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## **A novel bispecific DARPIn targeting FcγRIIB and FcεRI-bound IgE inhibits allergic responses**

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## Abbreviations:

|                  |                                                |
|------------------|------------------------------------------------|
| DARPIn           | Designed ankyrin repeat protein                |
| EC <sub>50</sub> | Half-maximal activation                        |
| ELISA            | Enzyme-linked immunosorbent assay              |
| ITAM             | Immunoreceptor tyrosine-based activatory motif |
| ITIM             | Immunoreceptor tyrosine-based inhibitory motif |
| NIP              | 4-Hydroxy-3-iodo-5-nitrophenylacetyl           |

|     |                           |
|-----|---------------------------|
| PE  | Phycoerythrin             |
| RU  | Response unit             |
| SPR | Surface plasmon resonance |

**Short Title:** Bispecific DARPIn protein to inhibit allergic reactions

## Abstract:

**Background:** Binding of allergen-specific IgE to its high-affinity receptor FcεRI on basophils and mast cells is a central event in the development of allergies. Exposure of these cells to allergens induces the release of soluble mediators causing allergic symptoms. The inhibitory low-affinity IgG Fc-receptor FcγRIIB is co-expressed on allergic effector cells and has been implicated in negative regulation of immediate hypersensitivity responses. In order to harvest the inhibitory function of this receptor we aimed to select specific binders against FcγRIIB and to generate a bispecific molecule simultaneously targeting FcγRIIB and FcεRI-bound IgE on the surface of allergic effector cells.

**Methods:** We selected FcγRIIB-specific binding molecules from a library of designed ankyrin repeat proteins using ribosome display technology. The bispecific binding modality was generated by molecular cloning and recombinant protein expression. We determined binding characteristics on molecular and cellular levels using SPR, ELISA and flow cytometry. The inhibitory potential of the newly described molecules was assessed in different cellular degranulation assays *ex vivo* and in a mouse model of passive systemic anaphylaxis.

**Results:** We demonstrate that the selected DARPIn<sup>®</sup> proteins recognize FcγRIIB with high affinity. Furthermore, the bispecific binding protein successfully interferes with allergen-induced cell degranulation and efficiently inhibits systemic anaphylaxis *in vivo*. Mechanistically, we report that FcγRIIB-mediated inhibition of effector cell activation requires direct ligation to an activating FcεRI receptor.

**Conclusion:** The described bispecific DARPIn protein has the ability to co-ligate FcγRIIB with FcεRI-bound IgE on allergic effector cells and represents an efficient dual-modality to interfere with allergic hypersensitivity reactions.

The worldwide prevalence of allergic disorders is steadily increasing and currently afflicts roughly one third of the human population in industrialized countries(1,2). People suffering from allergic conditions experience a marked reduction in quality of life and treatment options are still limited(3). In recent years, remarkable progress in the development of new intervention strategies has been made(3). However, due to limited treatment success patients still often have to rely on unspecific therapies such as corticosteroids(1,4). The continuously growing prevalence of allergies underlines the urgent need for more effective intervention strategies targeting basic mechanisms underlying disease pathogenesis.

Binding of allergen-specific immunoglobulin E (IgE) to its high-affinity receptor FcεRI on allergic effector cells, such as basophils and mast cells, is a central step for allergy induction. Due to the slow dissociation rate of IgE:FcεRI complexes, these cells are permanently sensitized with IgE and thus ready to respond immediately upon allergen challenge. On a molecular level, FcεRI-aggregation leads to trans-phosphorylation of its intracellular immunoreceptor tyrosine-based activation motifs (ITAMs) through receptor-associated kinases(5). Phosphorylated ITAMs recruit and activate downstream tyrosine kinases such as Syk, which further propagate the activation(5-7). This signaling cascade initiates the transcription of inflammatory genes and ultimately promotes degranulation of newly synthesized as well as pre-stored mediators inducing an allergic response(2,8,9).

Besides ITAM-containing activating receptors allergic effector cells co-express inhibitory receptors that are characterized by intracellular immunoreceptor tyrosine-based inhibition motifs (ITIMs)(5,6,10-12). FcγRIIB (CD32b) is such an inhibitory receptor that is expressed on a variety of immune cells including basophils as well as mast cells(13). It binds IgG with low affinity (14) and has been implicated in the regulation various cellular processes. There is convincing evidence that co-aggregation of FcγRIIB with FcεRI on the surface of allergic effector cells represents a promising approach to inhibit allergen-dependent immediate hypersensitivity reactions (15-25). Phosphorylation of the intracellular ITIM of FcγRIIB by FcεRI-associated Src family kinases has been shown to be crucial for the recruitment and activation of the SH2 domain-containing inositol 5'-phosphatase SHIP which dephosphorylates adaptor molecules downstream of FcεRI and thereby abrogates signaling pathways involved in the degranulation and mediator release(25-27).

Here we describe the selection and characterization of novel DARPin<sup>®</sup> proteins (designed ankyrin repeat proteins) targeting the inhibitory FcγRIIB. The DARPin protein platform represents a class of alternative binding molecules based on natural ankyrin repeat proteins(28). Specific binders can be selected from a DARPin library using ribosomal display and produced in high yields in a prokaryotic expression system using *E. Coli*(29). By joining one of the newly selected FcγRIIB binding proteins to a previously selected anti-IgE molecule we generated a bispecific modality with the capacity to co-aggregate FcγRIIB with FcεRI-bound IgE(16,17). We assessed binding characteristics of the newly selected and engineered DARPin proteins on a molecular and cellular level and demonstrate that they successfully interfere with IgE-dependent allergic reactions *ex vivo* in cellular activation tests as well

as *in vivo* in a mouse model of passive systemic anaphylaxis. Furthermore, we provide important mechanistic insight into FcγRIIB-mediated inhibition of allergic effector cells using the newly described DARPin proteins.

