

A novel IgE-neutralizing antibody for the treatment of severe uncontrolled asthma

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Abbreviations: IgE, Immunoglobulin E; IgG, Immunoglobulin G; mAb, monoclonal antibody; CDR, complementarity-determining region; Fab, antibody binding fragment; Fc, Fragment crystallizable; IC₅₀, half maximal inhibitory concentration; IC₉₀, 90% inhibitory concentration; K_d, equilibrium dissociation constant; scFv, single chain variable fragment; VH, variable heavy; VL, variable light; HC, heavy chain; LC, light chain; ICS, inhaled corticosteroids; LABA, Long acting β 2 agonists

The critical role played by IgE in allergic asthma is well-documented and clinically precedented, but some patients in whom IgE neutralization may still offer clinical benefit are excluded from treatment with the existing anti-IgE therapy, omalizumab, due to high total IgE levels or body mass. In this study, we sought to generate a novel high affinity anti-IgE antibody (MEDI4212) with potential to treat a broad severe asthma patient population. Analysis of body mass, total and allergen-specific IgE levels in a cohort of severe asthmatics was used to support the rationale for development of a high affinity IgE-targeted antibody therapeutic. Phage display technology was used to generate a human IgG1 lead antibody, MEDI4212, which was characterized in vitro using binding, signaling and functional assay systems. Protein crystallography was used to determine the details of the interaction between MEDI4212 and IgE. MEDI4212 bound human IgE with an affinity of 1.95 pM and was shown to target critical residues in the IgE C ϵ 3 domain critical for interaction with Fc ϵ RI. MEDI4212 potently inhibited responses through Fc ϵ RI and also prevented the binding of IgE to CD23. When used ex vivo at identical concentration, MEDI4212 depleted free-IgE from human sera to levels ~1 log lower than omalizumab. Our results thus indicate that MEDI4212 is a novel, high affinity antibody that binds specifically to IgE and prevents IgE binding to its receptors. MEDI4212 effectively depleted free-IgE from human sera ex vivo to a level (1 IU/mL) anticipated to provide optimal IgE suppression in severe asthma patients.

Introduction

Asthma is a chronic disorder characterized by airway inflammation, airways hyperresponsiveness and variable, reversible airway obstruction. Most patients have mild-to-moderate disease usually controlled by regular use of combined inhaled corticosteroids (ICS) and long-acting beta2 agonists (LABA) supplemented with short-acting beta2 agonists for symptomatic relief. However, asthma continues to be poorly controlled in a small subset of patients (~5%), who exhibit persistent symptoms, air-flow obstruction or frequent exacerbations despite aggressive

treatment including oral corticosteroids.¹ This has considerable effects on quality of life, disproportionate use of healthcare resources and adverse effects from regular systemic steroid use. Therefore, there is a substantial medical need for improved treatments in the poorly-controlled asthma patient population, including new biological agents.

The critical role played by IgE in type I hypersensitivity (allergic) responses is well documented² and beneficial effects of targeting the IgE pathway in asthma are clinically validated.³ Upon release from B lymphocytes IgE binds, via its Fc domain, to the high-affinity IgE receptor (Fc ϵ RI) present on mast cells

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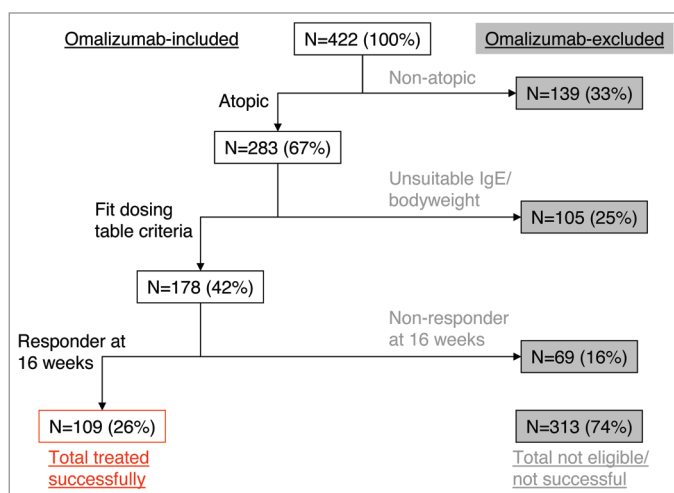


Figure 1. Hypothetical successful treatment with omalizumab in a severe asthma cohort. 424 severe asthma patients were stratified for hypothetical eligibility for omalizumab treatments based on atopic status [at least one allergen-specific IgE result ≥ 0.35 kU/L from ALT, ALTERNATA, CAT DANDER, COCKROACH,, D. FARINAE, D. PTERONYSSINUS, DOG DANDER and additional panel of allergens dependent on country (Table S1)], IgE/bodyweight eligibility (based on US dosing table; Table S2) and responder status after 16 wk by physician's global assessment (based on responder rate of 61% in Bousquet et al. 2007¹²).

and basophils. Cross-linking of receptor-bound IgE by allergen triggers cell activation and degranulation, resulting in release of histamine and other mediators of the allergic response. The role of the low affinity receptor, CD23, is complex, but consequences of CD23 interaction with IgE include regulation of IgE synthesis, allergen presentation,⁴ allergen transport^{5,6} and cell-mediated effector functions.⁷

Positive clinical experience with the IgE-specific antibody omalizumab (Xolair®, Genentech) in treatment of uncontrolled allergic asthma demonstrates the utility of targeting IgE. Omalizumab rapidly lowers serum free-IgE, preventing IgE binding to FcεRI and CD23. Omalizumab is indicated for adults and adolescents (≥ 12 y) with moderate-to-severe persistent asthma (US) or severe, persistent allergic asthma (EU) with positive skin test or in vitro reactivity to a perennial aeroallergen and symptoms inadequately controlled with ICS/LABA. Treatment of patients with omalizumab delivers both reduced exacerbations and reduced steroid requirements.^{8–10} In the EU, omalizumab is also approved for treating pediatric patients (6–12 y) with severe, persistent allergic asthma. In all patients, use of omalizumab is restricted by a complex dosing table incorporating baseline IgE and body mass.¹¹ In addition, the reported non-responder rate in eligible patients is 39%.¹² Thus, there remains need for optimized anti-IgE treatments that may overcome these disadvantages.

Here, we provide analysis of total and allergen-specific IgE levels in a severe asthma population to support rationale for a high affinity anti-IgE. We define the proportion of patients ineligible for omalizumab treatment, as well as possible causes of the sizable omalizumab-non-responding population. We also describe the generation and preclinical development of a novel high affinity anti-IgE antibody, MEDI4212, which has the potential to

overcome these challenges and provide options for treatment of individuals with high body mass or high total/specific IgE.

Results

Analysis of omalizumab eligibility in severe asthma patients

To define the need for alternative anti-IgE therapies, investigation was performed in a cohort ($n = 422$) of severe asthmatics, i.e., the target patient population for a therapeutic anti-IgE molecule (Fig. 1), of which 67% ($n = 283$) were determined as atopic (Table S1). After taking into account additional exclusions based on IgE/bodyweight (US dosing table; Table S2) and non-response after 16 wk,¹² only 39% (26% of the entire severe asthmatic population) were potentially eligible to benefit from long-term omalizumab treatment. Thus, in the severe atopic asthmatic population, there appears to be substantial unmet need for therapies that can target a broader IgE/bodyweight range and improve efficacy.

Antibody generation

Human IgE presented to a large phage library displaying human single-chain variable fragment (scFv)^{13,14} resulted in isolation of ENG085, an antibody specific for IgE that did not bind IgA, IgM, IgD or IgG. ENG085 demonstrated concentration-dependent inhibition of IgE binding to FcεRI (Fig. 2A). The potency of this scFv in neutralizing human IgE bioactivity mediated through FcεRI was barely detectable, but, upon conversion to a human IgG molecule, a mean IC_{50} of 91 nM ($n = 3$) in an FcεRI-dependent calcium-signaling assay (Fig. 2B) was achieved. To improve affinity and generate a potential drug candidate, targeted mutagenesis of V_H and V_L CDR3 loops of the scFv was performed. This led to identification of a V_H CDR3 able to pair with a variety of V_L CDR3 sequences and achieve potent neutralization of FcεRI-mediated calcium signaling. The parent scFv clone, ENG085, was aligned to known human V gene germline sequences, with VH1-f₁ (DP-3) and VL1-e₁ (DPL8) being the closest match. Of 12 non-Vernier¹⁵ amino acid differences, 7 were reverted back to germline without detrimental effect, while 5 that could not be reverted without significant loss of activity were left unchanged. The CDR3 loops were replaced with the affinity optimized V_H and V_L CDR3s to generate the final lead human IgG1, MEDI4212.

