

ORIGINAL ARTICLE

Basic and Translational Allergy Immunology

Structure of intact IgE and the mechanism of ligelizumab revealed by electron microscopy

Rasmus K. Jensen¹  | Frederic Jabs² | Michaela Mieke² | Brian Mølgaard² | Wolfgang Pfützner³  | Christian Möbs³  | Edzard Spillner²  | Gregers R. Andersen¹ 

¹Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark

²Immunological Biotechnology, Department of Engineering, Aarhus University, Aarhus, Denmark

³Clinical and Experimental Allergology, Department of Dermatology and Allergology, Philipps-Universität Marburg, Marburg, Germany

Correspondence

Edzard Spillner, Immunological Biotechnology, Department of Engineering, Aarhus University, Gustav Wieds Vej 10C, DK-8000 Aarhus, Denmark.
Email: e.spillner@eng.au.dk

Gregers R. Andersen, Department of Molecular Biology and Genetics, Aarhus University, Gustav Wieds Vej 10C, DK-8000 Aarhus, Denmark
Email: gra@mbg.au.dk

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Abstract

Background: IgE is the central antibody isotype in TH2-biased immunity and allergic diseases. The structure of intact IgE and the impact of IgE-targeting molecules on IgE however remain elusive. In order to obtain insights into IgE biology and the clinical impact, we aimed for structure determination of IgE and the complex of IgE with the anti-IgE antibody ligelizumab.

Methods: Structures of two distinct intact IgE with specificity for cross-reactive carbohydrate determinants and Der p 2 as well as complexes of ligelizumab-Fab with IgE and IgE Fc were assessed by negative stain electron microscopy and solution scattering. Inhibition of IgE binding and displacement of receptor-bound IgE were assessed using cellular assays, basophil activation testing and ELIFAB assays.

Results: Our data reveal that the investigated IgE molecules share an overall rigid conformation. In contrast to the IgE Fc fragment, the IgE Fc in intact IgE is significantly less asymmetrically bent. The proximal and the distal Fabs are rigidly tethered to the Fc. Binding of ligelizumab to IgE in a 2:1 stoichiometry induces an extended and twofold symmetrical conformation of IgE, which retains a rigid Fab-Fc architecture. Analyses of effector cell activation revealed that ligelizumab inhibits IgE binding without displacing receptor-bound IgE. Together with an interference of CD23 binding, the data underline a functional activity similar to omalizumab.

Conclusions: Our data reveal the first structures of intact IgE suggesting that the IgE Fab is fixed relative to the Fc. Furthermore, we provide a structural rationale for the inhibitory mechanism of ligelizumab.

KEYWORDS

antibody structure, electron microscopy, flexibility, IgE, therapeutic antibody

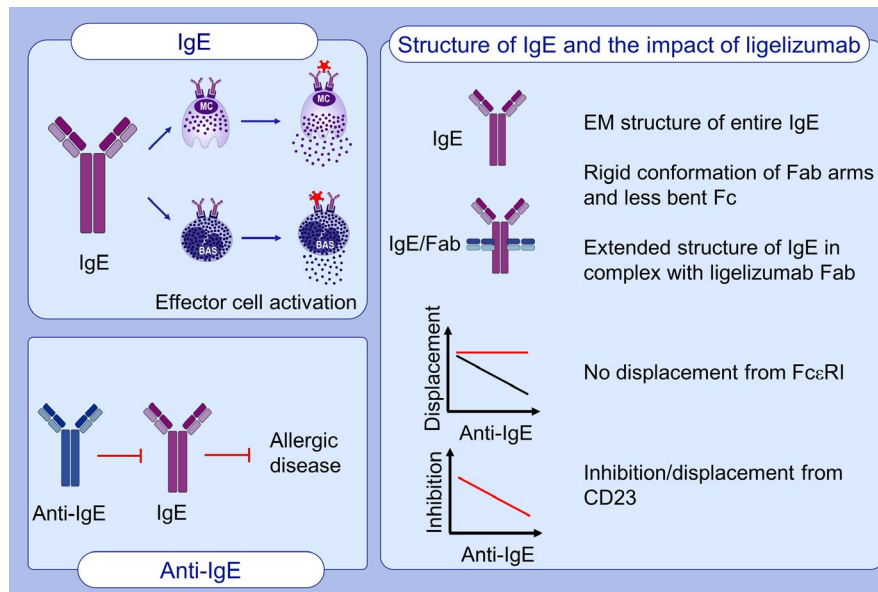
1 | INTRODUCTION

Immunoglobulin E (IgE) has a key role in allergic diseases and is found both in the circulation and on a variety of cell types.¹ It interacts directly with the high-affinity receptor FcεRI and the low-affinity

receptor CD23.² Upon cross-linking by allergens, FcεRI-bound IgE on mast cells and basophils triggers degranulation, release of mediators and immediate reactions.² CD23 forms a homotrimeric receptor crucial for facilitated antigen presentation, transport across the epithelium, amplification of IgE production and epitope spread.^{2,3}

Abbreviations: Fab, fragment antigen-binding; Fc, fragment crystallizable.

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GRAPHICAL ABSTRACT

Electron microscopy and solution scattering reveal that the structure of entire IgE is highly rigid with a spatial organization differing from current models. The Fab arms are rigidly tethered to the Fc and lack flexibility. Ligelizumab traps IgE in an extended conformation retaining the Fab arm assembly. The localization of the epitope enables inhibition of binding to both IgE receptors.