## Methods

See the methods section in this article's Online Repository for details about materials, inhibitors, ELISAs, BIAcore binding assays, cell isolation, basophil sensitization, basophil activation test, basophil inhibition assay and passive systemic anaphylaxis assay.

### *Human samples and animal experiments*

Human primary basophils were isolated from peripheral whole blood of allergic and healthy donors with approval from the local ethics committee. All donors provided informed consent in accordance with the Helsinki Declaration. Mice transgenic for human FcεRIα and knocked out for the human FcεRIα were kindly provided from Prof. J.-P. Kinet. All animal experimentation was approved by the local ethics committee.

### *Statistics*

Statistical analysis was performed using Prism 6.0 (GraphPad software, La Jolla, USA). Data are shown as means ± SEM. For comparison of treatments, ordinary One-way Anova analysis, using Tukey's multiple comparison test was performed. To compare concentration-dependent treatments, Two-way analysis was used. Statistical analysis of *in vivo* PSA assay was performed using Two-way Anova, Sidak's multiple comparison test. Statistical significance (*P value*) was reported if  $p < 0.05$  was achieved.

## Results

### *D11 specifically recognizes human FcγRII and inhibits binding of IgG*

In order to specifically target human FcγRIIB (huFcγRIIB) we selected binding molecules from a designed ankyrin repeat protein (DARPin) library using ribosome display (Supplementary Fig. 1)(16,30,31). Our selection strategy led to successful identification of multiple anti-FcγRII binders as shown in the enzyme-linked immunosorbent assay (ELISA) using *E. Coli* crude extracts (Supplementary Fig. 2A). The DARPin binding protein D11 (Supplementary Fig. 2B) showed the

highest absorption values on huFcγRIIB in ELISAs using either crude extracts or His-tag purified protein. Based on these data D11 has been chosen for further detailed analysis.

We determined the binding kinetics of D11 to immobilized human FcγRIIA (huFcγRIIA) and huFcγRIIB by surface plasmon resonance (SPR) (Fig. 1A). The overall affinity ( $K_D$ ) of D11 for huFcγRIIB was 6-times smaller ( $K_D = 1.9 \times 10^{-9}$  M) than for huFcγRIIA ( $K_D = 1.1 \times 10^{-8}$  M) indicating stronger binding to huFcγRIIB (Supplementary Table 1). The major kinetic difference was due to the 18-times lower dissociation constant on huFcγRIIB. Results from additional binding analysis by ELISA support the kinetic data indicating preferential binding of D11 to huFcγRIIB (Fig. 1B).

To obtain further information about the binding epitope of D11 on FcγRII, we tested the ability of the DARPin to block IgG binding using SPR (Fig. 1C). Pre-incubation of equimolar concentrations of D11 and huFcγRII resulted in 60-70% reduction of huFcγRII binding to immobilized IgG. A 10-fold molar excess of D11 was sufficient to fully block huFcγRII binding to IgG. These results provide evidence that D11 recognizes an epitope within or close to the IgG binding site. We further assessed binding of D11 to FcγRIIB expressing primary human basophils by flow cytometry. While D11 showed dose-dependent binding, only background signal was detectable for the unspecific control DARPin (Fig. 1D).

### ***Generation of a bispecific anti-FcγRIIB/ anti-IgE binding modality***

In order to generate a molecule that can be used to simultaneously target FcγRIIB and FcεRI via receptor-bound IgE we cloned the selected anti-FcγRIIB DARPin protein D11 to the previously described anti-IgE DARPin protein E53 via a flexible (Gly<sub>4</sub>-Ser<sub>1</sub>)<sub>4</sub>-linker(16). The bispecific DARPin has been termed D11\_E53. As an additional tool to co-ligate FcγRIIB without involvement of FcεRI we also generated the bivalent anti-FcγRIIB construct D11\_D11 (Fig. 2C). First, we analyzed the binding characteristics of purified DARPins D11\_E53 and D11\_D11 by SPR. D11\_E53 recognized huFcγRIIB and huFcγRIIA with slightly reduced affinity ( $K_D = 9.1 \times 10^{-9}$  M and  $K_D = 2.6 \times 10^{-8}$  M, respectively) as compared to monovalent D11 (Fig. 2A, Supplementary Table 1). This difference mainly originated in a reduced association constant, while there was no major difference observable in the dissociation constant. ELISA results confirmed specific binding of D11\_E53 to huFcγRIIB, while it showed no specific binding of other human Fc-receptors (Fig. 2B) such as FcγRI (CD64) or FcγRIIA (CD16A). We additionally assessed binding of the bispecific D11\_E53 to immobilized IgE(32) (Fig. 2A). Its affinity ( $K_D = 1.8 \times 10^{-7}$  M) is comparable to values we have previously determined for other molecules involving the anti-IgE DARPin protein E53(16). The bivalent FcγRIIB DARPin protein D11\_D11 benefits from an avidity effect and displays a 200-fold increased binding affinity to huFcγRIIA ( $K_D = 5.6 \times 10^{-11}$  M) and a 3000-fold increased binding affinity to huFcγRIIB ( $K_D = 6.3 \times 10^{-13}$  M) (Fig. 2C, Supplementary Table 1). Additionally, we measured binding of

D11\_E53 and D11\_D11 to FcγRIIB-expressing human primary basophils. Both divalent molecules showed specific and dose-dependent binding on these cells as measured by flow cytometry (Supplementary Fig. 3A-C). In order to assess whether D11 binds huFcγRII on other cells we tested binding of D11, D11\_D11 and D11\_E53 on B- and T-lymphocytes. As expected we observed dose-dependent binding on B-cells, which express huFcγRII, but not on T-cells, which lack this receptor (Supplementary Fig. 4A-C).