MEDI4212 inhibits IgE-mediated responses through FcεRI and CD23

MEDI4212 was characterized in a panel of in vitro assays reflecting the proposed mechanism of action. Omalizumab, an anti-IgE antibody approved for clinical use, was included as a comparator.

First, binding studies indicated an overall K_D value for the MEDI4212-human IgE interaction of 1.95 pM, indicating 106-fold higher affinity than omalizumab (Table 1).

Second, the ability of anti-IgE antibodies to neutralize human IgE bioactivity mediated through FcεRI was assessed. MEDI4212 inhibited human IgE-mediated calcium signaling in RBL-ER51 cells (Fig. 2B) and LAD2 degranulation (Fig. 2C) with IC_{50} s of 84 pM and 20 pM, respectively, indicating ~1000-fold improved potency over parent antibody ENG085 and 30- to 100-fold higher potency than omalizumab (Table 2).

Finally, the ability of MEDI4212 to inhibit IgE interaction with CD23 was also investigated. In a flow cytometry-based cell

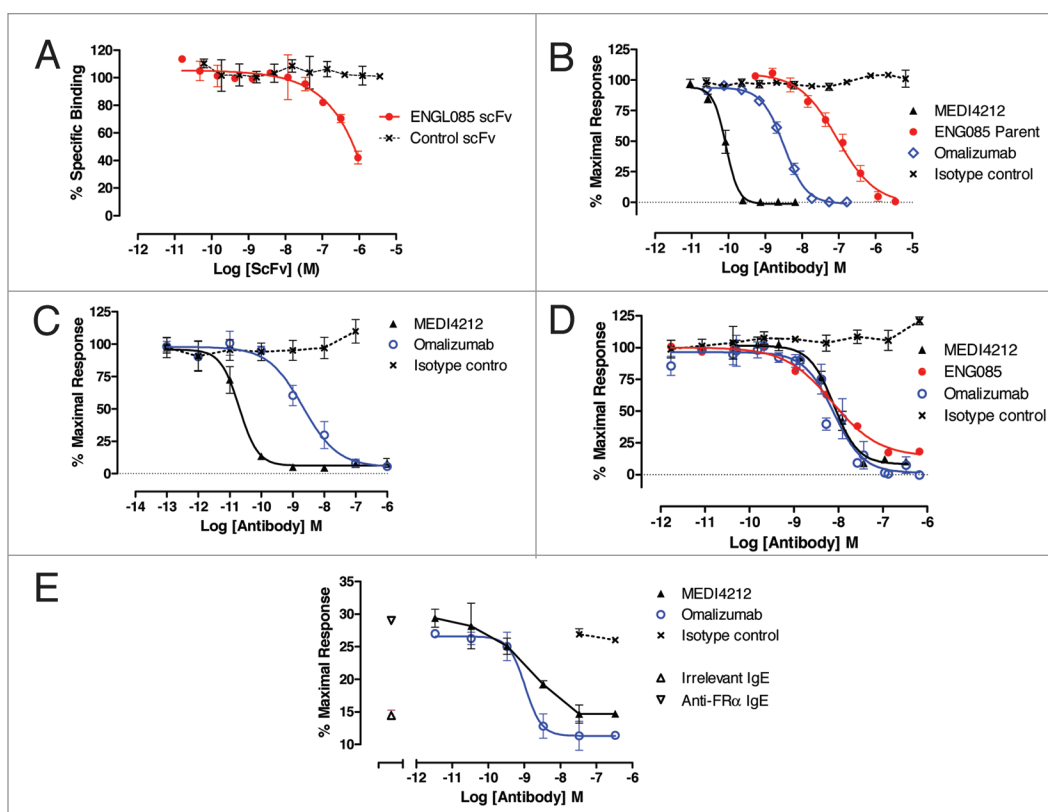


Figure 2. In vitro characterization of MEDI4212. Neutralization of human IgE was assessed in FcεRI-dependent (**A, B and C**) or CD23-dependent (**D and E**) assays. (**A**) Human IgE-FcεRI binding assay; (**B**) RBL-ER51 calcium signaling; (**C**) LAD2 β-hexosaminidase release; (**D**) IM9 binding; (**E**) U937 phagocytosis of IGROV1 cells. Folate receptor α (FRα). Data points represent average of duplicate determinations from a representative experiment (**A**) or mean ± SEM of combined data from 3–11 experiments (**B–E**). All test compounds are IgG unless stated otherwise.

Table 1. Equilibrium dissociation constant (K_D) measured by KinExA

Molecule	K_D (95% CI) pM		
	MEDI4212 Fab	MEDI4212	Omalizumab
Human IgE	26 (9–60)	1.95 (1.5–2.5)	226 (163–312)

CI, confidence interval; IgE, immunoglobulin E; K_D , initial equilibrium dissociation constant

binding assay using IM9 B-lymphoblast cells expressing CD23 but not FcεRI on the cell surface, MEDI4212 inhibited IgE binding (IC_{50} 8 nM; **Fig. 2D**). To measure functional responses through CD23, a cell-mediated killing assay was used.¹⁶ IL-4 pre-treated monocytic cells (U937) were shown to mediate killing of anti-folate receptor α (FRα) IgE antibody-labeled IGROV1 target cells (expressing FRα) by CD23-mediated phagocytosis. MEDI4212 reduced phagocytosis (IC_{50} 1.3 nM; **Fig. 2E**). These studies confirmed that MEDI4212 reduced IgE responses through CD23. In contrast to FcεRI-dependent assays, MEDI4212 demonstrated comparable potency to parent antibody ENG085 and omalizumab in blocking IgE binding and responses through CD23 (**Table 2**).

MEDI4212 does not trigger receptor-bound IgE

To avoid induction of inflammation, it was critical that MEDI4212 did not cross-link and activate IgE already bound to its receptors. To test this concept, we examined several different experimental systems that measured IgE-mediated activation of cells. The positive control anti-IgE polyclonal antibody,

but not MEDI4212, triggered calcium signaling in IgE-primed RBL-ER51 cells (**Fig. 3A**) and histamine release in human whole blood (**Fig. 3B**). These studies indicate that MEDI4212 does not cross-link or activate IgE bound to FcεRI. To determine if MEDI4212 could bind (and would therefore have potential to cross-link) IgE already bound to CD23, FITC-labeled test antibodies were assessed for binding to IgE-loaded RPMI-8866 B-lymphoblast cells by flow cytometry. MEDI4212 showed minimal binding to IgE-loaded cells relative to the commercially-available positive control (**Fig. 3C**).

Structural characterization of human IgE bound by MEDI4212

Protein crystallography was used to define the interaction between MEDI4212 and IgE. Because binding assays confirmed that MEDI4212 bound to the Cε3-Cε4 domain of the IgE Fc region with similar affinity to full-length protein, this domain was used to form a complex with MEDI4212 Fab and crystallized. The overall structure showed that one Cε3-Cε4 bound

Table 2. MEDI4212 Inhibits IgE-mediated functional responses

Assay	IgE concentration (MW = 180kDa)	IC ₅₀ (95% CI) ¹	
		MEDI4212 (pM)	Omalizumab (pM)
FcεRI-mediated calcium signaling in RBL-ER51 cells	139 pM (10 IU/mL)	84 (73–96) (n = 11)	3254 (2764–3830) (n = 11)
Beta-hexosaminidase release from LAD2 mast cell line	150 pM (11 IU/mL)	20 (12–35) (n = 4)	2116 (981–4564) (n = 4)
IM9 (CD23) cell binding assay	56 nM (4167 IU/mL)	7893 (6075–10260) (n = 3)	7814 (5206–11730) (n = 5)
CD23-mediated phagocytosis assay	28 nM (2083 IU/mL)	1337 (192–9316) (n = 3)	1044 (300–3627) (n = 3)

CI, confidence interval; FcεRI, high affinity receptor for IgE; IC₅₀, half maximal inhibitory concentration; IgE, immunoglobulin E; MW, molecular weight; ¹IC₅₀ fit of combined data from separate experiments.