Anti-IgE antibodies interfere with FcεRI binding and typically bind in proximity to its binding site. The only approved anti-IgE antibody omalizumab primarily prevents interaction of free IgE with FcεRI and CD23^{4,5} and eventually reverses phenotypic and functional effects of IgE such as enhanced FcεRI levels on effector cells.^{6,7} The second generation anti-IgE antibody ligelizumab (QG031) has a higher affinity to IgE and an increased potency compared with omalizumab⁸ leading to longer and greater suppression of IgE.⁹ Although some clinical trials showed limited benefit, for example in asthma and atopic dermatitis, ligelizumab exhibited improved efficacy compared with omalizumab in treatment of chronic spontaneous urticaria.¹⁰ Ligelizumab has now entered a phase III trial (NCT03580356). Structural and functional aspects of its mode of action however remain unclear.

Antibodies are commonly perceived as flexible molecules due to the presence of their hinge region.¹¹ The hinge region in IgE is small compared with the IgG isotypes and followed by an additional pair of Ig domains. This feature is believed to reduce IgE flexibility compared with IgG^{12,13} (Figure 1A-B). There is, however, an understanding of the IgE Fabs as flexible relative to the Fc.¹⁴⁻¹⁶ Recent structural studies on

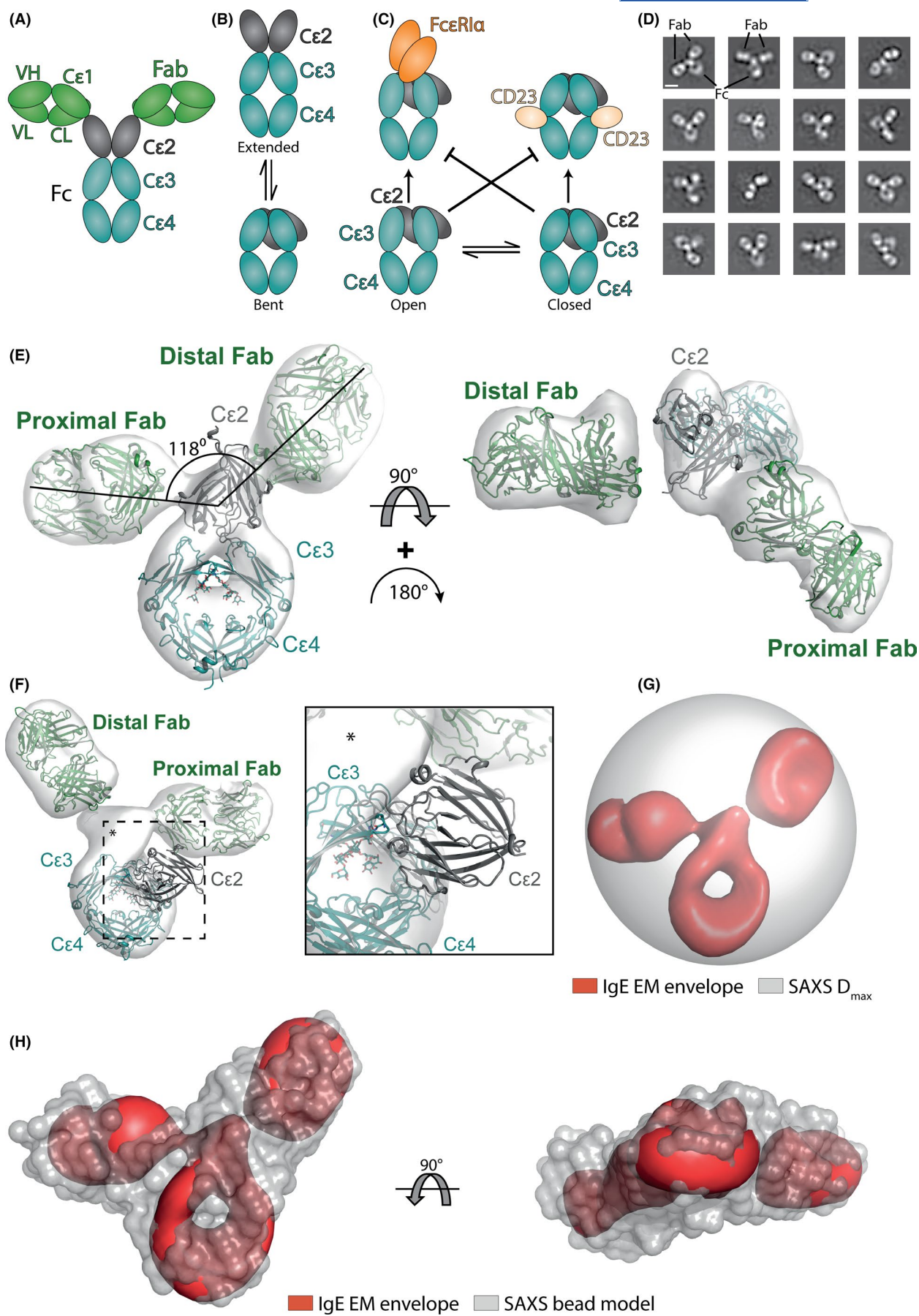
IgM¹⁷ and chicken IgY,¹⁸ also having an additional Ig domain in the Fc moiety, similarly suggested a flexible attachment of the Fabs to the Fc.

A number of structural studies have investigated the conformational properties of the IgE Fc (Figure 1C), but scarce structural work has been done on intact IgE. In this study, we use negative stain electron microscopy (nsEM) and small-angle X-ray scattering (SAXS), which are orthogonal techniques that both provide structural information in the 15–30 Å resolution range. Thereby, we show that IgE adopts one major conformation with defined Fab and Fc organization deviating from the multiple crystal structures of isolated Fc. We furthermore show that ligelizumab-bound IgE exhibits a significantly re-organized conformation, which is incompatible with both CD23 and FcεRI binding. Hence, our data provide a new understanding of the general principles of IgE structure and inhibitory mechanism of ligelizumab.