### ***Inhibition of basophil degranulation by D11\_E53***

To test whether D11\_E53 or D11\_D11 have the ability to inhibit allergic effector cell degranulation in a FcγRIIB-dependent manner and to compare their inhibitory potential to the therapeutic anti-IgE antibody omalizumab we recruited grass-pollen allergic volunteers (Supplementary Fig. 5A) and isolated primary human basophils from their whole blood. We used a functional basophil activation test (BAT) in which surface expression of CD63 is measured as a marker for cell degranulation (Fig. 3A)(33). Additionally, we measured leukotriene C4 release (LTC<sub>4</sub>) from the cells. First, we tested D11\_E53 and D11\_D11 for their ability to activate the cells in the absence of allergen challenge. Neither one of them induced allergen-independent basophil degranulation (Supplementary Fig. 5C). Incubation of the cells with D11\_E53 one hour prior to allergen stimulation, however, resulted in a significant dose-dependent inhibition of basophil degranulation (Fig. 3B and C). Results are shown as percentage of half-maximal activation (EC<sub>50</sub>) of each donor using specific allergen extracts (Supplementary Fig. 5B). Interestingly, we did not observe any inhibitory effect for D11\_D11 or omalizumab (Fig. 3B). Additionally, we also tested the inhibition of allergen-dependent basophil activation in whole blood. At a concentration of 100 nM D11\_E53 displayed time-dependent inhibition on basophil degranulation with a fast onset of action already at 5 minutes of pre-incubation (Figure 3C and D). Suboptimal concentrations of D11\_E53 required prolonged incubation to inhibit basophil activation. Together these data suggest that D11\_E53-mediated co-ligation of FcγRIIB with FcεRI-bound IgE induces inhibition of allergen-induced basophil activation while homo-aggregation of FcγRIIB by D11\_D11 does not exert any inhibitory effect. Strikingly, D11\_E53 was superior to the therapeutic antibody omalizumab in these inhibition assays.

In order to exclude that the observed inhibitory effect of D11\_E53 might be due to a decrease in cell viability we determined basophil survival by measuring apoptosis markers Annexin V and Propidium Iodide (PI) as well as by counting cell numbers (Supplementary Fig. 6A and B). Primary human basophils were cultured *in vitro* over a period of four days in the presence of D11\_E53. Both Annexin V / PI staining as well as absolute cell counting clearly indicated that D11\_E53 does not alter the survival of primary human basophils in culture.

### ***Abrogation of intracellular activation signaling by D11\_E53***

To further decipher D11\_E53 induced intracellular events leading to the abrogation of allergen-induced cell degranulation we assessed the phosphorylation state of FcγRIIB and known downstream adaptor molecules in human primary basophils. Western blot analysis of D11\_E53 treated cells revealed dose-dependent phosphorylation of FcγRIIB in the absence of allergen stimulation (Fig. 4A). FcγRIIB phosphorylation was completely inhibited when cells were pre-treated for 30min with the chemical inhibitor AZD-0530 (Saracatinib) to block the Src kinases Lyn and Fyn (Fig. 4B). Pan-inhibition of FcεRI-associated kinases Lck/Fyn and Syk with PP1 and specific inhibition of Syk with R406 resulted only in a minor reduction of FcγRIIB phosphorylation. These data provide strong evidence and confirm previous findings in mast cells that trans-phosphorylation of FcγRIIB in primary human basophils is mainly dependent on the FcεRI-associated Src family kinase Lyn(25).

Even though we observed D11\_E53 mediated phosphorylation of FcγRIIB in the absence of additional stimuli the degree of phosphorylation was markedly increased when basophils were challenged with the anti-FcεRI antibody 29C6(16) (Fig. 4C and Supplementary Fig. 7A and B). Recruitment and phosphorylation of the SH2 domain-containing inositol 5'-phosphatase SHIP via activated FcγRIIB has been described to abrogate FcεRI-induced cell degranulation through de-phosphorylation of adaptor molecules downstream of FcεRI(34-37). As expected, D11\_E53 in combination with anti-FcεRI challenge induced phosphorylation of SHIP (Fig. 4D). Additionally, we observed decreased phosphorylation levels for tyrosine kinase Syk as well as for protein kinase B (Akt) (38). While sole receptor cross-linking with anti-FcεRI antibody 29C6 induced phosphorylation of Syk and Akt, treatment with D11\_D11 did not significantly change this activation pattern (Fig. 4D).