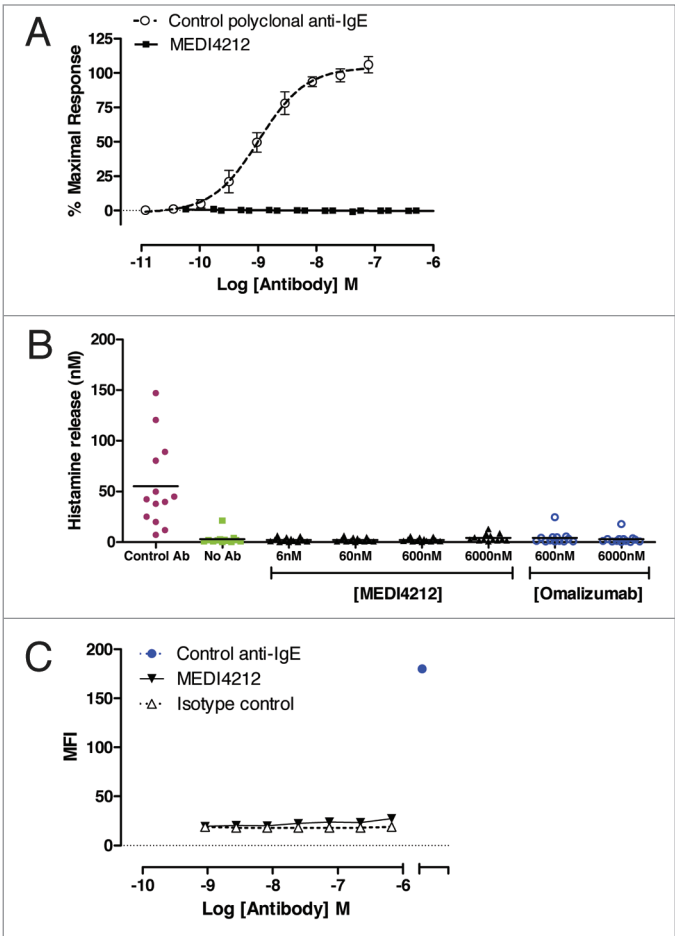


Figure 3. MEDI4212 does not trigger receptor-bound IgE. Cross-linking of FcεRI-bound IgE by MEDI4212, in comparison with a commercially available polyclonal anti-IgE, was measured by (A) calcium signaling in RBL-ER51 cells (B) human whole blood histamine release assay. (C) Binding to the IgE-loaded CD23 on RPMI-8866 cells was determined for test antibodies in comparison to a commercially available positive control anti-IgE. Data points represent mean ± SEM of combined data from 4–13 experiments (A and B) or average of duplicate determinations from a representative experiment (C).

two Fab molecules in close to symmetric fashion, and that each Fab engaged both IgE chains in binding (Fig. 4A). The Cε3–Cε4

domain adopted an open conformation, similar to that capable of binding FcεRI.^{17,18}

The structural epitope of IgE Cε3–Cε4 recognized by MEDI4212 was made up by amino acid residues Leu221, Arg223, Glu271 through to Asn275 inclusive, Ala309 to Thr315 inclusive, Thr317, Ser318 in Cε3, and Glu353 in Cε4. The sugar moieties GlcNAc1 and Man6 attached via Asn275 were in contact with the MEDI4212 heavy chain (data not shown). The total interaction area was 1150 Å². Interestingly a large fraction, 57%, of the buried paratope surface constituted non-CDR residues (Fig. 4B). It was clear that antibody framework residues contributed significantly to binding as full germlining of the V_H resulted in loss of activity. Figure 4C shows how Ser75 and Arg77, two residues that could not be germlined without affecting potency, directly contributed to binding of IgE. It was also apparent that the V_L CDR3 did not contribute to direct interaction with IgE, which may explain the ability of MEDI4212 V_H to pair with multiple V_L CDR3s without significant effects on potency.

Comparison of the MEDI4212 epitope with the FcεRI binding site on IgE¹⁷ indicated overlap within Cε3 at residues Arg274 and Asn275, suggesting MEDI4212 directly competes with FcεRI for IgE binding. In addition, when overlaying the two complex structures, there is clear spatial overlap between the MEDI4212 Fab and FcεRI (Fig. 4D).

In contrast, comparison with the recently published structure of the CD23:IgE Cε3–Cε4 complex¹⁹ revealed that, although MEDI4212 binds in close proximity to the CD23 binding site on IgE, it does not directly overlap this site. Instead, MEDI4212 prevents binding of CD23 by locking IgE in an open conformation incompatible with CD23 binding (Fig. 4E). In addition, a minor steric clash between MEDI4212 and CD23 was identified (not shown).

Theoretical prediction of IgE suppression in humans

Having shown improved affinity and in vitro potency for MEDI4212 over omalizumab, it was important to understand how this might translate to potential benefits in the clinic.

It is reported that omalizumab may have altered affinity and form different antibody-antigen complexes in serum compared with PBS.²⁰ Thus, it was critical to confirm the high potency of MEDI4212 within this context. To investigate the ability of MEDI4212 to deplete free-IgE in human serum, we used an ex

vivo IgE suppression assay with IgE levels spanning a broad range (25–1339 IU/mL). The omalizumab target free-IgE concentration (10 IU/mL, the level shown to relate to clinical efficacy²¹) was achieved with only 2–3-fold molar excess of MEDI4212, whereas omalizumab required 100-fold excess (Fig. 5A). Alternatively, for the same concentration of MEDI4212 as omalizumab, free-IgE levels could be suppressed ~10-fold lower. These data support concepts of greater coverage of high IgE patients and greater suppression of free-IgE with MEDI4212 compared with omalizumab.

A mechanistic pharmacokinetics/pharmacodynamics (PK/PD) model (Fig. 5B) was then constructed and translational simulations performed to predict the IgE response in asthma patients following anti-IgE treatment. Simulated serum free-IgE levels following repeated omalizumab and MEDI4212 dosing in a typical asthma patient (61 kg, 200 IU/mL baseline IgE) are shown in Figure 5C. The improvement in binding affinity is anticipated to translate to more substantial free-IgE suppression by MEDI4212.

Further simulations were performed to evaluate potential use of MEDI4212 in asthma patients ineligible for omalizumab treatment. MEDI4212 is predicted to suppress free serum IgE to the 10 IU/mL target level even in patients with extremely high baseline IgE (1500 IU/mL) and high body weight (150 kg) (Fig. 5D), as well as eliminate antigen-specific IgE on basophils in patients with low baseline IgE (13.5 IU/mL) but high allergen specificity (12.5%) (Fig. 5E). The target level (200 specific IgE per basophil) corresponded to IC₉₀ value determined from in vitro histamine release experiments.²²

Analysis of allergen-specific IgE in severe asthma patients

As in vitro and in silico data showed the capacity for MEDI4212 to suppress free-IgE considerably lower than omalizumab, we investigated the potential value of this feature in severe allergic asthma patients. Serum levels of specific IgE against a panel of perennial allergens were measured (Fig. 6 and Table S1). Atopic status was determined by a test result ≥ 0.35 IU/mL of any single allergen-specific IgE and hypothetical suppression of total and specific IgE was investigated. At the omalizumab-defined target free-IgE level of 10 IU/mL,²¹ individuals with > 3.5% specific IgE did not fall below the 0.35kU/L threshold, resulting in a high proportion of patients (57%; $n = 160/283$) that would still test positive for atopic status. Omalizumab has a reported non-responder rate of 39%,¹² surprisingly close to the 57% with > 3.5% specific IgE in this cohort. We therefore speculate that a significant proportion of omalizumab non-responders may be due to insufficient IgE suppression.

In contrast, a free-IgE level of 1 IU/mL captured > 99% of patients in our cohort below the allergen-specific IgE atopy threshold, suggesting this is the optimal ‘target’ free-IgE in a severe asthma population. MEDI4212 reduced serum free-IgE levels 10-fold lower than omalizumab (Fig. 5A), reaching the new 1 IU/mL target. We propose that by fully suppressing IgE, MEDI4212 may provide improved efficacy compared with omalizumab in up to 55% of severe allergic asthma patients.