2 | METHODS

Proteins were expressed in HEK293 or insect cells and purified to homogeneity.¹⁹ For EM grid preparation, IgE and IgE-ligelizumab

FIGURE 1 The structure of intact IgE HMM5. A, The domain structure of IgE. B, Representation of the extended and bent conformations previously observed in crystal structures of antibody bound and free IgE Fc, respectively. C, Representation of the open and closed conformation of the Cε3-4 fragment and the mutual allosteric inhibition of FcεRI (orange) and CD23 (light-yellow). D, The 16 most populated 2D class averages observed in single particle nsEM analysis of IgE HMM5. The white scale bar corresponds to 50 Å. E, The nsEM 3D reconstruction of intact IgE HMM5. The Fc density is fitted with the two IgE Fc fragments Cε3-4 and Cε2 (From RCSB entry 2WQR), and the Fab densities are fitted with an IgG Fab (RCSB entry 4X7S). F, The nsEM 3D density of intact IgE HMM5 fitted with the bent conformation of IgE Fc (RCSB entry 2WQR), showing that the major conformation of IgE HMM5 is much less bent than previously suggested. G, Comparison of the IgE HMM5 nsEM density and a sphere with a diameter of 170 Å corresponding to the D_{max} obtained by SAXS. H, The average of 17 ab initio reconstructions of IgE HMM5 calculated from the SAXS data in grey aligned to the nsEM density of IgE in red