### ***FcγRIIB-mediated regulatory function is restricted to direct receptor ligation***

Since we have observed that D11\_E53 induces phosphorylation of FcγRIIB in the absence of FcεRI stimulation, we were wondering whether such an inhibitory priming of the cell might abrogate subsequent activation with allergen or whether phosphorylated FcγRIIB needs to be directly ligated to the allergen cross-linked FcεRI in order to exert its inhibitory function (Fig. 5A and B). Due to the low dissociation constant of IgE:FcεRI complexes most FcεRI on primary human basophils are permanently occupied by endogenous IgE. However, a small fraction of FcεRI remains free and can be sensitized by addition of exogenous antigen-specific IgE. For this experiment we sensitized isolated primary human basophils either with NIP-specific human IgE (huIgE) or TNP-specific mouse IgE (mIgE). Even though mIgE has a slightly lower affinity than huIgE for the human FcεRI (Supplementary Fig. 8, Supplementary Table 2) both IgEs show steady binding (mIgE ΔMFI: 157, huIgE ΔMFI: 191) to primary human basophils in our experimental setup as assessed by flow cytometry (Fig. 5C). Antigen induced stimulation of huIgE sensitized cells with NIP<sub>7</sub>-BSA and mIgE

sensitized cells with TNP<sub>21</sub>-BSA resulted in dose-dependent cellular activation (Fig 5D). We then treated cells carrying either huIgE or mIgE with D11\_E53 that cannot recognize mIgE (Fig. 5E), in order to assess whether direct ligation of FcγRIIB to the allergen cross-linked FcεRI is required to prevent activation. Interestingly, we only observed a dose-dependent inhibition of allergen-induced degranulation for basophils sensitized with huIgE (Fig. 5F). These results clearly indicate that FcγRIIB requires direct ligation to the activating receptor for successful inhibition of antigen-dependent basophil degranulation.

### ***D11\_E53 cross-reacts with mouse FcγRIIB***

Based on the fact that human and mouse FcγRIIB (mFcγRIIB) share approximately 50% homology in their protein sequence we tested whether D11 containing DARPin formats cross-reacts with mFcγRIIB by SPR. Indeed, monovalent D11 ( $K_D = 1.5 \times 10^{-6}$  M) as well as bispecific D11\_E53 ( $K_D = 2.6 \times 10^{-6}$  M) and bivalent D11\_D11 ( $K_D = 5.1 \times 10^{-8}$  M) recognized mouse FcγRIIB (Fig. 6A). However, the affinities for mFcγRIIB were clearly lower than for its human counterpart (Supplementary Table 1). In line with this finding, D11, D11\_E53 and D11\_D11 also recognized mFcγRIIB on bone marrow-derived mast cells (mBMMC) of human FcεRIα-transgenic mice (huFcεRIα) (Figure 6B)(39).

In order to assess whether D11\_E53 is able to inhibit allergen-induced degranulation of murine allergic effector cells *in vitro* we used huFcεRIα-mBMNCs passively sensitized with NIP-specific huIgE. As a read-out for degranulation we measured the released β-hexosaminidase in the cell culture supernatant after antigen challenge. In line with the results we received for the inhibition of primary human basophils, D11\_E53 also showed FcγRIIB-dependent inhibition of passively sensitized huFcεRIα-mBMNCs (Figure 6C and D). Again, D11\_D11 showed no effect further supporting our initial finding for human primary basophils that homo-ligation of FcγRIIB does not abrogate antigen-induced cell degranulation.

To test *in vivo* functionality of D11\_E53 we performed a passive systemic anaphylaxis model using huFcεRIα transgenic mice(40-42). The mice were intravenously injected with NIP-specific huIgE. The next day D11\_E53 was intravenously applied 6h prior to intravenous challenge with NIP<sub>7</sub>-BSA antigen in 0.9% saline. Mice receiving systemic D11\_E53 did not show any sign of abnormal behavior or adverse reaction as judged by continuous observation. Upon antigen challenge D11\_E53 treated mice underwent a significantly less pronounced change in body core temperature compared to saline treated mice (Fig.6E). The data clearly indicate that the selected DARPin molecule can be safely applied to animals and that despite its low-affinity for mFcγRIIB D11\_E53 remarkably decreases antigen-induced systemic anaphylaxis *in vivo*.

## Discussion

Here we described and characterized a new class of non-immunoglobulin based binding proteins targeting the regulatory IgG receptor FcγRIIB on a molecular, cellular and systemic level. The selected DARPin protein D11 recognizes huFcγRIIB with high affinity and specificity. It binds huFcγRIIB with 6-fold higher affinity than huFcγRIIA despite the >90% amino acid sequence identity in the extracellular domain of the two receptor variants. By generating the bispecific binding modality D11\_E53 to simultaneously target huFcγRIIB and FcεRI-bound IgE on allergic effector cells we propose an alternative immunoglobulin-free approach to harvest the inhibitory capacity of FcγRIIB and thereby to interfere with allergen-induced cell degranulation. We chose this strategy since FcγRIIB is a low-affinity receptor for IgG and therefore remains mostly unoccupied on the cell surface. On the other hand, the majority of FcεRI is constantly complexed with IgE due to their high-affinity interaction. Thus, both targets are readily available for binding by the bispecific DARPin D11\_E53 on the cell surface and there is only marginal competition with endogenous receptor ligands. Consequently, the onset of inhibitory action is remarkably fast and happens within minutes after application of D11\_E53.