Discussion

IgE is a critical mediator of allergic responses.² Powerful actions through FcεRI, triggering degranulation and release

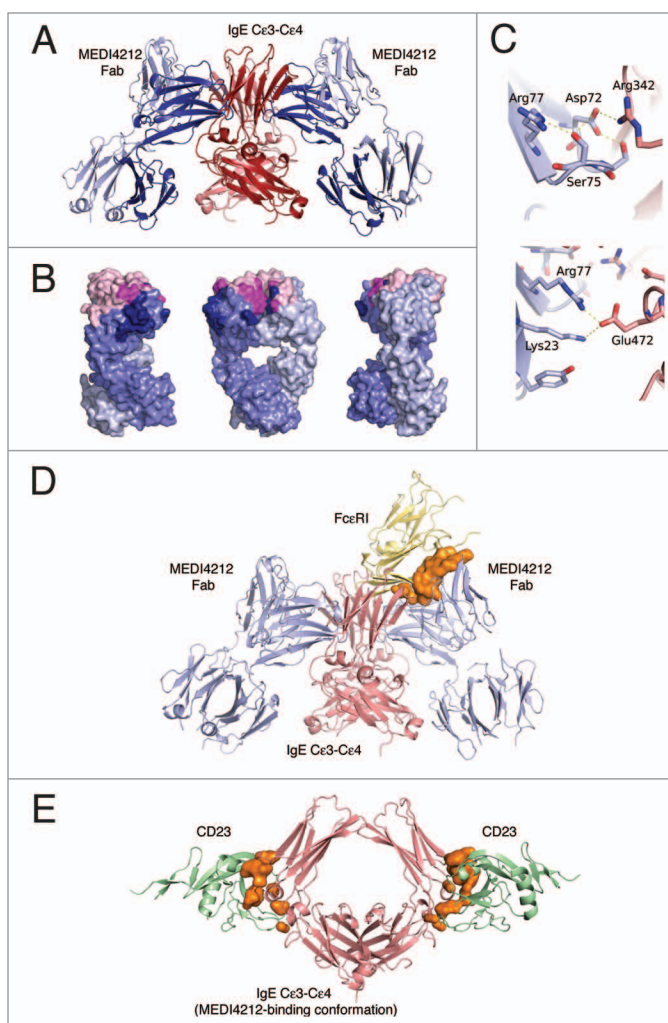


Figure 4. Structural definition of MEDI4212-IgE interaction. (A) Overview of the MEDI4212 Fab and IgE Cε3-Cε4 complex. The two identical IgE chains are colored dark red and salmon and the MEDI4212 Fab domains blue, with the heavy chain dark blue and the light chain light blue. (B) A surface representation of MEDI4212 Fab illustrating the large fraction of framework residues in the paratope for IgE. MEDI4212 colored as previously, CDR- not engaged in IgE binding light pink, CDR engaged in binding magenta and framework/Vernier residues engaged in IgE binding in dark blue. (C) Framework residues in MEDI4212 (blue) involved in binding IgE (light red). Arg77 (right panel) forms a salt bridge directly with Glu472 in IgE. Ser75 (left panel) is involved in a stabilizing H-bonding network at the interface to IgE, positioning Asp72 in position to form a salt bridge with Arg342 of IgE. (D) Mode of action of MEDI4212 with respect to FcεRI binding. Overlay of the MEDI4212:IgE complex with the structure of FcεRI:IgE shows a direct overlap of the binding site of MEDI4212 and FcεRI. In addition a large volume, illustrated in orange, describes the steric clash between the two molecules trying to bind simultaneously. (E) Mode of action of MEDI4212 with respect to CD23. Overlay of the CD23:IgE complex structure¹⁹ with the MEDI4212:IgE structure shows how the open conformation which IgE adopts in complex with MEDI4212 is incompatible with CD23 binding. The clash volume between CD23 and IgE is illustrated as an orange surface. For clarity, MEDI4212 and IgE of the CD23:IgE complex have been omitted in the picture.

of inflammatory mediators from mast cells and basophils, are well defined. Furthermore, clinical experience with IgE-specific

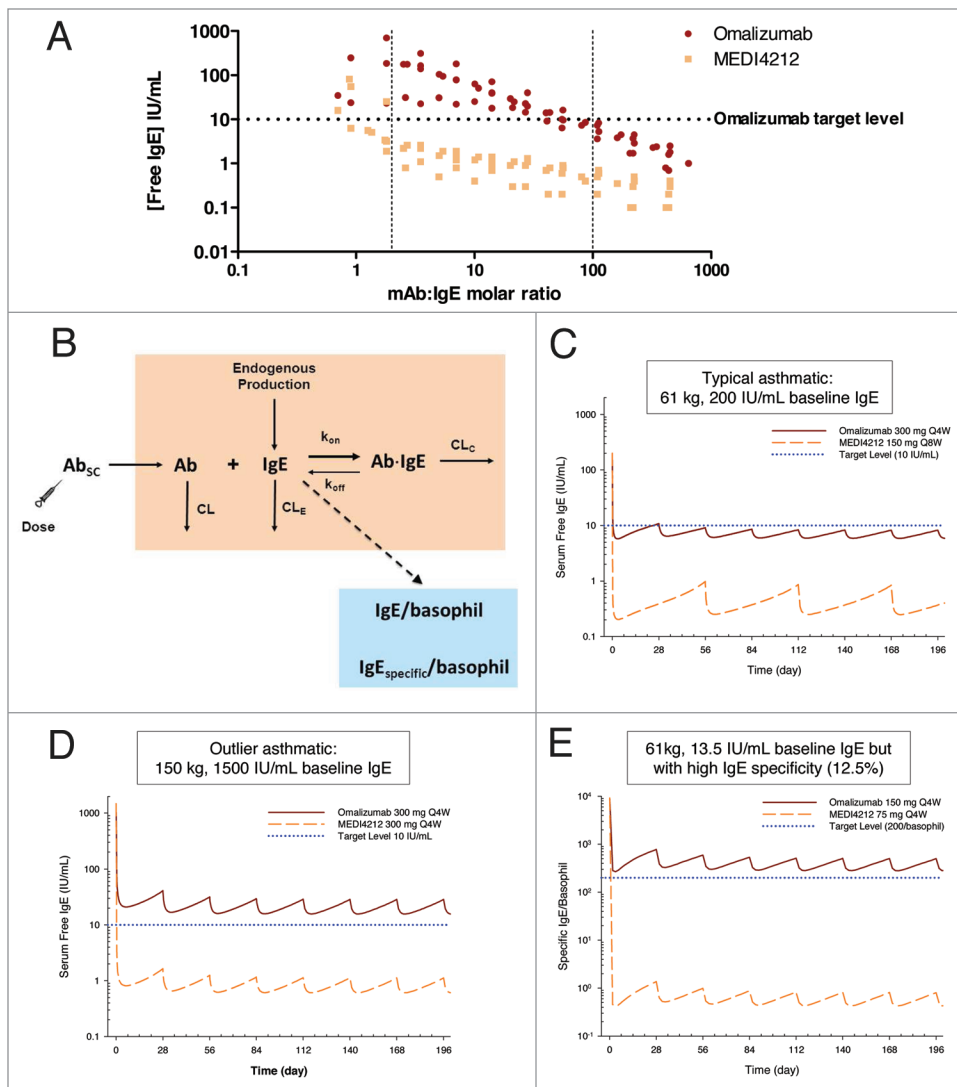


Figure 5. IgE suppression in humans. (A) Suppression of free-IgE in ex vivo human sera from 7 donors with baseline IgE levels 25–1339 IU/mL. Data from each donor at each antibody concentration are plotted as separate data points. (B) Mechanistic PK/PD model. Simulated serum free IgE or specific IgE per basophil profiles following administrations of MEDI4212 and omalizumab in (C) typical asthma patient; (D) High IgE / bodyweight; (E) Low total IgE with high IgE specificity.

antibody omalizumab demonstrates the benefits of suppressing IgE in treatment of atopic asthma.^{8–10} However, the modest affinity of omalizumab for IgE prevents its use in patients with high IgE levels.¹¹ Even within the eligible population, there is evidence that IgE responses may not be completely eliminated in many patients.^{23,24} Published studies of total and specific IgE levels in a severe asthma population to investigate these issues are few.²⁵ Here, we include such an analysis, which defines numbers of severe asthma patients ineligible for treatment with omalizumab and may also shed light on the sizable non-responding population. In addition, we describe a high affinity anti-IgE antibody, MEDI4212, with potential to enable treatment of individuals with higher body mass or total/specific serum IgE than currently possible with omalizumab.

was shown by crystallography studies to target critical residues in the IgE Cε3 domain essential for interaction with FcεRI.^{17,28} Consistent with this, MEDI4212 is more efficient at inhibiting IgE binding to, and subsequent activation of, FcεRI than omalizumab.