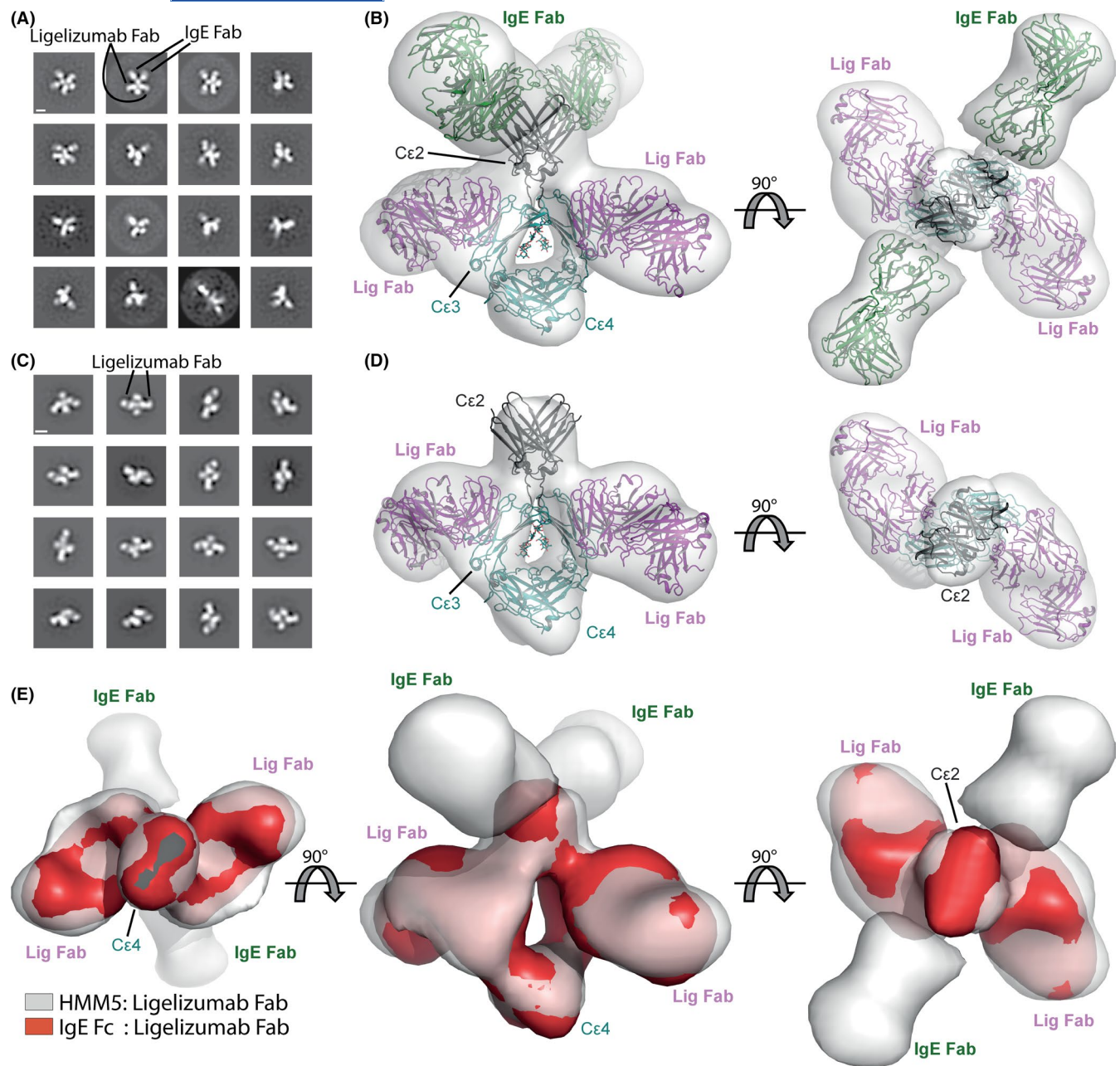


FIGURE 2 Single particle nsEM analysis of the HMM5 IgE in complex with ligelizumab-Fab. A, The 16 most populated 2D class averages observed in nsEM analysis of HMM5 IgE in complex with ligelizumab-Fab. The ligelizumab-Fabs are interacting with the Fc in a 2:1 ratio. The scale bar corresponds to 50 Å. B, The 3D reconstruction of IgE HMM5 in complex with the ligelizumab-Fab. Ligelizumab induces an extended conformation of the Fc leading to IgE having global twofold rotational symmetry. The major epitope of ligelizumab is located in the Cε3 domain. The Fc is fitted with an extended IgE Fc fragment Cε2-4 (RCSB entry 4J4P), and both the ligelizumab and IgE Fabs are fitted with an IgG Fab (RCSB entry 4X7S). C, The 16 most populated 2D class averages observed in nsEM analysis of IgE Fc in complex with ligelizumab-Fab. The white scale bar corresponds to 50 Å. D, The 3D reconstruction of IgE Fc in complex with ligelizumab-Fab, confirming the findings with intact IgE HMM5. E, Alignment of the 3D reconstructions of IgE HMM5 and IgE Fc in complex with ligelizumab-Fab. The reconstructions are overall very similar, but slight differences can be observed for the densities corresponding to the Cε2 domain pair

complexes were purified by size exclusion chromatography using a Superdex Increase 200 20/300 GL column (GE Healthcare) equilibrated in 20 mmol/L HEPES, 50 mmol/L NaCl, pH 7.2. Carbon-evaporated copper grids (G400-C3, Gilder) were glow-discharged for 45 seconds at 25 mA using an easiGlow (PELCO). Three μL of IgE, IgE:ligelizumab-Fab or IgE Fc:ligelizumab-Fab at 10–20 μg/mL were adsorbed to the grid for 5 seconds. The grids were washed in

3 μL of 20 mmol/L HEPES, 50 mmol/L NaCl, pH 7.2 followed by a 3 μL drop of 2% (w/v) uranyl formate which was immediately blotted away. The grids were finally stained in a 3 μL drop of 2% (w/v) uranyl formate for 45 seconds. The stain was blotted away and the grids were air-dried before being imaged on a 120 kV Tecnai G2 spirit electron microscope. Automated data collection was performed at a nominal magnification of 67,000x and a defocus

ranging from -0.5 to -1.5 μm using Legikon.²⁰ Particles were picked using DoG picker²¹ (HMM5:ligelizumab-Fab complex) or cis-TEM²² (unbound HMM5, unbound H10 and IgE Fc:ligelizumab-Fab complex). The steps of classification, initial model generation using stochastic gradient descent and 3D refinement were performed in RELION.²³ For the IgE-ligelizumab complexes, the final 3D refinement was performed with C2 symmetry imposed. Docking of IgE fragments was done in Coot.²⁴ Focused classifications for each of the IgE Fabs in both unbound HMM5 and HMM5:ligelizumab were performed in RELION using spherical soft-edged binary masks with radii of 50 Å. To assess the uncertainty in the docking of our models, we generated models of the IgE Fc C ϵ 3-4 fragment (RCSB entry 2WQR) translated 0-10 Å and rotated 0-10° in 2.5 Å/° steps. The cross-correlation between the resulting models and the nsEM map was calculated using Chimera, and revealed that the uncertainty in the docking is approximately ± 5 Å and $\pm 5^\circ$. For extended information, see the Methods section in the SI Appendix.

3 | RESULTS

3.1 | IgE adopts a single major conformation

NsEM can provide valuable structural information based directly on images of single macromolecules and classification of different conformational states, but it is limited to a resolution of ~ 15 -20 Å due to the grain size of the stain. To establish the foundation for investigating the influence of anti-IgE antibodies on IgE by nsEM, we first studied unbound IgE HMM5 (Figure S1). This IgE has specificity for α -1,3-fucosylated N-glycan core structures, a prominent cross-reactive carbohydrate determinant on plant- and insect-derived allergens such as grass pollen and insect venom allergens.¹⁹ Micrographs were subjected to single particle EM analysis using RELION²³ (Figure S2A-B). In the resulting 2D class averages (Figure 1D, Figure S2C-D), both the Fc and the two Fabs were well resolved indicating rigid conformations of IgE HMM5. To investigate whether these class averages represent a finite number of distinct conformations or a single conformation, we performed a 3D classification followed by 3D refinement. In the resulting 3D reconstruction (Figure 1E), the Fc moiety can clearly be distinguished from the Fab moieties due to the low level of density observed between the two heavy chains at the location of two N-glycans on Asn394. The Fab moieties are also well resolved, and the densities containing the variable and constant domains are of comparable strength. This suggests that the rotational freedom of the Fabs relative to the C ϵ 2 domains is minor; otherwise, the variable domains furthest from the Fab-C ϵ 2 junction would vary significantly in position between IgE molecules and the density for the variable domains would average out in the 3D reconstruction. Importantly, as 46% of the picked particles contributed, the 3D reconstruction must be very common amongst the IgE molecules.