Even though D11 was selected against huFcγRIIB it features cross-reactivity with the murine homologue. This allowed us to investigate the inhibitory capacity of D11\_E53 on cells derived from both species. Our data clearly indicate that D11\_E53 inhibited allergen-induced cell degranulation on primary human basophils as well as on passively sensitized bone marrow-derived murine mast cells derived from huFcεRI transgenic mice *ex vivo*. The observed inhibitory effect was still considerable given the low affinity of D11\_E53 for mFcγRIIB.

Bispecific DARPin D11\_E53 represents a valuable tool to investigate intracellular events underlying FcγRIIB-mediated inhibition of allergen-induced cell degranulation. Using D11\_E53 we found that low level FcγRIIB phosphorylation is induced upon co-ligation with FcεRI even in the absence of allergen stimulus. In line with previous findings we could confirm that this FcγRIIB trans-phosphorylation is greatly dependent on the FcεRI-associated Src kinase Lyn(25). The question whether phosphorylation of FcγRIIB may translate into general basophil anergy has not been answered so far. Here, we demonstrated that FcγRIIB has to be directly ligated or at least in close proximity to the antigen cross-linked FcεRI in order to efficiently inhibit IgE-dependent cell degranulation. Our data suggest, that the amount of inhibition is greatly dependent on the ratio between FcεRI occupied with antigen-specific IgE and FcγRIIB that is co-expressed on the cell surface. Previous reports indicate that the level of FcγRIIB on human basophils is highly variable and that its expression is dependent on IL-3(18). Also, FcεRI surface levels show high variability on human basophils and correlate with the total amount of IgE present in the serum(42). This variability in receptor expression might explain why D11\_E53 treatment did not result in full inhibition in our *in vitro* experiments. Nevertheless, systemic *in vivo* application of D11\_E53 greatly reduced antigen-

mediated passive anaphylaxis in huFcεRI transgenic mice sensitized with human IgE highlighting the therapeutic potential of this DARPin-based macromolecular inhibitor. Thus, the prove-of-concept DARPin molecule D11\_E53 may serve as a valuable tool guiding the future development of efficient allergy therapeutics.

There are a variety of additional immune cells such as B-cells, macrophages, dendritic cells or neutrophils that also express the inhibitory FcγRIIB. However, only B-cells and dendritic cells simultaneously display IgE on their cell surface. While dendritic cells also express FcεRI, B-cells carry IgE either as B-cell receptor (IgE-BCR) or bound via CD23 on their surface. Due to increased avidity we assume that the bispecific format of D11\_E53 will preferentially bind to cells carrying both targets on their surface. However, we cannot exclude off-target effects at this stage. Further investigations assessing the effect of D11\_E53 on other FcγRIIB-expressing cells will be required. Whether D11\_E53 has the ability to aggregate CD23-bound IgE or IgE-BCR with FcγRIIB on the surface of IgE switched B-cells and whether this might result in a down-regulation of IgE synthesis as previously shown for other molecules will be of particular interest in the context of allergy treatment (42).

Therapeutic monoclonal antibodies (mAb) are a huge success for the treatment of various immunologic disorders. However, in recent years several limitations of mAb have come to light (43). Amongst those are the high production costs, the rather low molecular stability, and issues related to the wide expression of Fc-receptors, which can significantly reduce treatment specificity and efficacy *in vivo*. DARPin proteins represent an interesting alternative to mAb. They feature a small molecular size (12-18 kDa as monomer), high thermodynamic stability and they can be produced in high yields using prokaryotic expression systems. DARPin proteins against various targets (e.g. anti-VEGF) have recently shown promising results in clinical studies(44,45). Clearly, the new anti-FcγRIIB DARPin as well as the concept of bispecific targeting of FcγRIIB and FcεRI-bound IgE that we present in this work has to undergo further detailed evaluation before therapeutic application can be considered. However, our promising results together with previous studies strongly indicate that targeting of FcγRIIB is an efficient way to interfere with allergic reactions. Furthermore, we speculate that DARPin D11 might also represent an interesting candidate to block the inhibitory FcγRIIB to increase treatment efficacy and specificity of certain therapeutic mAb that are already in clinical use (46).