Omalizumab is licensed for use in atopic asthma, which in our cohort accounts for ~two-thirds of severe asthmatics. Analysis of this population shows that 38% of allergic asthma patients are ineligible for omalizumab treatment due to low IgE < 30 IU/mL (3.3%) or high IgE / bodyweight (34.3%). Thus, a significant potential for expansion of the patient population exists beyond those served by omalizumab. In silico translational simulations using a mechanistic PK/PD model suggested that MEDI4212, based on free-IgE levels reported to correlate with efficacy,²¹ could achieve adequate suppression in individuals with high (700–1500 IU/mL) baseline IgE. In support of these assumptions, ex vivo

MEDI4212 is a human IgG1λ monoclonal antibody generated by phage-display technology that selectively binds to the Cε3-Cε4 domain of human IgE. The initial lead antibody, ENG085, isolated from a naïve antibody library had relatively low starting potency (91 nM). A combinatorial mutagenesis strategy taking advantage of CDR loop synergy²⁶ was successfully employed and improved affinity of the final IgG by > 45,000 fold over the starting parent antibody. Investigation of the paratope through protein crystallography suggested this gain was achieved through optimization of V_H CDR3 alone. Interestingly, besides V_H CDR3, the MEDI4212 V_H framework regions also appeared to play a significant role in the interaction with IgE, explaining why attempts to revert certain framework residues to germline significantly affected activity. Framework regions are commonly present in antigen recognition to a variable degree and can comprise up to 15% of the buried surface area of an antibody-antigen complex.²⁷ The presence of 57% buried surface area of non-CDR residues in the MEDI4212-IgE interface represents a novel observation and suggests that mutagenesis of these non-CDR regions could contribute to further affinity improvements for this antibody.

MEDI4212 selectively binds human IgE with affinity of 1.95 pM, more than 100-fold higher than the affinity of omalizumab for IgE, and

treatment of human sera with MEDI4212 resulted in potent suppression of endogenous IgE levels at concentrations of MEDI4212 50-fold lower than omalizumab.

In addition to expanding the patient population beyond the current IgE/bodyweight limits of omalizumab, a crucial question is whether there is potential for better efficacy with MEDI4212. Patients in our severe asthma cohort with < 76 IU/mL serum IgE (corresponding to a subgroup with lower omalizumab efficacy described by Bousquet et al.¹²) had higher proportions of allergen specific IgE (67%, $n = 29/43$) with > 3.5% specific IgE for at least 1 allergen compared with 55% ($n = 131/240$) in the subgroup with serum IgE ≥ 76 IU/mL. As our study as well as others^{24,29-31} suggest the inability of omalizumab recommended doses to fully suppress IgE activity in patients with > 3–4% of specific IgE to a single allergen, these data highlight the possibility that reduced efficacy with omalizumab in this case may be due to incomplete target suppression. With adequate IgE suppression, there may also be no need to exclude atopic patients with low total IgE (< 30 IU/mL) that often have a high fraction of specific IgE²⁹⁻³¹ (87.5%, $n = 7/8$ in our cohort).

The ability to treat low baseline IgE (greater probability of high specific IgE) patients may be even more important if potential treatment of non-atopic asthmatics is considered. Here a much higher proportion (51%) have low total IgE levels (< 76 IU/mL). Although omalizumab has never been fully evaluated and is not currently licensed in this patient subgroup, emerging data³²⁻³⁶ suggests that IgE may play a pathogenic role.

Most importantly, analysis of total and specific IgE in severe asthmatics suggests that at 5–10 IU/mL of free serum IgE, the lowest levels reached in omalizumab clinical studies,³⁷ 38–57% of patients would not have their free allergen-specific IgE suppressed below the ‘atopy’ threshold of 0.35 IU/mL for every allergen. It is therefore interesting to note that reported non-responder rates following omalizumab treatment are 30–39%,^{12,38} a very similar number. There is also a spectrum of efficacy level reported³⁸ and perhaps some patients classified as responders might not have achieved maximal response. Our data suggest that a target free-IgE level of 1 IU/ml appears optimal to achieve maximal efficacy in severe asthma. Furthermore, in vitro and in silico analysis highlight that MEDI4212 should achieve this 1 IU/mL target. We speculate that MEDI4212 may lead to an increase in responder rate or efficacy compared with omalizumab by improving IgE suppression in the significant number of severe asthma patients with > 3–4% allergen-specific IgE.

In conclusion, we used an integrated approach to drug discovery²⁶ that led to the generation of MEDI4212, a novel anti-IgE antibody that has been evaluated in a Phase 1 clinical study (NCT01544348). The data presented here suggest that MEDI4212 may reduce free-IgE to a level (1 IU/mL) anticipated to provide optimal IgE suppression in severe asthma patients.

Materials and Methods

Subjects

Subjects (18–75 y, body mass index 16–40 kg/m²) with physician-diagnosed asthma, asthma controller regimen consistent with

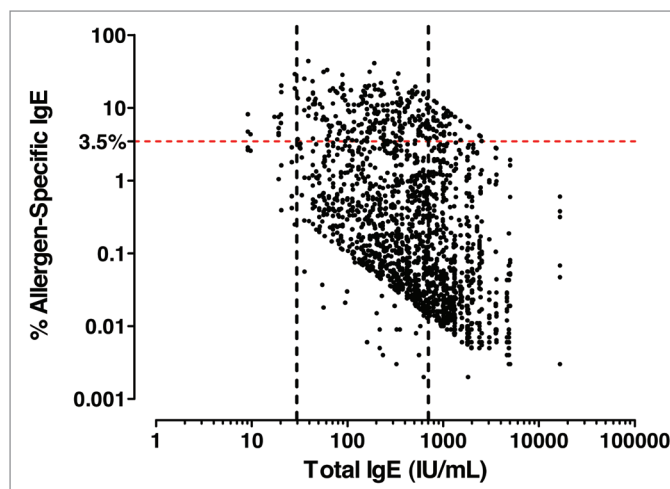


Figure 6. Allergen-specific IgE analysis in severe atopic asthma patients. Total vs. allergen-specific IgE levels in severe atopic asthma. Atopic was defined as at least one allergen-specific IgE result ≥ 0.35 kU/L from ALT. ALTERNATA, CAT DANDER, COCKROACH, D. FARINAE, D. PTERONYSSINUS, DOG DANDER and additional panel of allergens dependent on country (Table S1).

Step 4 or 5 of the Global Initiative for Asthma guidelines (GINA Report, Global Strategy for Asthma Management and Prevention, updated December 2009. Available from: www.ginasthma.org) for at least 6 mo and 2–6 documented asthma exacerbations in the past year were included in the analysis. Key exclusion criteria were additional respiratory pathology, cigarette smoking ≥ 10 pack-yrs, recent infection or treatment with immunosuppressive medication (> 10 mg oral prednisone or equivalent per day) or any biologic agent.

The entire cohort included 452 severe asthmatics. Only subjects with complete data for atopic status, total and specific IgE, and bodyweight ($n = 422$) were included in the analysis reported here. Sensitivity analysis revealed that the missing values had little effect on the results presented here and will not invalidate the results.

This study was conducted in accordance with principles of the Declaration of Helsinki and International Conference on Harmonisation Guidance for Good Clinical Practice. Independent ethics committee approval was obtained. All subjects provided written informed consent.

IgE assays

Total, allergen-specific and free-IgE levels were determined by ImmunoCAPTM (Phadia AB) according to manufacturer’s instructions.

Recombinant proteins

IgE was purified from U266 cells.³⁹ C ϵ 3–C ϵ 4 expression and purification is provided in supplementary methods. IgG⁴⁰ and scFv¹³ were expressed and purified as previously described. Omalizumab was sourced from a pharmacy, reconstituted according to the package insert and further diluted as required in PBS.

MEDI4212 generation and characterization assays

Generation of MEDI4212, affinity measurements, RBL-ER51 calcium signaling, LAD2 degranulation, IM9 and RPMI-8866

IgE binding, and basophil histamine release assays are described in supplementary methods. Data from cell-based assays were analyzed using Microsoft Excel and Graphpad Prism software. Data are expressed as geometric mean (95% confidence intervals) unless stated otherwise.

