE levels of untreated cells were set to 100% for each time point. Consistent with the previous results we did not observe any change in IgE levels for the non-disruptive anti-IgE binder E3_58 over time. However, DARPin E2_79 as well as omalizumab, were able to remove receptor-bound IgE from the cells in a concentration and time-dependent manner. IgE removal was already detectable within minutes of incubation with E2_79, whereas it took more than 1 hour to see an effect with omalizumab (Fig 3, A–B and Fig E2). This is in line with the higher potency of E2_79 to actively dissociate IgE-Fc:Fc ϵ RI complexes in SPR experiments. Half-maximal stripping of IgE from the cell surface receptors was observed after 15–30 minutes with E2_79 and 4–6 hours with omalizumab using a concentration of 50 μM inhibitor.

This novel method to remove IgE from the cell surface allows studying the role of receptor-bound IgE on cellular functions of human cells *ex vivo* under physiologic conditions. Since

it is known that IgE stabilizes FcεRI on the cell surface we assessed how the disruption of IgE:receptor complexes would translate into receptor down-regulation. To investigate changes in the amount of FcεRI on the surface of isolated primary human basophils we treated these cells with 50 μM of E2_79, omalizumab or medium only and determined receptor levels by flow cytometry every 48 hours (Fig 3, C and E). Simultaneously we quantified IgE levels for each time point (Fig 3, C and D). As expected, no more receptor-bound IgE was detectable after 24 hours of incubation with E2_79 or omalizumab and thereafter. Receptor levels simultaneously decreased for E2_79 and omalizumab treated cells starting at day three, indicating that receptor turnover and not IgE-stripping is the rate-determining step in this experiment. Intrinsic dissociation of IgE without the influence of any inhibitor led to a 50% reduction of FcεRI cell surface levels within 9 days whereas the same extent of receptor decrease was reached already after 5 days by treating the cells with either E2_79 or Xolair. These data suggest that enhanced FcεRI down-regulation on allergic effector cells in patients receiving omalizumab treatment might be due to accelerated dissociation of IgE from the cell surface *in vivo* ^{25,27,30}.

The possibility of actively removing receptor-bound IgE from the surface of basophils sets the stage for new approaches to interfering with allergen-induced hypersensitivity reactions. We therefore determined whether active desensitization of highly responsive IL-3 primed human blood basophils will abrogate IgE-dependent activation ^{31,32}. Degranulation of pre-formed mediators was indirectly probed as previously described by flow cytometry measuring the percentage of CD63 positive basophils (Fig 3, F) ³³. Triggering with the monoclonal anti-IgE antibody Le27 resulted in concentration-dependent basophil degranulation, whereas IgE-dependent activation was completely abrogated after desensitization of the cells overnight with 50 μM E2_79 prior to challenge (Fig 3, G). In parallel, we assessed secretion of *de novo* synthesized mediators by ELISA measuring the amount of leukotriene (LTC4) in the cell supernatants upon activation (Fig 3, H). LTC4 secretion nicely corresponded to the CD63 measurements and therefore correlated with the degranulation data. Namely, desensitized basophils did not release any LTC4 upon IgE-dependent stimulation, whereas untreated cells induced leukotriene synthesis and release dose-dependently. Taken together, these data suggest that both E2_79 as well as omalizumab accelerate IgE dissociation from its high-affinity receptor FcεRI on primary human basophils and subsequently thereby induce a decrease in surface FcεRI levels. This active desensitization fully abrogates IgE-dependent cell activation and release of pro-inflammatory mediators.