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## Figures

Figure 1

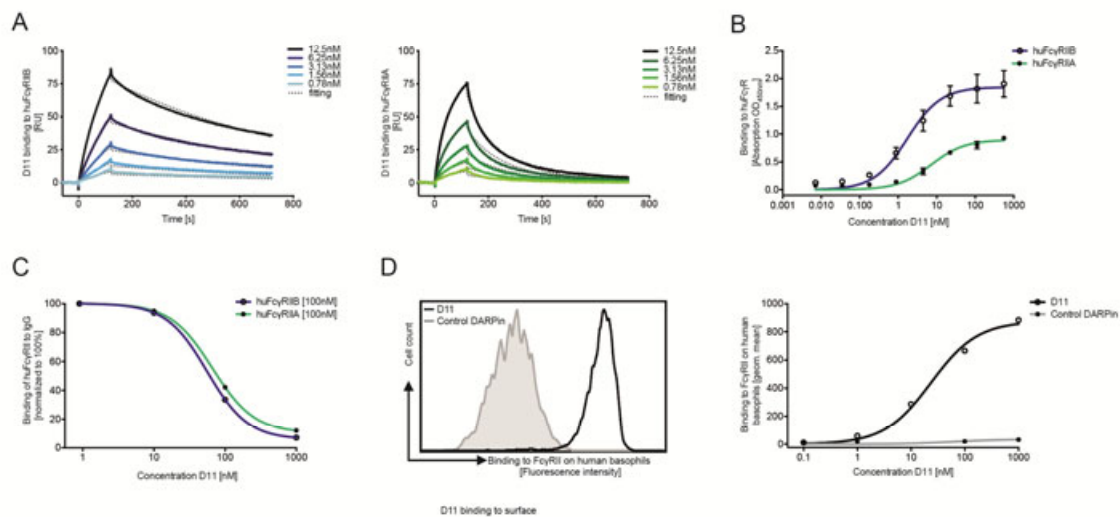


Figure 2

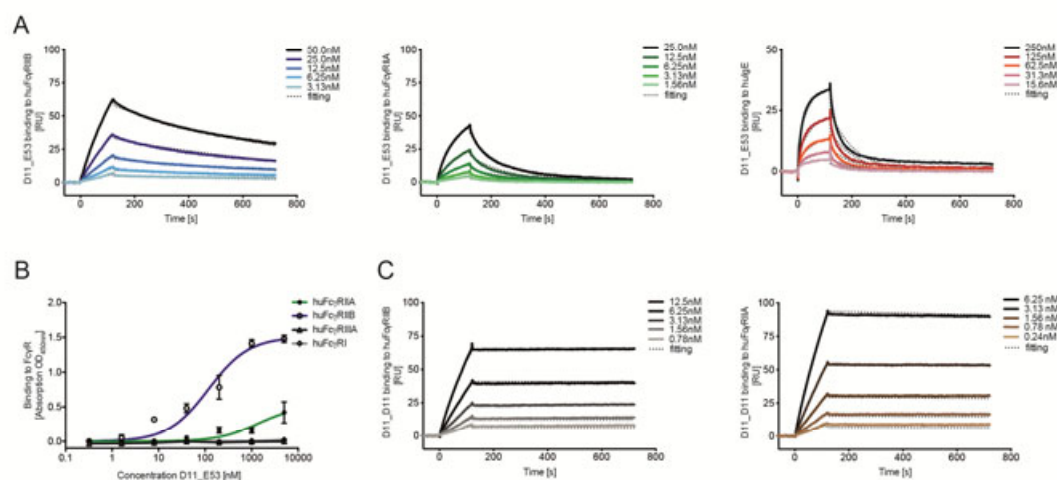


Figure 3

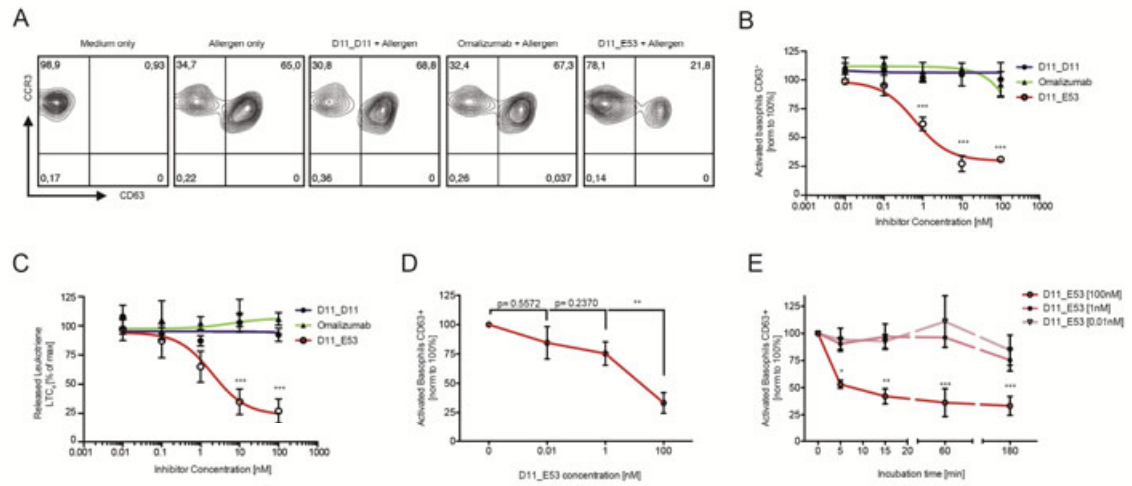


Figure 4

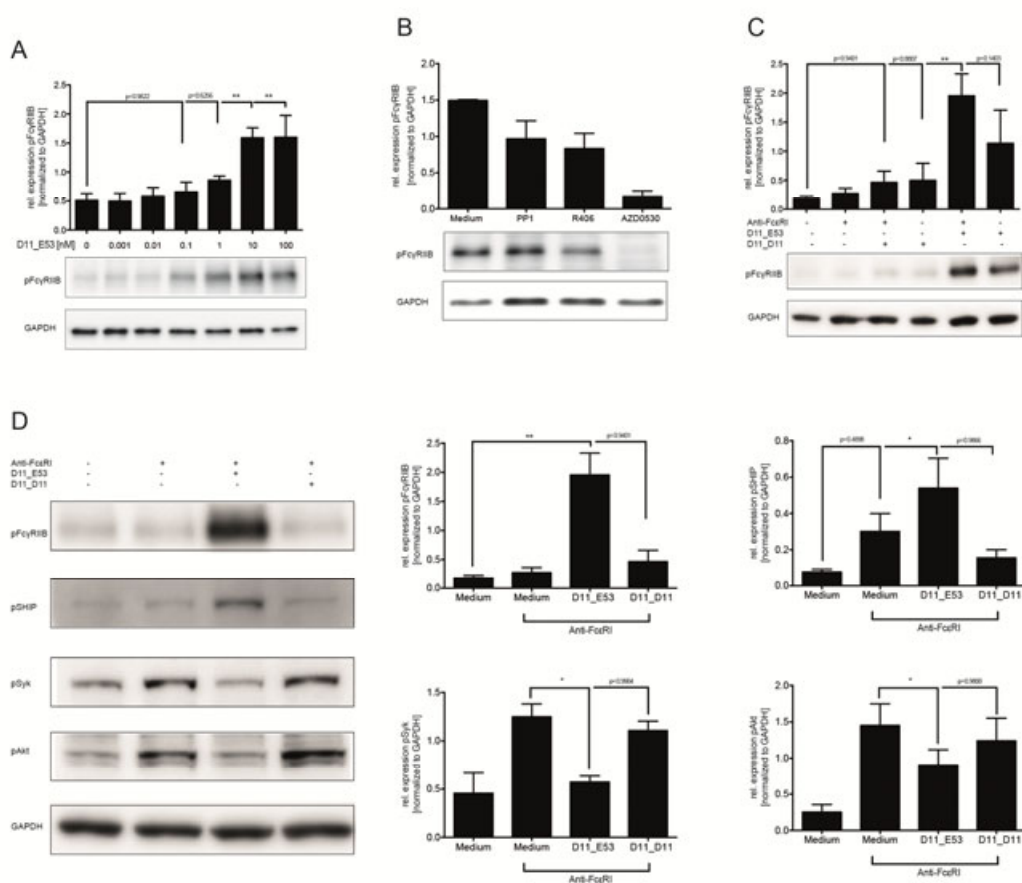
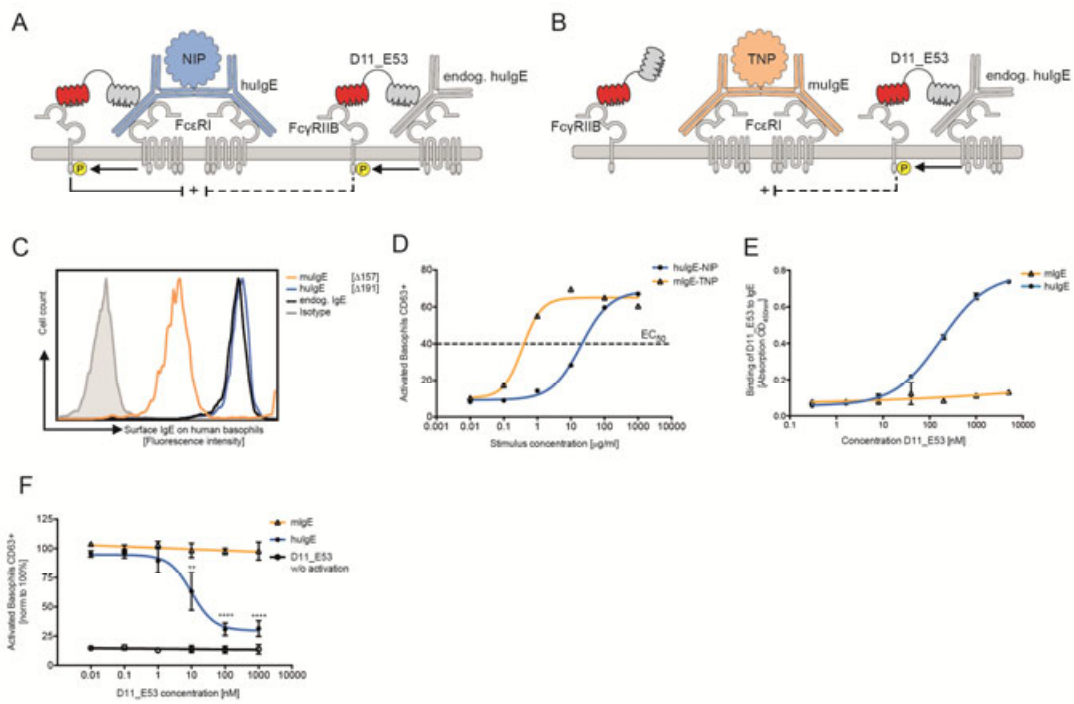


Figure 5





## Figure Legends

### *Figure 1*

DARPin protein D11 recognizes huFcγRII and blocks IgG binding to the receptor. **A** SPR sensorgram and **B** ELISA for the binding of D11 to immobilized huFcγRIIA and huFcγRIIB. **C** Preincubation of huFcγRIIA and huFcγRIIB with increasing molar concentration of D11 blocks the binding of the receptor to immobilized IgG. Epitope blocking was determined by SPR. **D** Binding of D11 to isolated peripheral blood basophils was determined by flow cytometry measurement. D11 binds to surface receptor in a dose-dependent manner.

### *Figure 2*

Receptor specificity and binding of divalent DARPin binders D11\_E53 and D11\_D11 to human IgG receptors. **A** SPR sensorgram of bispecific anti-FcγRII/anti-IgE DARPin D11\_E53 to immobilized huFcγRIIA, huFcγRIIB and huIgE. **B** D11\_E53 specifically binds to huFcγRII and shows no cross-reactivity to human high- and medium-affinity receptor FcγRI, FcγRIIA respectively. **C** SPR sensorgram of bivalent D11\_D11 to FcγRIIA/B.