The IgE adopts an asymmetric conformation with one "proximal" Fab located close to the Fc, and a "distal" Fab located further

away (Figure 1E). This conformation is not a classical antibody Y- or T-shape but rather a hybrid T-Y shape with an estimated Fab-C ϵ 2-Fab angle of 118° . Comparison of our EM density with the crystal structure of IgE Fc reveals dramatically less bending of the C ϵ 2 domains in intact IgE (Figure 1F). We observe an estimated bending angle for IgE of $\sim 30^\circ$ as compared to the 110 - 120° bending observed in the Fc crystal structures.²⁵

Several crystal structures demonstrated that the conformation of the C ϵ 3-4 domains varies between an open and a closed conformation differing by the distance between the N-termini of the two C ϵ 3 domains.¹³ Although the nsEM 3D reconstruction is of high quality, as seen from the Fourier shell correlation and orientational distribution of particles (Figure S2E-F), it is insufficient to indisputably distinguish the open and closed conformation.

SAXS is a technique which can provide low resolution information on the molecular shape under near physiological conditions²⁶ and therefore complements nsEM. We collected synchrotron SAXS data on IgE HMM5 which revealed a radius of gyration (R_g) of 51.6 Å (Figure S3A-D). This is similar to what has earlier been observed for intact rat IgE²⁷ and comparable to that of IgG1.²⁸ A pair distribution function calculated from the SAXS data indicated that IgE has a maximum extent (D_{max}) of 170 Å (Figure S3E). This estimate of D_{max} is in excellent agreement with the size of the nsEM density (Figure 1G). We next generated 20 ab initio structures from the SAXS data and compared an average model generated from the major cluster containing 17 of the 20 models to the nsEM density (Figure 1H). The resulting model shows a central core with three protruding densities, with two of the protrusions in close proximity and the third pointing away from the molecule, similar to our EM 3D reconstruction.

We further asked whether our data obtained with IgE HMM5 represent general features of IgE. Therefore, we produced another IgE, called H10 with specificity for Der p 2 (Figure S4).²⁹ The protein sequences of the two IgE molecules are identical except for the variable domains (Figure S1). Moreover, both HMM5 and H10 IgE have the glycosylation sites found in human IgE,³⁰ but due to the use of different expression hosts, their glycan profile differs. EM analysis of IgE H10 yielded 2D class averages indistinguishable from those observed for IgE HMM5, and the final nsEM 3D density exhibited the same features with a proximal Fab bent in over the IgE Fc and a distal Fab pointing away from the IgE Fc (Figure S5). Overall, our nsEM and SAXS data are consistent with a general model of IgE, in which the IgE Fc is less bent than expected from previous crystal structures and the Fabs have fixed positions relative the C ϵ 2 domains.

3.2 | Ligelizumab induces an extended IgE conformation

We next investigated the complex between IgE HMM5 and ligelizumab. In order to use a sterically defined and monovalent antibody moiety, that allows analysis of single particles, the ligelizumab-Fab was expressed in insect cells and assessed for immunoreactivity and complex formation with IgE HMM5 (Figures S6 and S7A-B). The

IgE-ligelizumab-Fab complex was subjected to nsEM single particle analysis as above (Figure S8A-B). The resulting nsEM 2D classes revealed that ligelizumab-Fab binds IgE in a 2:1 ratio and forces the Fc into an extended conformation (Figure 2A and Figure S8C-D). The 3D reconstruction obtained after 3D classification suggested that the complex exhibited twofold rotational symmetry across the C ϵ 3-4 symmetry axis. In the final 3D reconstruction obtained after imposing twofold rotational symmetry (Figure 2B), there is clear density for the IgE Fc, the IgE Fabs and the ligelizumab-Fabs. The 3D reconstruction is of decent quality, but at a slightly lower resolution compared with the 3D reconstruction of unbound IgE, due to a tendency towards preferred orientations (Figure S8E-F). As for unbound IgE, the 3D reconstruction of the IgE-ligelizumab complex must represent a common conformation as 55% of the particles remaining after 2D classification contributed to the 3D reconstruction.

The major epitope of the ligelizumab-Fab is in the C ϵ 3 domain, but ligelizumab is also in close proximity to the C ϵ 2 domain and the opposing C ϵ 3 domain. Interaction with the C ϵ 2 domain or possibly the linker between C ϵ 2 and C ϵ 3 would rationalize how binding of ligelizumab-Fab can induce an extended IgE Fc conformation. The two IgE Fabs are pointing away from the Fc, and due to the twofold symmetry, the two Fab arms are equivalent in the ligelizumab-bound IgE HMM5, in contrast to the proximal and distant Fabs in unbound IgE HMM5 and H10. As in unbound IgE, the densities for the variable and constant domains are comparable indicating that the rotational freedom at the Fab-C ϵ 2 junction is limited.

To investigate whether the IgE Fabs obey strict twofold symmetry, we performed focused classification on the two Fabs independently. The resulting Fab reconstructions were of higher quality than the full reconstruction, and changes in the refined particle orientations were observed (Figure S9A-D). For unbound IgE, focused classification did not change the quality of 3D reconstruction significantly (Figure S9E-H). This shows that in the IgE:ligelizumab complex, the IgE Fabs do not obey strict overall C2 symmetry, and minor independent movements relative to the Fc appear to be possible.

To assess the importance of the IgE Fabs for the extended conformation, we also formed and purified the complex between IgE Fc and ligelizumab-Fab (Figure S7C-D). The negative stain 2D class averages corroborated that IgE Fc in complex with ligelizumab-Fab adopts an extended conformation (Figure 2C-D). The 3D reconstruction showed clear density for the C ϵ 2-4 domains. Superposition of

the 3D reconstructions of intact IgE and IgE Fc in complex with ligelizumab-Fab revealed that the C ϵ 3-4 domain pairs adopt a very similar conformation in the complexes and that the ligelizumab binding site is the same (Figure 2E). Overall, the nsEM analyses revealed the same binding site for ligelizumab and induction of twofold symmetry in both intact IgE and its Fc fragment.