Antigen-specific re-sensitization of basophils

Considering the observed receptor kinetics of E2_79 or omalizumab treated basophils we assessed whether IgE-stripped cells may be re-sensitized with allergen-specific IgE and challenged accordingly. For this purpose we added 100 nM 4-Hydroxy-3-iodo-5-nitrophenylacetyl (NIP) specific IgE for one hour to primary human basophils that were treated overnight with 50 μM E2_79. In this short period of re-sensitization surface IgE levels were restored close to their initial values (Fig 4, A–B) while we did not observe any major changes in receptor levels (Fig 4, C–D). Re-sensitization with NIP-specific IgE fully restored IgE-dependent activation (Fig 4, E) and release of LTC4 (Fig 4, F) rendering the

cells responsive to low concentrations of antigen. These results might pave the way for the establishment of novel cellular allergy diagnostics to test patient sera on actively desensitized primary cells from any given donor and compare the reactivity of different sera in a standardized manner on the same cells.

Biparatopic anti-IgE targeting increases disruptive efficacy

In order to further improve the disruptive efficacy of E2_79 we generated bivalent DARPins as previously described¹⁷. By fusing E2_79 to the non-disruptive anti-IgE DARPIn E3_53, which recognizes receptor-bound IgE¹⁷ (Fig E3, A) we aimed to increase IgE targeting and the local concentration of disruptive DARPIn in close proximity to FcεRI. We have previously reported that the molecular N- to C-terminal orientation in which two DARPins are joined via a flexible glycine-serine linker may affect their monovalent binding behavior¹⁷. Therefore, we generated constructs in both directions and expressed bispecific DARPIn E2_79-linker-E3_53 (bi79_53) as well as bispecific DARPIn E3_53-linker-E2_79 (bi53_79). As a control we also produced both bivalent constructs DARPIn E3_53-linker-E3_53 (bi53_53) as well as DARPIn E2_79-linker-E2_79 (bi79_79). Using the same SPR measurements described for the monovalent DARPins we measured kinetics and compared the disruptive efficacy of the bivalent anti-IgE DARPins to E2_79 (Fig 5, A–D and Fig E3, C–G and Table E1). As expected bi53_53 recognized receptor-bound IgE and formed ternary complexes without removing IgE from the receptor (Fig E3, B). Interestingly, both bi79_79 and bi79_53 showed no improvement in IgE:FcεRI complex dissociation. Rather, bi79_53 appears to be noticeably less effective at stimulating complex disruption. However, we observed remarkably (>10-fold) increased disruptive efficacy for bi53_79 compared to E2_79 in the SPR experiments. We confirmed these results by measuring IgE surface levels on DARPIn treated primary human basophils by flow cytometry (Fig 5, E–F). Compared to the monovalent disruptive E2_79, bi53_79 showed significantly enhanced IgE dissociation as a function of DARPIn concentration, whereas all other bivalent DARPins performed worse (Fig 5, F).

In general bivalent anti-IgE molecules targeting non-FcεRI epitopes might cross-link receptor-bound IgE and thus induce basophil or mast cell activation and degranulation³⁴. To probe whether the bivalent disruptive anti-IgE DARPins might trigger such anaphylactic reactions, we tested them on isolated primary human basophils, again measuring CD63 as an activation marker (Fig 3, F). The bivalent anti-IgE DARPIn bi53_53 that we used as a positive control activated basophils in a dose-dependent manner, whereas we did not observe any activation above baseline level for the other bivalent DARPins containing at least one E2_79 subunit (Fig 5, G).

bi53_79 is more potent than omalizumab in blocking anaphylaxis in vivo

Omalizumab as well as the anti-IgE DARPins used in this study show very high specificity for human IgE and do not cross-react with murine IgE. Moreover, human IgE does not bind to murine FcεRI. Thus, in order to test *in vivo* functionality and compare the different inhibitors side-by-side we used transgenic mice expressing the human FcεRI alpha-chain instead of the murine receptor^{3,35,36}. We injected these mice intradermally with human NIP-specific IgE to passively sensitize cutaneous mast cells at different sites on the back