Epitope mapping

X-ray crystallography was used to visualize the MEDI4212-IgE interaction. Monomerized Fab was mixed with IgE domain Cε3-Cε4, resultant complex purified by size exclusion chromatography and crystals obtained belonging to the trigonal space group P3221. Diffraction data to 2.85Å resolution were collected at the European Synchrotron Radiation Facility. The structure was solved by Molecular Replacement.⁴¹ Details are provided in supplementary methods.

In silico translational simulations

A model was constructed to describe the PK of MEDI4212, binding of MEDI4212 to endogenous IgE and disposition of MEDI4212-IgE complex based on a study of omalizumab in asthma patients.⁴² Model parameters were adjusted to incorporate KinExA-determined affinity values for MEDI4212 and omalizumab (Table 1). MEDI4212 affinity was adjusted upward by 6.7 pM, a theoretical component of in vivo affinity associated with the elimination kinetics of IgG-IgE immunocomplex calculated as the ratio of reported elimination rate of omalizumab-IgE immunocomplex⁴² and association rate constant to IgE.⁴³ All other PK and PD parameters were assumed identical for omalizumab and MEDI4212. The model was extended to simulate amount of specific IgE on basophils, with assumptions that

MEDI4212 does not alter the fraction of antigen-specific IgE, and, at low serum IgE levels, specific IgE/basophil correlates with serum IgE.

Disclosure of Potential Conflicts of Interest

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/mabs/article/28394

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1 **Supplementary Table 1. Determination of atopic status: allergen panel by country**

	Argentina	Chile	Czech Republic	France	Germany	Japan	Mexico	Philippines	Poland	Russia	South Korea	Spain	United Kingdom	USA
ACACIA														
ALT. ALTERNATA														
ASP. FUMIGATUS														
ASPERGILLUS NIGER														
B. TROPICALIS														
BAHIA GRASS														
BERMUDA GRASS														
C. HERBARUM														
CAT DANDER														
COCKROACH														
COMMON PIGWEED														
COMMON RAGWEED														
D. FARINAE														
D. PTERONYSSINUS														
DOG DANDER														
ELM														
JAPANESE CEDAR SERUM														
JOHNSON GRASS														
MUGWORT														
OAK														
OLIVE														
ORCHARD GRASS														
PARIETARIA JUDAICA S														
PRIVET														
RUSSIAN THISTLE														
RYE GRASS														
TIMOTHY														
TREE POLLEN MIX														
WEED MIX														

2 Shading represents inclusion in testing panel

Supplementary Table 2. Atopic subjects (N=283) omalizumab eligibility using US dosing table

IgE level (kU/L)	Weight category (kg)			
	30-60	60-70	70-90	90-150
<30	2	1	5	0
30-100	11	7	18	11
>100-200	8	10	19	11
>200-300	8	8	15	3
>300-400	8	5	11	3
>400-500	7	5	1	3
>500-600	5	5	5	3
>600-700	2	2	9	1
>700-1500	16	8	16	4
>1500	9	3	14	1

* Shading indicates omalizumab ineligible

*Atopic status defined by at least one positive allergen-specific IgE result (≥ 0.35 kU/L) from allergen panel described in Supplementary table 1.

10 **Supplementary Table 3. Non-atopic subjects (N=139) by weight and IgE level**

IgE level (kU/L)	Weight category (kg)			
	30-60	60-70	70-90	90-150
<30	7	4	19	6
30-100	11	10	22	4
>100-200	2	7	14	4
>200-300	4	4	3	1
>300-400	0	0	1	0
>400-500	0	1	1	1
>500-600	0	1	2	0
>600-700	0	1	1	1
>700-1500	1	3	1	2
>1500	0	0	0	0

11 * Shading matches Table E2

12 *Non-atopic status defined by lack of positive allergen-specific IgE result (≥ 0.35
 13 kU/L) from allergen panel described in Supplementary Table 1.

14

15

Supplementary Methods

Antibody generation

Anti-IgE-specific scFv antibodies were isolated from naive phage display libraries^{E1,E2} using alternating selection cycles on human IgE-lambda (Bioscience Resource Project) and IgE-kappa (Calbiochem). Following selections, individual scFv were tested for their ability to inhibit the binding of europium-labelled human IgE-kappa binding to recombinant FcεRI in a LANCETM assay (Perkin Elmer).

Large mutant scFv phage libraries derived from an initial IgE-binding clone ENG085 were created by oligonucleotide-directed mutagenesis of the V_H and V_L CDR3^{E3}. Separate libraries were made from blocks of six fully randomised codons (NNS randomisation) comprising V_H95–100, V_H101-106, and V_L89-94 and V_L94-99 (numbered according to Kabat^{E4}) and subjected to affinity-based selections with biotinylated IgE^{E5}. Following five rounds of selection the V_H and V_L libraries were recombined^{E6} and affinity based selections continued over a further three rounds.

Over expression of IgE Cε3-Cε4.

Cell lines and culture medium.

In this work the original adherent cell line HEK293-EBNA (Invitrogen, Stockholm, Sweden) stably expressing the Epstein Barr virus Nuclear Antigen-1 gene were used. Cells were adapted to suspension growth before transferred into DHI medium by stepwise medium replacement^{E7}. The DHI medium used deviated from the original description slightly by being CA2+-free. After adaptation a working cell bank was made and both cell lines were grown routinely in CA2+-free-DHI medium supplemented with 4 mM Glutamine, 2% v/v ultra-low IgG foetal bovine serum, 250

40 $\mu\text{g/ml}$ G418 (all from Invitrogen, Stockholm, Sweden) and 0.1% w/v Pluronic F68
41 (Sigma-Aldrich, Stockholm, Sweden) to a maximum of 20 passages.

42 **Transfection procedure**

43 The 1 mg/ml stock solution of linear 25 kDa polyethylenimin (Polysciences Europe,
44 Eppenheim, Germany) was prepared in water, pH adjusted to 7.0, sterile filtered and
45 stored in small aliquots at -80°C until use. The transfection cocktail was prepared
46 shortly before transfection in non-supplemented DHI media in a volume equivalent to
47 one-tenth of the transfection volume. For preparing the transfection cocktail the DHI
48 media was divided into two halves. $0.8\mu\text{g}$ DNA per ml transfection volume was added
49 to one half of the DHI medium and into the other half $2\mu\text{g}$ PEI per ml transfection
50 volume was added. After shaking the two solutions briefly and incubating them for 5
51 min the DNA solution was slowly added to the PEI solution. The transfection cocktail
52 was incubated for 20-30 min at room temperature before addition to the Wave
53 bioreactor (Wave Biotech AG, Tagelswangen, Switzerland). Four hours post
54 transfection the culture was fed to the final production volume with supplemented
55 DHI medium and HypPep1510 (Kerry Bio-Sciences, Almere, the Netherlands) to a
56 final concentration of 0.3% (w/v).

57 **Seeding cultures**

58 For expansion of the seeding culture the cells were grown in plastic shake bottles at
59 37°C in 5% CO_2 atmosphere placed in an orbital shaker incubator (Infors AG,
60 Bottmingen, Switzerland). The cells were routinely passaged twice a week reaching
61 approximately 2×10^6 cells/ml before splitting. Cell density and viability were
62 assessed using a Cedex automatic cell counter (Innovatis AG, Bielefeld, Germany).
63 For Wave cultures the cells were split to 1×10^6 cells/ml one day before transfection to

ensure that they were in logarithmic growth phase at the start of the experiment. Wave cultures were inoculated directly from shakers. All seeding cultures were concentrated by centrifugation and resuspended in fresh culture medium before addition to the bioreactors.

Wave cultures

Expression was performed in Wave bioreactors (Wave Biotech AG, Tagelswangen, Switzerland) at a working volume of 10 L. The wave bioreactors were seeded to 1×10^6 cells/ml in 4.5 L supplemented DHI medium. After a 2 h adaptation phase the culture was transfected with 0.5 L transfection cocktail. Four hours post transfection the culture was fed to 10 L total volume with supplemented DHI medium and HyPep1510 to a final concentration of 0.3% (w/v). Samples were taken daily to determine cell density, viability and protein concentration.

Expression vector

The vector expressing the human IgE C ϵ 3-C ϵ 4, with C-terminal Flag tag and 10-histidine tag, was derived from a vector described by Persic et al.^{E8}. The system is under the control of the EF1- α promoter.