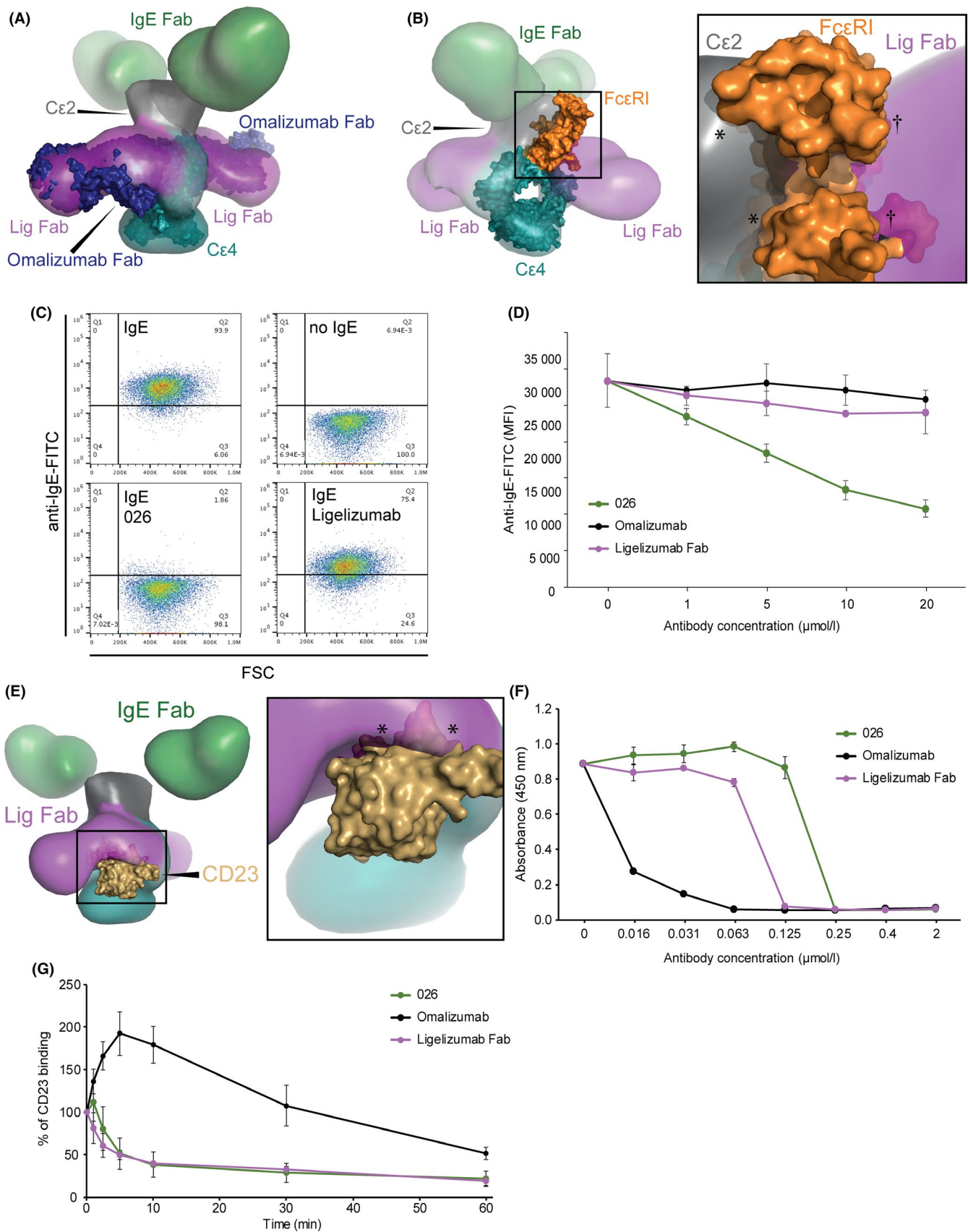
3.3 | Functional effects of ligelizumab binding to IgE

Our results indicate that ligelizumab has a unique binding mode, although its epitope also appears to show considerable overlap with those of known anti-IgE binders. Alignment of the structure of omalizumab Fab-IgE Fc complexes to our model of the IgE-ligelizumab complex (Figure 3A) indicates that the ligelizumab and omalizumab binding sites overlap substantially, although omalizumab does not induce the extended conformation.³¹ Hence, similarities in the inhibitory activity of the two anti-IgE molecules could be expected.

Ligelizumab prevents binding of IgE to Fc ϵ RI. This can be rationalized by aligning the Fc ϵ RI-IgE Fc crystal structure to the IgE Fc density in our IgE HMM5-ligelizumab complex (Figure 3B). The Fc ϵ RI receptor overlaps not only with the ligelizumab-Fab density, but also with the C ϵ 2 domains in the extended conformation of ligelizumab-bound IgE. Recently, the single domain antibody (sdab) O26 and even omalizumab at very elevated concentrations were shown to mediate dissociation of IgE from Fc ϵ RI.⁵ Therefore, we assessed the effect of ligelizumab-Fab on Fc ϵ RI-bound IgE. Flow cytometry analysis of surface IgE on RBL-SX38 cells expressing the Fc ϵ RI and passively sensitized with IgE as well as basophils from an allergic patient showed that ligelizumab, in contrast to the monovalent sdab O26, did not efficiently reduce the amount of surface-bound IgE (Figure 3C-D).