flank. The following day the disruptive anti-IgE inhibitor E2_79, the improved disruptive anti-IgE inhibitor bi53_79, the non-disruptive anti-IgE E3_58 or the therapeutic anti-IgE antibody omalizumab were applied to the same injection sites intradermally. Six hours later the mice were challenged by intravenous injection of NIP₂₅BSA antigen in saline containing 0.5% Evans Blue dye. We quantified dye leakage caused by the local allergic reaction visually on the inner skin (Fig 6, A and C) as well as by dimethylformamide extraction of the blue dye from the skin and measuring absorbance at 620 nm wavelength (Fig 6, B and D). Consistent with the *in vitro* and *ex vivo* data E2_79 and omalizumab performed similarly. They both completely inhibited passive cutaneous anaphylaxis at concentrations of 50 μ M, whereas inhibition was much less pronounced at 5 μ M. However, the improved disruptive inhibitor bi53_79 was significantly more effective and sufficient to achieve full inhibition of this IgE-mediated allergic response at a concentration of 5 μ M. Thus, disruptive IgE inhibitors based on DARPin scaffolds might represent an attractive alternative to antibodies such as omalizumab for intervention in acute allergic reactions.

Discussion

Here we demonstrated that disruptive IgE inhibitors not only accelerate dissociation of IgE from Fc ϵ RI *in vitro* but that these inhibitors may also be used to actively remove IgE from the surface of allergic effector cells *ex vivo* and *in vivo*. The efficacy of IgE removal is not dependent on the affinity of the inhibitor but rather on its epitope specificity. We calculated the relative efficiencies of E2_79, bi53_79 and omalizumab in dissociating IgE complexes as the ratio of their affinity for IgE to the half maximal concentration in stimulating accelerated complex dissociation (K_D/ID_{50}), as measured by SPR (Fig E4 and Table E2). Based on this calculation, bi53_79 is two orders of magnitude more efficient than E2_79 and >10,000 fold more efficient than omalizumab. For bi53_79, the fusion of a non-inhibitory DARPin (E3_53) to the disruptive DARPin (E2_79) enabled tethering of E2_79 to IgE:Fc ϵ RI complexes, increasing its local concentration in close proximity to the receptor. The improved ability of bi53_79 to actively dissociate IgE:Fc ϵ RI complexes is consistent with the proposed mechanism of facilitated dissociation²⁸, where complexes must partially open to expose the E2_79 binding site, allowing DARPin insertion into the IgE:Fc ϵ RI interface. Compared to classic biparatopic approaches, where targeting of two functional epitopes increases biological efficacy^{37,38}, here a non-inhibitory anchor DARPin (E3_53) increases biological efficacy through co-localization of the active, disruptive inhibitor (E2_79). This approach emphasizes the advantage of bispecific over monospecific anti-IgE binders and might foster the development of novel inhibitors specifically targeting receptor-bound IgE. The increased potency of bi53_79 is not due to IgE binding affinity, as bi53_79 binds ~40-fold more weakly (K_D ~12 nM) than omalizumab (K_D ~0.3 nM) to IgE (Table E1). Instead, the increased potency appears more consistent with the increased ability of bi53_79 to actively dissociate preformed IgE:Fc ϵ RI complexes, as revealed in the SPR and cell-based assays. Taken together these data provide evidence that accelerated disruption of pre-formed IgE:Fc ϵ RI complexes on allergic effector cells may provide greater therapeutic potential than neutralization of free IgE alone.

In this study we also discovered an additional but so far overlooked molecular mechanism of the therapeutic anti-IgE antibody omalizumab. We observed that at high concentrations

omalizumab accelerates the dissociation of pre-formed IgE:FcεRI complexes on the surface of allergic effector cells in addition to its ability to inhibit the interaction of free IgE with FcεRI. These findings add valuable information to previous omalizumab studies, potentially explaining the rapid therapeutic effects of omalizumab in IgE-related conditions such as chronic urticaria^{39,40}.