### *Figure 3*

Inhibition of allergen induced basophil activation by D11\_E53. Isolated basophils of allergic donors were incubated with increasing concentration of D11\_D11, omalizumab or D11\_E53 prior to allergen stimulation. Allergen was titrated for each donor to determine half-maximal effective allergen concentration ( $EC_{50}$ ). **A** Flow cytometric gating strategy for inhibition of basophil activation at 100 nM inhibitor concentration. **B** Dose-dependent inhibition of basophil degranulation. Results are expressed as percentage of CD63<sup>+</sup> basophils relative to allergen alone (100%). **C** Dose-dependent inhibition of basophil degranulation. Results are expressed as percentage of LTC<sub>4</sub> release relative to allergen alone (100%). **D** Basophils activation test using whole blood samples of three different donors. Half-maximal effective activation was determined and stimulus alone was set to 100%. **D** Different concentrations of D11\_E53 were incubated for 5, 15, 60 and 180 min previous to stimulation. Values represent means  $\pm$  SEM (n=3).

#### Figure 4

D11\_E53-mediated phosphorylation of FcγRIIB inhibits basophil activation signaling downstream of FcεRI. **A** Western blot shows one representative of three independent experiments. Phosphorylation of FcγRIIB increases dose-dependently upon receptor ligation. Bars represent mean values  $\pm$  SEM (n=3) of FcγRIIB phosphorylation according to treatment concentration of D11\_E53. **B** Kinase blocking experiments were performed by pre-incubation of basophils with kinase inhibitor previous to D11\_E53-mediated receptor ligation. Western blot represents one of two independent experiments. Trans-phosphorylation of FcγRIIB is primarily induced by kinase Lyn. **C** D11\_E53-mediated receptor ligation induces phosphorylation of FcγRIIB independent of activation stimulus. Bars represent mean values  $\pm$  SEM (n=3-4). Western blot shows one representative of four independent experiments. **D** Western blot shows the relative phosphorylation of FcγRIIB, SHIP, Syk and Akt upon receptor ligation of stimulated basophils in the presence and absence of the divalent DARPin constructs. Graph bars summarize the result of four independent experiments. Phosphorylation of FcγRIIB by D11\_E53 in activated basophils initiates recruitment and phosphorylation of phosphatase SHIP. Recruited SHIP dephosphorylates FcεRI-associated kinase Syk and PI(3)K downstream activation pathway Akt. Western blot shows one representative of four independent experiments. Bars represent mean values  $\pm$  SEM (n=3-4).

#### Figure 5

FcγRIIB requires ligation to the activating FcεRI receptor to regulate basophil degranulation. **A** Human peripheral blood basophils were sensitized with NIP-specific human IgE previous to treatment with D11\_E53 and stimulation. D11\_E53 treatment results in ligation of FcεRI:FcγRIIB, which is involved in activation (blue) and FcεRI:FcγRIIB, which only phosphorylates FcγRIIB **B** Basophils were sensitized with murine TNP-specific IgE previous to treatment with D11\_E53 and stimulation. D11\_E53 does not recognize murine IgE (orange) and therefore only ligates to FcεRI, that is bound to endogenous IgE, which is not involved in cell activation. **C** Isolated peripheral human blood basophils were sensitized with human JW8 IgE against NIP ( $\Delta$ MF1 191) or mouse MEA-36-IgE against TNP ( $\Delta$ MF1 157). One representative of three independent experiments is shown to demonstrate similar amount of IgE-binding to free surface FcεRI. **D** IgE-specific antigen was titrated for each donor to determine half-maximal effective allergen concentration ( $EC_{50}$ ) for basophil activation. **E** Binding of D11\_E53 to human and mouse IgE was determined on ELISA. DARPin D11\_E53 binds to human IgE and does not recognize mouse IgE. **F** FcγRIIB only inhibits basophil activation when directly ligated to the activating FcεRI. Sensitized basophils were treated with increasing concentration of D11\_E53 previous to specific antigen-challenge at  $EC_{50}$ . Results are expressed as percentage of CD63<sup>+</sup> basophils relative to allergen alone (100%). Values represent means  $\pm$  SEM (n=3).

**Figure 6**

DARPin protein D11 recognizes mFcγRIIB and inhibits *in vitro* and *in vivo* IgE-induced degranulation of allergic effector cells. **A** SPR sensorgram of monovalent and divalent DARPin formats to immobilized mFcγRIIB. **B** Human IgE-sensitized femoral mBMMC from huFcεRIα-transgenic mice were stained with the DARPin formats to demonstrate binding of the constructs to cell surface expressed mFcγRIIB. **C** D11\_E53 inhibits degranulation of mBMMC's. mBMMC's from huFcεRIα-transgenic mice were o/n sensitized with human JW8-IgE and washed previous to DARPin treatment and following antigen-challenge. Results show duplicates of one representative of four independent experiments. **D** BMMC's were treated with increasing concentration of D11\_E53 previous to maximal stimulation. The bispecific DARPin construct inhibits BMMC degranulation in a concentration-dependent manner. **E** huFcεRIα-transgenic mice were sensitized o/n with human JW8-IgE. Sensitized mice were treated with D11\_E53, 6 hours before challenge with NIP-antigen. Body core temperature was determined every 10 min and expressed as Δcore body temperature [°C] (n=3).