Purification of IgE C ϵ 3-C ϵ 4

20L of cell supernatant were concentrated five times and diafiltered to 2xPBS (308mM NaCl, 20mM phosphate, pH 7.4) with a 10kDa molecular weight cut-off cross-flow membrane (Pellicon 2, Millipore). The medium was batch bound with 30mL NiSepharose (GE Healthcare) for two hours at 4°C, washed with five volumes 2xPBS and packed into an XK26 column. The column was then washed with five column volumes 40mM imidazole in 2xPBS to wash away contaminating proteins. IgE was finally eluted with 400mM imidazole in 2xPBS. The pool contained IgE with

high purity and was concentrated about four times (to ~5mg/mL) before it was run over a Superdex 200 50/60 SEC-column (1200 mL, GE Healthcare) with 2xPBS used as running buffer. Some larger proteins were separated out and IgE was found in the main peak. Only the main peak fractions were pooled because of contamination in the other two fractions. This step increased the purity of the sample to ~99%. The total amount produced was 42mg and the purified IgE had a concentration of 2mg/mL.

Analysis of glycosylation of IgE Cε3-Cε4

In-solution digestion with Trypsin

Human IgE minimal domain, IgE Cε3-Cε4, 2 mg/ml, in 2 x PBS (composition 308 mM NaCl, 20 mM phosphate, pH 7.4) was mixed with 100μl trypsin 0.02 mg/ml in 25 mM NH₄HCO₃. Digestion proceeded overnight in 37°C and was stopped with 2μl formic acid (67%) in H₂O.

Nano-LC MS/MS:

Analysis was performed on a 20 cm x 50μm i.d. fused silica column packed with ReproSil-Pur C18-AQ 3μm porous particles, connected to an LTQ-Orbitrap mass spectrometer (Thermo). 8μl sample injection was made (Agilent autosampler) and the peptides were trapped on a precolumn, 4.5 cm x 100μm i.d., before separation. After 5 min linear run with 0.1% formic acid, the gradient was 10-50% acetonitril during 5-30 min (Agilent), 200nl/min, and the eluent was electrosprayed from the emitter tip. The instrument was operated in data-dependent mode to switch between Orbitrap (FT-MS) survey scan and ion trap (IT-MS/MS) of the three most abundant multiply protonated ions.

Static electrospray MS/MS:

To verify the charge state of the glycopeptide fragments, selected precursors were analyzed with ESI needle at 1.6 kV, fragmented and detected in the Orbitrap opposed to the linear ion trap in the nano-LC analysis.

Glycopeptide data analysis:

the calculated MH⁺ masses of possible glycopeptides were examined for the presence of glycosylation by use of the GlycoMod tool (<http://expasy.org/tools/glycomod>)^{E9}. The protein sequence and a mass tolerance of 10ppm was entered. All suggested glycopeptides were checked for the presence of glycan containing fragments.

Production of MEDI4212 Fab.**IgG purification**

MEDI4212 was purified from CHO-EBNA transient material using MabSelect SuRe (GE Healthcare) protein A chromatography media. The protein A eluate was buffer exchanged into PBS, pH7.2 using PD-10 columns (GE Healthcare) then 0.22µm filtered using a Millex-GP syringe tip filter (Millipore).

Fab digest and purification

A digest buffer of 30mM DL-cysteine hydrochloride dissolved in GIBCO PBS (Invitrogen) was prepared. Papain from papaya latex (Sigma) was reconstituted in digest buffer to give a 10mg/mL solution and kept at room temperature for a minimum of 30 minutes before use. Cysteine was added to MEDI4212 IgG to give a 30mM solution and papain was added at a ratio of 1mg papain to 100mg IgG. The

digest was terminated after 90 minutes by the addition of 0.5M iodoacetamide (Sigma) to give 50mM iodoacetamide in the final digest mixture. The Fab was purified from the digest mixture using MabSelect SuRe (GE Healthcare) protein A chromatography media in a non-binding mode. The Fab fraction from the MabSelect SuRe step was buffer exchanged into 50mM sodium acetate/100mM NaCl, pH5.5 using PD-10 columns (GE Healthcare) and then concentrated to ~10mg/mL using Amicon Ultra-15 5kDa MWCO centrifugal filter devices (Millipore). The final product was further purified using a Mustang Q acrodisc (Pall) and then 0.22µm filtered using a Millex-GP syringe tip filter (Millipore).

Generation of the IgE Cε3-Cε4 E85 50 Fab complex.

A solution containing 2 mg/mL of IgE Cε3-Cε4 in buffer 308 mM NaCl and 20 mM phosphate, pH 7.4, was mixed with a solution containing 10.6 mg/mL of MEDI4212 Fab in 50 mM sodium acetate, 100 mM NaCl, pH 5.5 at a stoichiometric ratio of 1 to 1.1 of IgE Cε3-Cε4 homodimer and MEDI4212 Fab heterodimer, respectively. The mix was left on ice overnight followed by gel filtration on a HiLoad Superdex200 16/60 column (GE Healthcare), equilibrated in 20 mM Tris HCl pH 7.6 and 0.15 M NaCl. The main peak containing the complex was collected and concentrated to 10.4 mg/mL before it was used in crystallization experiments.

Crystallization of the IgE Cε3-Cε4 E85 50 Fab complex.

Crystallisations were carried out according to the method of sitting drop vapour diffusion. The drops contained an equal volume of protein and reservoir solution (150+150 nL) and were set up in a Crystal Quick 96 well plate (Greiner Bio-one) with

reservoir volumes of 80 μ L. The complex crystals grew in drops with a reservoir solution of 100mM MgCl₂, 100mM sodium citrate pH 5.0 and 15% PEG 4000 over a period of 2-3 weeks at 4°C. The crystals were harvested into a cryoprotectant solution (100mM MgCl₂, 100mM sodium citrate pH 5.0 and 15% PEG 4000 made 20% glycerol by addition of 100% glycerol) and cooled rapidly in liquid nitrogen.

Data collection and Structure solution of the IgE C ϵ 3-C ϵ 4 MEDI4212 Fab complex.

Diffraction data were collected from single crystals at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France at beam line ID-29. An initial dataset (data set 1, table 3) was recorded to 3 Å resolution and later a higher resolution data set was collected to 2.85 Å resolution, both of which belong to space group P3221. The data was processed with autoPROC (Global Phasing Limited GPhL, Cambridge, UK). Statistics from the data processing is presented in table 3. The asymmetric unit contains three Fab molecules and three molecules of IgE C ϵ 3-C ϵ 4 corresponding to a solvent content of 54 %. The structure was solved by the method of molecular replacement using the program PHASER^{E10-E12}. Initial models for the Fab fragment and the IgE Fc domain was generated from the previously reported structures 1AQK^{E13} and 1FP5^{E14}.

Use of the entire Fab as the search model failed, due to variations of angles in the hinge region between the variable domain and the constant domains. Instead two separate search models consisting of the variable domains and constant domains respectively were prepared and identified in the initial run of PHASER¹⁰⁻¹². These were later joined to complete Fab fragments. In total two Fab fragments and one

variable domain was identified in this fashion. Subsequent runs of molecular replacement located three IgE C ϵ 3-C ϵ 4 molecules, of which one had to be trimmed back to comprise only domain 4. At this stage better quality data had been collected (dataset 2, table 3) and this model was refined against the new data using the program autoBUSTER (Global Phasing Limited GPhL, Cambridge, UK). Subsequently the amino acids of the Fab molecules were manually altered to the correct sequence of MEDI4212 using the graphical program COOT^{E15}. After a second round of restrained maximum-likelihood refinement using isotropic B factors refinement in Refmac (CCP4 1994) the remaining domains of the last Fab and IgE molecules were manually fitted into the electron density. Two additional features of elongated electron density was observed protruding from amino acid residue Asn394 into the cavity between the IgE molecules. This was interpreted as glycosylations and therefore two N-acetylglucosamine and three to four mannose units were added to the IgE models. Further rounds of refinement included manual rebuilding of loop regions in COOT^{E15} intervened by refinement in either autoBUSTER (Global Phasing Limited GPhL, Cambridge, UK) or Refmac5^{E16} applying TLS for individual domains and non-crystallographic symmetry (NCS) restraints for the IgE molecules. In total 213 waters were built in using the water picking option in Refmac5^{E16} followed by manual inspection. In the final refinement round the NCS restraints were released resulting in a final model with R=20.0 % and Rfree=27.0%.