Our results together with previous studies mapping binding sites of E2_79 and omalizumab offer an explanation as to why the efficiencies by which these two IgE inhibitors accelerate the dissociation of IgE from FcεRI are different^{19,28}. The binding-site of omalizumab shows major overlap with the FcεRI-binding residues on IgE. On the other hand we previously reported that E2_79 shows only minor overlap with receptor binding to IgE and that it disrupts IgE:FcεRI through a facilitated dissociation mechanism²⁸. The reduced efficacy of omalizumab-induced IgE dissociation is likely due to a larger steric overlap between omalizumab and FcεRI when bound to IgE, creating greater direct steric constraints for insertion into partially dissociated intermediates. The two distinct inhibitor geometries may result in the different receptor complex dissociation rates of E2_79 and omalizumab in SPR measurements, as well as in cellular IgE-removal assays *ex vivo*.

It has been previously widely accepted that omalizumab does not directly interfere with or engage receptor-bound IgE because its epitope overlaps with the FcεRI binding-site in the Cε3 domains of IgE^{12,19,29}. However, we observed direct binding of omalizumab to IgE-Fc₃₋₄:FcεRI but not full-length IgE:FcεRI complexes, indicating that the lack of binding to receptor bound IgE is in part due to steric hindrance of the IgE Cε2 domains and not the receptor. The asymmetric binding of IgE to FcεRI clearly leaves one of the Cε3 domains exposed and available for omalizumab binding¹ in the absence of the Cε2 domains. Whether truncated IgE molecules such as IgE-Fc₃₋₄ are generated physiologically and whether they might be involved in rare cases of omalizumab-induced anaphylaxis requires further investigation⁴¹.

Different clinical studies have previously reported that the FcεRI density on basophils and dendritic cells in patients receiving anti-IgE treatment considerably decreases within the first week of omalizumab application^{25,27,30}. However, the published half-life of receptor-bound IgE on basophils *ex vivo* has been described to be on the order of 8 days. Furthermore, reduction of FcεRI levels to 50% has been shown to occur within the range of 13 days of culture in the absence of free IgE⁴². Our findings now indicate that the remarkable decline of FcεRI levels on basophils of omalizumab treated patients might not only be due to the neutralization of free IgE but additionally could result from omalizumab induced accelerated dissociation of IgE from FcεRI. Peak serum concentrations of omalizumab treated patients have been described to reach > 7 μM (1,100 μg/ml) in dosage studies during clinical development⁴³. These *in vivo* concentrations are comparable to the amounts of omalizumab we used in this study. Even though lower concentrations are currently used for treatment, omalizumab induced accelerated IgE:FcεRI complex dissociation might still be of physiological relevance.

So far, it has been difficult to assess the functional role of IgE on primary human allergic effector cells, since IgE-removal could only be achieved by lactic acid treatment (pH 3.9) of

the cells *ex vivo*. These harsh stripping conditions may cause cellular stress or damage and have complicated the interpretation of results obtained. A more physiological stripping method through facilitated dissociation will enable the investigation of functional consequences of IgE-removal from allergic effector cells under physiologic circumstances *in vivo* as well as *ex vivo*. Furthermore, this novel stripping approach that we describe here opens the possibility of establishing standardized assays for cellular allergy diagnostics. Activation tests using desensitized basophils passively re-sensitized with sera from allergic patients might help differentiate between cell intrinsic and extrinsic factors contributing to the disease state, which could be important for choosing the best treatment approach.

Our results using DARPin-based IgE inhibitors *ex vivo* as well as *in vivo* provide the basis for the development of novel drug candidates interfering with allergic hypersensitivity reactions. Further studies addressing immunogenicity and pharmacokinetics are required in order to develop DARPins as human therapeutics. DARPins are produced in remarkably high yields in bacterial expression systems and feature several favorable molecular characteristics such as small size, exceptional stability and high solubility facilitating manufacturing, storage and therapeutic application⁴⁴. Based on these considerations, the disruptive DARPin inhibitors may be particularly suitable and promising for topical therapy of allergic disease.