Alignment of the crystal structure of CD23-bound IgE Fc on our 3D reconstruction of IgE HMM5 bound to ligelizumab suggested that ligelizumab like omalizumab⁵ also inhibits IgE binding to this receptor (Figure 3E). We therefore investigated whether ligelizumab interferes with CD23 binding of IgE (K_D 2 μ mol/L) in ELIFAB assays that enable detection of preformed oligovalent IgE:allergen complexes bound to CD23.³² Serum of a patient with an elevated sIgE level (>100 kUA/L) against the honeybee venom allergen Api m 1 was incubated with Api m 1. Ligelizumab-Fab as well as sdab O26 and omalizumab prevented binding of the IgE:allergen complexes

FIGURE 3 Impact of ligelizumab binding on interaction with the IgE receptors. A, Omalizumab and ligelizumab have very similar epitopes on IgE, as observed by comparison of the IgE-omalizumab (RCSB entry 5HYS, blue) structure to the IgE-ligelizumab density. In panels A, B and E, the density suggested to contain the ligelizumab-Fab is coloured magenta while the remaining density encompassing IgE Fab, C ϵ 2 and C ϵ 3-4 is coloured green, grey and cyan, respectively. B, Alignment of the IgE-ligelizumab density with the IgE-Fc ϵ RI structure (RCSB entry 1F6A, orange) shows that Fc ϵ RI sterically overlaps with both the C ϵ 2 domain (marked *) and the ligelizumab-Fab (marked †). C, Displacement of IgE from the surface of RBL-SX38 cells providing the human Fc ϵ RI and sensitized with IgE by ligelizumab-Fab, and O26 sdab was analysed by surface staining of IgE using flow cytometry. D, Displacement of IgE from the surface of basophils of an allergic patient by ligelizumab-Fab, omalizumab and O26 sdab was analysed as in panel C. E, Ligelizumab inhibits CD23 binding to IgE sterically according to a comparison of the IgE Fc-CD23 structure (RCSB entry 4EZM, yellow) to the ligelizumab-IgE density. Overlap between ligelizumab-Fab and CD23 is marked by *. F, Inhibition of IgE binding to CD23 by ligelizumab-Fab, omalizumab and O26 sdab was analysed by using an Api m 1-specific human serum in ELIFAB. G, The displacement of preformed IgE:allergen complexes from CD23 using serum as in panel F was analysed by detecting remaining binding of IgE-allergen complexes after incubation with the anti-IgE antibodies



to CD23 (Figure 3F). While the bivalent omalizumab IgG inhibited at lower concentrations, monovalent ligelizumab-Fab and sdab 026 showed inhibition at comparable concentrations. When applied

after binding of the complexes to CD23, all three anti-IgE molecules displaced IgE:allergen complexes from CD23 efficiently within minutes (Figure 3G). In summary, the IgE-ligelizumab density compared

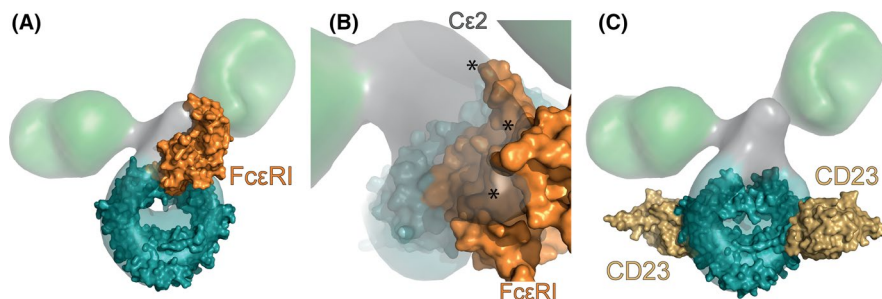


FIGURE 4 Implications for IgE Fc receptor binding to intact IgE. A, Alignment of the IgE HMM5 density with the structure of the IgE Fc Cε3-4:FcεRI complex (RCSB entry 1F6A, orange), showing that FcεRI overlaps with the Cε2 domain. The envelope suggested to contain the IgE Fc Cε3-4, Cε2 and IgE Fabs is coloured cyan, grey and green, respectively. B, Close up of the clash (marked with *) between FcεRI and the density for the Cε2 domain. C, Alignment of the IgE density with the IgE Fc Cε3-4-CD23 complex (RCSB entry 4EZM, yellow)

with structures of IgE Fc in complex with FcεRI and CD23 as well as functional analyses revealed that ligelizumab and omalizumab act functionally in a similar manner by inhibiting IgE binding to both FcεRI and CD23.

4 | DISCUSSION

With its central role in both immunity and response to allergens, IgE is a double-edged immune molecule and a crucial target for intervention. Understanding the structure-function relationship of IgE is therefore critical. So far, there are no structures available describing intact IgE. Prior nsEM, studies of IgE have been conducted,^{15,33,34} but to the best of our knowledge, no prior single particle analyses have been presented. Based on single particle nsEM analysis, we conclude that for intact IgE, one very frequent conformation is highly rigid with very little rotational freedom of the Fab arms.

The current model of IgE conformations and dynamics is heavily based on crystal structures^{25,35} and solution scattering³⁶ of IgE Fc fragments. Our nsEM data for two intact IgE molecules suggest differences from these crystal structures, since the Cε2 domain tandem is less strongly bent towards the Cε3 and Cε4 domains in our 3D reconstructions. This discrepancy may be due to a reduced flexibility of the Cε2-Cε3 linkage in intact IgE originating from altered dynamic and conformational properties of the Cε2 domains due to their stable association with the Fabs. In contrast, our results do not conflict with the current perception of IgE Fc dynamics stating that the Fc can transiently adopt an extended conformation with global twofold symmetry.¹⁴ It can either revert or bend towards the other symmetry-related side of the molecule (Figure 1B). Since we do not observe unbent IgE in nsEM and obtain excellent density for both Fabs in our two 3D reconstructions, the extended conformation must be rare, but similar to the aeFab¹⁴ and the 8D6-Fab,³⁷ ligelizumab clearly stabilizes this conformation.