Table 3: Crystal Parameters and X-ray Data-Processing and Refinement Statistics

	Data set 1	Data set 2
Space group	<i>P3₂21</i>	<i>P3₂21</i>
Wavelength (Å)	<i>0.976</i>	<i>0.976</i>
Cell constants a (Å)	<i>140.62</i>	<i>141.562</i>
b (Å)	<i>140.62</i>	<i>141.562</i>
c (Å)	<i>244.65</i>	<i>245.562</i>
Resolution range (Å)	<i>2.93 - 35.16</i>	<i>2.85 – 109.11</i>
Resolution highest shell (Å)	<i>2.93 – 3.01</i>	<i>2.85 – 2.92</i>
Completeness overall (%)	<i>99.9</i>	<i>100.0</i>
Completeness highest shell (%)	<i>100.0</i>	<i>100.0</i>
Reflections, unique	<i>60601</i>	<i>66338</i>
Multiplicity	<i>6.6</i>	<i>10.7</i>
Multiplicity highest shell	<i>6.8</i>	<i>11.0</i>
Rmerge _{overall} (%) ¹	<i>0.133</i>	<i>0.099</i>
Rmerge highest shell (%)	<i>0.914</i>	<i>0.75</i>
Mean(I)/sd(I)	<i>13.1</i>	<i>20.2</i>
Mean(I)/sd(I) highest shell	<i>2.0</i>	<i>3.4</i>
Rvalue _{overall} (%) ²	<i>N/A</i>	<i>20.0</i>
Rvalue _{free} (%)	<i>N/A</i>	<i>27.0</i>

$$^1 R_{\text{merge}} = \sum_{hkl} [(\sum_i |I_i| - \langle I \rangle) / \sum_i I_i]$$

$$^2 R_{\text{value}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$$

R_{free} is the cross-validation R factor computed for the test set of 5 % of unique reflections

Characterization Assays

KinExA: Monomerized Fab or IgG was allowed to come to equilibrium with human IgE. Fully equilibrated solutions were analyzed using KinExA 3200 technology. In brief, for each sample, the KinExA 3200 instrument automatically packed a fresh column of epsilon C3-C4 minimal domain fragment conjugated azlactone micro-beads. The sample containing antibody (or Fab fragment), antigen, and Ab/antigen (or Fab/antigen) complex (in Dulbecco's PBS, 1 mg/mL bovine serum albumin, 0.02% [w/v] sodium azide) was flowed through the column (0.25 mL/min). Free antibody/Fab bound to the human Fc epsilon Cε3-Cε4 fragment-beads. DyLight650 fluorescently labelled secondary detection reagent (DyLight650-Protein G' for intact IgG or DyLight649-mouse anti human heavy and light chain specific antibody for Fab fragment) was then passed through the column. A buffer wash removed excess label, leaving fluorescence signal on the bead column directly proportional to the amount of free antibody (IgG) in the original sample. KD was estimated for the antibody binding to the IgE (least squares fitting, using a 1:1 reversible bimolecular interaction model within the supplied KinExA Pro software).

RBL-ER51 calcium signaling assay

Human FcεRI was cloned from human peripheral blood lymphocytes into the pcDNA3.1 vector and transfected, using a standard electroporation method, into RBL-2H3 cells. Transfected cells were cloned by limiting dilution and analysed for surface FcεRI expression. The resulting RBL-ER51 cells were maintained in media (DMEM with 9% v/v FBS and 400μg/mL G418) (Invitrogen). RBL-ER51 cells were seeded at 5×10^4 /100ul/well into 96 well black-walled, flat-bottomed, tissue culture-treated

plates (Costar). After 24 hours, media was replaced with test antibodies followed by addition of IgE to a final concentration of 25ng/mL. Following a 4 hour incubation at 37°C, antibody/IgE mixture was aspirated, leaving the cell monolayer intact, and replaced with 100uL/well of FLUO-4AM loading buffer [DMEM with 0.1% FBS, 20mM HEPES, 2.5mM probenid and 2µg/mL FLUO-4AM (Invitrogen)] for 1-2 hours at 37°C. Cells were washed with PBS and placed in 100uL/well of FLIPR buffer [125mM NaCl₂, 5nM KCl, 1mM MgCl₂, 1.5mM CaCl₂, 30mM Hepes, 2.5mM Probenid, 5mM glucose, 0.01% v/v FCS] for 5-45min at 37°C. To measure calcium mobilisation following addition of cross-linking anti-IgE, the FLIPR (Molecular Devices) was calibrated for suitable exposure according to the manufacturer's instructions. Anti-IgE (Biosource AHI0501), diluted in FLIPR buffer, was added to the assay plates to a final concentration of 2.3µg/mL. Fluorescence of the FLUO-4AM dye was recorded over 2 mins and the peak response exported for analysis using Graphpad Prism software. For mAb cross-linking experiments RBL-ER51 were cultured in the presence of 1µg/mL IgE for 4 hours before loading with FLUO4-AM and cross-linking with anti-IgEs as above.

LAD2 β-hexosaminidase assay

LAD2 cells^{E17} were maintained in serum-free media (StemPro-34, Life Technologies) supplemented with StemPro-34 nutrient supplement, 2 mM L-Glutamine and 100 ng/mL recombinant human stem cell factor (rhSCF, R&D). Cells were seeded at a density of 2.5×10^4 cells/well and incubated in a 96-well polypropylene plate with test antibodies for 30 min at 37°C before addition of IgE to a final concentration of 0.15nM. Following a 4 hour incubation at 37°C, cells were washed with buffer to remove any excess IgE, and IgE bound to the FcεRI on the LAD2 cells was cross-

linked with α IgE (600 μ g/mL goat-anti human IgE, Sigma) for 30 min at 37°C. The incubation was stopped by at 4°C centrifugation and the cell supernatants analysed for β -hexosaminidase content^{E18}.

Basophil histamine release assay

Human whole blood from 14 donors was independently incubated with various concentrations of goat-anti human IgE, as a positive control, or with MEDI4212 at 37°C for 60 minutes. After the incubation, samples were analysed for histamine release. For total release blood was diluted 1:20 in water followed by two freeze-thaw cycles. All samples were centrifuged and the plasma collected for further analysis of histamine content, following the protocol provided in the Histamine EIA kit (BD Biosciences). In addition, serum from each donor was collected and analysed for total IgE content (Phadia, Uppsala).

CD23 binding assay

IM9 cells were maintained in RPMI 1640 glutamax with 9% v/v heat-inactivated FBS (Invitrogen) and pre-treated with 25ng/ml human IL-4 (Peprotech) for 72 hours to up-regulate CD23 expression^{E19}. The binding assay was performed in Flow buffer [PBS, 1% Goat serum (Sigma) and 0.1% BSA fraction V (Sigma) and 5 μ g/mL Fc fragments (TEBU-bio)]. Antibody dilutions were prepared in U-bottomed polypropylene plates (Greiner) and mixed with IgE to a final IgE concentration of 10 μ g/mL before incubating with 1x10⁵ cells on ice for 1 hour. Cells were washed in Flow buffer, and IgE bound to the cell surface was detected with anti-IgE-phycoerythrin (Caltag). Following fixation (cell fix; BD biosciences), cells were analysed using a

FACSCalibur (BD Biosciences) to quantify FL2 Geomean fluorescence.
Alternatively, RPMI-8866 cells were incubated with 56nM (4167IU/mL) IgE to allow
IgE binding. Test antibodies directly labeled with FITC (FluoroTag FITC
Conjugation kit – Sigma) were assessed for binding to the IgE-loaded cells by flow
cytometry.

Cell mediated killing assay

The assay was essentially as described in ^{E20}. In brief, titrations of test antibodies were
mixed with a target-specific MOv18 (folate receptor) or irrelevant (NIP) IgE prior to
incubation with carboxyfluorescein succinimidyl ester (CFSE)-labeled IGROV1
target cells and IL-4-stimulated U937 effector cells. Following a 2.5-hour incubation,
the cells were stained with an anti-CD89-phycoerythrin antibody (to identify effector
cells) and propidium iodide (to identify dead cells). Flow cytometry analysis was used
to differentiate live cells (CFSE only) from cells killed by cytotoxicity (CFSE and PI
double stained) and cells killed by phagocytosis (CFSE and CD89-PE double stained)
as previously described.

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