ONLINE METHODS

Materials

Anti-IgE DARPins were selected using ribosomal display and produced in *E. coli* as previously described^{1,2}. Recombinant FcεRIα-HSA-FcεRIα fusion protein, soluble FcεRIα (sFcεRIα) as well as recombinant human IL-3 was kindly provided by Novartis. IgE-Sus11³ and IgE-JW8 as well as anti-IgE antibody Le27⁴ were isolated from hybridoma and transfectoma cells respectively in our lab. NIP-BSA (Biosearch Technology), poly-HRP conjugated streptavidin (Thermo Scientific) and omalizumab (Novartis) were purchased. Anti-FcεRI antibody 29C6 was kindly provided by Roche. IgE-Fc₃₋₄ was expressed in insect cells as previously described⁵. Anti-IgE antibody Le27 and anti-FcεRI antibody 29C6 used for flow cytometry were labeled using LYNX conjugation kits (AbD Serotec). IgE-Sus11 was biotinylated using the EZ-Link Sulfo-NHS-Biotinylation kit (Thermo Scientific). Anti-CD63-FITC/anti-CCR3-PE antibody staining mix and leukotriene CAST ELISAs were kindly provided by Bühlmann Laboratories AG.

Inhibition ELISAs

ELISAs were performed using 96 half-well Maxisorp plates. Between each step the plate was washed 2 times with 150 µl of PBS and 2 times with 150 µl PBS/0.01% Tween-20. FcεRIα-HSA-FcεRIα was immobilized overnight at 4°C at 30 nM in 50 µl PBS. The next day wells were blocked with PBS/0.5% casein for 2 hour at 25 °C. In order to assess IgE:FcεRIα complex disruption 30 nM biotinylated IgE-Sus11 were first pre-incubated with 30 nM immobilized FcεRIα-HSA-FcεRIα for 1 h at 25°C. Subsequently different concentrations (0–100 µM) of DARPins E2_79, E3_58 or omalizumab were added for 30 minutes at 25°C. Supernatants were removed and remaining receptor-bound IgE was detected using

Le27-POX. The supernatants were diluted 1:10 in PBS/0.5% casein, incubated for 1 hour in a new 96 half-well Maxisorp plate with 30 nM immobilized Le27, and developed with poly-HRP conjugated streptavidin. 3,3',5,5'-tetramethylbenzidine was used as substrate for HRP. The enzymatic reaction was stopped using 1 M sulfuric acid, and the absorbance was measured at a wavelength of 450 nm with an EL808 plate reader (BioTek).

BIAcore binding assays

SPR measurements were conducted on a BIAcore X100 device (GE Healthcare) and evaluated with BIAevaluation Software. Individual sensorgram curves were exported to Excel for further data mining and graphs were prepared using GraphPad Prism 5.0. In all experiments unspecific binding to flow cell 1 was subtracted from the signal on flow cell 2. Unspecific signal peaks at the beginning and the end of injections, which are due to buffer changes and a short lag phase between Flow cell 1 and 2 were removed.

For kinetic analysis of different IgE binders 1000 response units (RU) IgE-Sus11 were immobilized in acetate pH5.0 buffer on flow cell 2 of a CM5 chip (GE Healthcare). Flow cell 1 was activated and deactivated without immobilization according to the manufacturer protocol. IgE binders were diluted in HBS-EP⁺ running buffer (GE Healthcare) and injected for 120 seconds at a constant flow rate of 10 μ l/min. Dissociation was assessed for 240 seconds under constant buffer flow.

For IgE removal experiments Fc ϵ RI α -HSA-Fc ϵ RI α was immobilized on flow cell 2 at 1000 RU. Flow cell 1 was immobilized with 500 RU human serum albumin (Sigma). 10 nM IgE-Sus11 or IgE-Fc₃₋₄ was injected for 120 seconds. Within the following 60 seconds almost no off-rate was observable due to the remarkably strong interaction of IgE with Fc ϵ RI α . All tested IgE binders were diluted in HBS-EP⁺ running buffer, except for omalizumab, which was first dialyzed, and where 1.75% Tween-20 (Sigma) was added to the samples and the running buffer. The indicated concentrations of anti-IgE molecules were injected for 180 seconds and IgE dissociation was monitored for an additional 180 seconds under constant buffer flow. For each measurement the chip surface was regenerated with 50 mM NaOH and new IgE was loaded onto the immobilized Fc ϵ RI α .

Cell isolation

Human basophils were isolated from different donors with total IgE levels ranging from 11 to 326 kU/l using percoll density centrifugation of dextran sedimented supernatants with further purification using Miltenyi's basophil isolation kit II as previously described⁶. Total IgE levels of all donors were determined using ImmunoCAP[®] (Phadia).

Basophil de- and resensitization

Purified human basophils were seeded at 0.5×10^5 cells/well in a 96-well plate in 100 μ l RPMI containing 10% heat-inactivated FCS, 100 IU/mL penicillin and 100 μ g/mL streptomycin (medium). For desensitization experiments cells were centrifuged at $500 \times g$ and 4 °C for 8 min and the supernatants were discarded. The different IgE binders (1–50 μ M) were diluted in medium and 100 μ l was added to the cells for the indicated durations. The cells were centrifuged and washed two times with 150 μ l PBS to remove any

dissociated IgE and anti-IgE molecules in the supernatant. 1 μ l FITC labeled Le27 and 0.5 μ l APC labeled 29C6 were used to stain 0.5×10^6 cells in PBS containing 2% heat-inactivated FCS and 0.05% NaN_3 (staining buffer) for 15 minutes at 25°C. Le27 detects surface bound IgE, while 29C6 recognizes IgE-bound as well as free Fc ϵ RI α . Cells were centrifuged, washed as above and resuspended in 300 μ l staining buffer. At least 3×10^3 basophils were acquired on a FACS Calibur device. Data were analyzed using FlowJo 9.6 software.

For resensitization the purified primary human basophils were stripped overnight and were washed two times with 150 μ l PBS to remove any dissociated IgE and anti-IgE molecules. Then the cells were incubated in medium containing 100 nM 4-Hydroxy-3-iodo-5-nitrophenylacetyl (NIP) specific IgE-JW8 for one hour at 37°C. The staining and acquisition protocol was the same as for desensitization.

Receptor timecourse

For the quantification of Fc ϵ RI α 0.25×10^5 cells/well were seeded in medium containing 10 ng/ml IL-3. Every 5 days new IL-3 (10 ng/ml) was added. Cells were cultured in the presence or absence of 50 μ M E2_79 or omalizumab until IgE and Fc ϵ RI α levels were quantified by flow cytometry using the same antibodies and staining procedure as for the desensitization experiments. Samples were measured every 48 hours over a time period of 11 days. The first quantification was performed after 24 hours of culture. At least 1×10^3 basophils were acquired.

Basophil activation test

Purified human basophils were seeded at 0.5×10^5 cells/well in a 96-well plate in 100 μ l medium. Cells were either not stripped, stripped or stripped and resensitized. In order to desensitize the cells they were incubated for 14 h with 50 μ M DARPIn E2_79 as described above. To optimally prime the cells for activation 50 ng/ml IL-3 was added to each well overnight. The next day cells were washed twice with 150 μ l PBS. For resensitization 100 nM 4-Hydroxy-3-iodo-5-nitrophenylacetyl (NIP) specific IgE-JW8 was added for 1 hour at 37 °C. After an additional wash step the cells were triggered either with Le27 (10–300 ng/ml) or NIP-BSA (0.01–10 ng/ml) for 20 minutes in the presence of IL-3 and 10 μ l CD63-FITC/CCR3-PE antibody mix. Supernatants were removed and the cells washed twice and resuspended in 300 μ l staining buffer. Again, at least 3×10^3 basophils were acquired. Supernatants were used for LTC4 quantification with the commercially available CAST-ELISA. The ELISA was performed according to manufacturer instructions.

For the assessment of anaphylactogenicity of the different divalent DARPins 0.5×10^5 cells/well were cultured in a 96-well plate in 100 μ l medium containing 50 ng/ml IL-3 overnight. The next day the indicated concentrations (5 μ M, 500 nM, 50 nM, 5 nM and 0 nM) of the different divalent DARPins (bi53_53, bi79_79, bi53_79 and bi79_53) were added to the cells for 10 minutes and the cells were simultaneously stained in the same way as for the regular basophil activation test using 10 μ l CD63-FITC/CCR3-PE antibody mix.

Passive cutaneous anaphylaxis

Mice that are transgenic for human FcεRIα and that have the murine FcεRIα knocked out were used to assess the *in vivo* efficacy of the different IgE inhibitors. During manipulation the mice were kept anesthetized under a constant flow of isoflurane. At each injection site 25 µl of 5 µg/ml human 4-Hydroxy-3-iodo-5-nitrophenylacetyl (NIP) specific IgE-JW8 or PBS was applied intra-dermally. The next day the mice were again anesthetized and different concentrations (5 µM or 50 µM) of DARPin E3_58, E2_79, bi53_79 or omalizumab were applied at the same injection sites. Six hours later multivalent allergen (NIP₂₅ BSA, Biosearch Technologies) was applied intravenously in combination with 0.5% Evans blue dye in 200 µl saline. Dye leakage into the skin was visually analyzed. For absolute quantification the dye was extracted from the skin using dimethylformamide (EMD Chemicals) and absorbance was measured at a wavelength of 620 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by a grant from the Fondation Acteria to A.E., a Swiss National Science Foundation Ambizione grant to A.E. (PZ00P3_148185), a Swiss National Science Foundation grant to C.A.D. (310030_127350), an NIH research grant (AI-18939) and an American Asthma Foundation Senior Investigator Award to T.S.J.

We thank past and present members of the Jardetzky, Dahinden and Stadler Lab, especially M.J. Baumann for DARPin selection and A. Odermatt, M. Udovicic, E. Keller-Gautschi as well as M. Zwicker for technical support. We also acknowledge Molecular Partners AG, in particular P. Amstutz, M. T. Stumpp and D. Steiner, for placing DARPin libraries at our disposal and for providing scientific input. Further we extend our acknowledgements to Bühlmann Laboratories AG for providing material. We also thank Prof. J.-P. Kinet for providing the transgenic FcεRIα mice.

Abbreviations

IgE	Immunoglobulin E
FcεRIα	high-affinity IgE receptor
DARPin	designed ankyrin repeat proteins
SPR	surface plasmon resonance
NIP	4-Hydroxy-3-iodo-5-nitrophenylacetyl
LTC4	leukotriene C4

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FIG 1.

Omalizumab accelerates IgE dissociation in vitro. **(A)** Increasing concentrations of non-disruptive DARPin E3_58 (upward triangles), disruptive DARPin E2_79 (downward triangles), and omalizumab (circles) dissociate pre-formed IgE:FcεRI complexes in ELISA. **(B)** Removed IgE is quantified in the supernatants of E3_58, E2_79 and omalizumab treated IgE:FcεRI complexes by ELISA. Values represent means ± SD of technical duplicates in the ELISAs. **(C–F)** The potency of E2_79, E3_58 and omalizumab to disrupt IgE:FcεRI complexes is assessed by SPR. FcεRIα is immobilized and injection of 10 nM full length IgE is followed by the addition of E2_79 **(C)**, E3_58 **(D)**, omalizumab **(E)** and sFcεRIα **(F)**.

FIG 2. Omalizumab recognizes receptor-bound IgE-Fc₃₋₄. Binding of omalizumab to receptor-bound full length IgE (A) or IgE-Fc₃₋₄ (B) complexes is measured by SPR. FcεRIα is immobilized and injection of 10 nM full length IgE is followed by the addition of omalizumab. The structures of IgE-Fc₂₋₄:FcεRIα (C) and IgE-Fc₃₋₄:FcεRIα complexes (D) from PDB ID 1F6A are shown. Residues 421–432 are represented as red spheres and correspond to F strand and FG loop residues in the IgE Cε3 domain. Site 2 is directly involved in FcεRIα binding and likely blocked from omalizumab binding. Site 1 is accessible to solvent. In the IgE-Fc₂₋₄:FcεRIα complex, the Cε2 domains sterically block omalizumab Site 1.

FIG 3.

E2_79 and omalizumab accelerate IgE:FcεRI complex dissociation on primary basophils ex vivo. (A) Surface IgE staining of isolated primary human blood basophils from one donor after incubation with E3_58 (first row), omalizumab (second row) or E2_79 (third row). Histograms show surface IgE levels compared to untreated cells. (B) IgE levels on the surface of primary human basophils after incubation with 50 μM of E3_58 (white bars), omalizumab (grey bars) or E2_79 (black bars) for the indicated durations. The geometric mean fluorescence intensity for surface IgE on untreated cells was set to 100%. (C) Surface IgE (columns one and two) and FcεRIα (columns three and four) staining of isolated primary human blood basophils after incubation without (grey area) or with omalizumab (dashed line), E2_79 (black line) over 11 days. Surface IgE (D) and FcεRIα (E) levels on primary human basophils after incubation without (squares) or with 50 μM omalizumab (downward triangles), E2_79 (circles). The geometric mean of the fluorescence intensity for IgE and FcεRIα levels of untreated cells were set to 100%. (F) Flow cytometric gating strategy for basophil activation test using isolated primary human basophils. Cells were treated without (white bars) or with 50 μM E2_79 (black bars) overnight. Activation by anti-IgE antibody Le27 is shown by surface CD63 (G) as well as secreted LTC4 quantification (H). Values represent means ± SD; (n = 3–4).

FIG 4.

Antigen-specific resensitization of desensitized primary basophils ex vivo. Surface IgE (A) and FcεRIα (B) stainings of isolated primary human basophils after overnight incubation without (grey area) or with E3_58, E2_79 or omalizumab (black line). Resensitization of E3_58, E2_79 or omalizumab treated cells with NIP-specific IgE is shown (dashed lines). Surface IgE (C) and FcεRIα (D) levels on primary human basophils after desensitization with E3_58, E2_79 or omalizumab (black bars) and resensitization (grey bars). The geometric mean of the fluorescence intensity for IgE and FcεRIα levels of untreated cells were set to 100%. Antigen-specific activation test with desensitized (black bars) or resensitized (grey bars) cells measuring CD63 (E) or secreted LTC4 (F). Values represent means ± SD; (n = 3).

FIG 5.

Biparatopic anti-IgE targeting increases disruptive efficacy. **(A–D)** Surface plasmon resonance to compare bivalent anti-IgE DARPins with E2_79. FcεRIα was immobilized and injection of 10 nM full length IgE was followed by different concentrations of E2_79 **(A)**, bi79_79 **(B)**, bi79_53 **(C)** or bi53_79 **(D)**. **(E)** Surface IgE staining on primary human blood basophils incubated with E2_79, bi79_79, bi79_53 or bi53_79. **(F)** Surface IgE level on primary human basophils from three donors after desensitization with 50 μM E2_79 (dark grey bars), bi79_79 (black bars), bi79_53 (white bars) or bi53_79 (light grey bars). The geometric mean of the fluorescence intensity for IgE of untreated cells was set to 100%. **(G)** Flow cytometric anaphylactogenicity analysis of bivalent DARPins using primary human blood basophils. CD63 was used as activation marker. Values represent mean ± SD; (n = 3).

FIG 6.

Disruptive IgE inhibitors block anaphylaxis in vivo. Transgenic mice expressing human FcεRIα were passively sensitized with intradermal injections of NIP-specific IgE. The next day 50 μM (A) or 5 μM (C) E2_79, E3_58, omalizumab and 5 μM bi53_79 (A and C) were applied at the same site intradermally. Six hours later the mice were intravenously challenged with antigen solution containing 0.5% Evans Blue. Representative pictures of the skin were taken to visualize the local allergic reaction (A and C). Additionally the reactions were quantified by extraction of the blue dye from the skin (B and D). Values represent mean ± SD; (n = 3–4). ***p* < 0.01, *****p* < 0.0001.