Classic fluorescence spectroscopy experiments suggested a distance of 71 Å between the antigen-binding site and the C-terminus of the IgE heavy chain, which is shorter than the distance estimated from our docked model of unbound IgE (Figure S10A). However,

this study used a molecule, in which the Cε4 was replaced by a Cγ3 domain.³⁸ A hybrid Cε3-Cγ3 dimer may not fully recapitulate the properties of the Cε3-Cε4 dimer in terms of Fc structure and Fab arm flexibility. Titration of an anti-DNP IgE with bivalent antigens suggested a separation of 13 nm between the DNP sites in the two Fab arms which is only 2 nm less than estimated from our docked model (Figure S10B). nsEM analysis of IgE immune complexes also suggested an angle of 141° between the Fab arms and a variability within this angle comparable with that of IgA and IgG2.¹⁵ Thus, engagement of IgE into immune complexes may unlock the Fab arms.

Our findings also pose the question how IgE binding to FcεRI affects the Fc conformation and whether receptor binding unlocks the Fab arms. Upon alignment of the IgE Fc-FcεRI structure to our nsEM reconstruction (Figure 4A-B), we observe that FcεRI would overlap with the Cε2 domain in the conformation we observe for unbound IgE. Hence, in line with the strongly bent Fc of the IgE Fc-FcεRI complex, FcεRI binding appears to impose a more strongly bent conformation of the Fc as compared to the IgE conformation we observe. For the Fab arms, molecular dynamics simulation of intact IgE bound to FcεRI indeed suggested significant flexibility,¹⁶ but as there is no atomic structure of intact IgE available, such simulations must be interpreted with care. Further structural and biophysical studies of IgE-FcεRI complexes are needed to address IgE structure and dynamics in bound state. Likewise, structural investigations of the intact IgE-CD23 complex may extend the current interaction model based on IgE Fc-CD23 crystal structures³⁹ and elucidate whether receptor recognition alters IgE conformation and dynamics.

We also investigated the structures of IgE and IgE Fc in complex with ligelizumab-Fab. This Fab binds IgE in a 2:1 ratio and locks IgE Fc in an extended, twofold symmetrical conformation, similar to the extended structure previously described for IgE Fc in complex with the 8D6-Fab³⁷ and the anti-ε-chain-Fab.¹⁴ We revealed a considerable overlap between the binding sites of FcεRI and ligelizumab as expected. Likewise, we observed an overlap between the binding sites of ligelizumab and CD23, but whether there is a conformational component in the mechanism of CD23 inhibition awaits a more accurate determination of the Fc conformation (open or closed) in ligelizumab-bound IgE. The reduced inhibition of CD23 binding by ligelizumab-Fab as compared to omalizumab IgG is in line with

current understanding of its mode of action. It is important to mention that the inhibition probably is not only driven by the epitope, but also by the antibody size, valency and architecture in the complex.

Notably, omalizumab prevents complex formation of IgE with both Fc ϵ RI and CD23.^{5,34} Although the binding sites of omalizumab and ligelizumab appear to overlap heavily, omalizumab does not induce the extended conformation of IgE Fc.³¹ Both the extended conformation of ligelizumab-bound IgE and the higher affinity towards IgE as compared to omalizumab may explain the improved suppression of basophil-bound and free IgE.⁹ We recently showed that sdab 026 inhibits Fc ϵ RI binding by locking IgE in the closed conformation.⁴⁰ We also showed that 026 leads to a slight unbending of the IgE Fc, but whether sdab 026 has the same effect on intact IgE remains open. In contrast to monovalent sdab 026, neither monovalent ligelizumab-Fab nor omalizumab efficiently reduce surface levels of IgE on basophils.

In conclusion, our study suggests that the IgE Fabs are crucial determinants of the structural characteristics of IgE. With the stable association of the Fab arms and the C ϵ 2 domains, IgE is unique amongst the mammalian antibody isotypes in adopting a rigid conformation. Notably, IgE readily forms structured complexes with antigen. Mounting an immediate reaction demands a sensitive, fast-acting mechanism. A very limited rotational freedom of the Fab arms and a spatially more defined correlation of allergen-binding and receptor cross-linking appears compelling, but future studies are needed to assess the impact of rigidity on cellular activation. Finally, our results pave the way for an improved understanding of the mechanisms of anti-IgE molecules and development of novel therapeutics.

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CONFLICT OF INTEREST

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Foundation and the Danish Foundation for Independent Research (DFF). All other authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

RKJ purified complexes and conducted all steps of the EM and SAXS analyses. FJ, MM and BM produced and characterized the proteins. MM, WP and CM assessed the inhibitory and displacement activity. EZ and GRA supervised research, analysed data and wrote the paper together with RKJ.

ORCID

Rasmus K. Jensen  <https://orcid.org/0000-0001-5472-4862>

Wolfgang Pfützner  <https://orcid.org/0000-0002-8721-724X>

Christian Möbs  <https://orcid.org/0000-0002-5197-7669>

Edzard Spillner  <https://orcid.org/0000-0003-0999-5254>

Gregers R. Andersen  <https://orcid.org/0000-0001-6292-3319>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Figures S1-S7

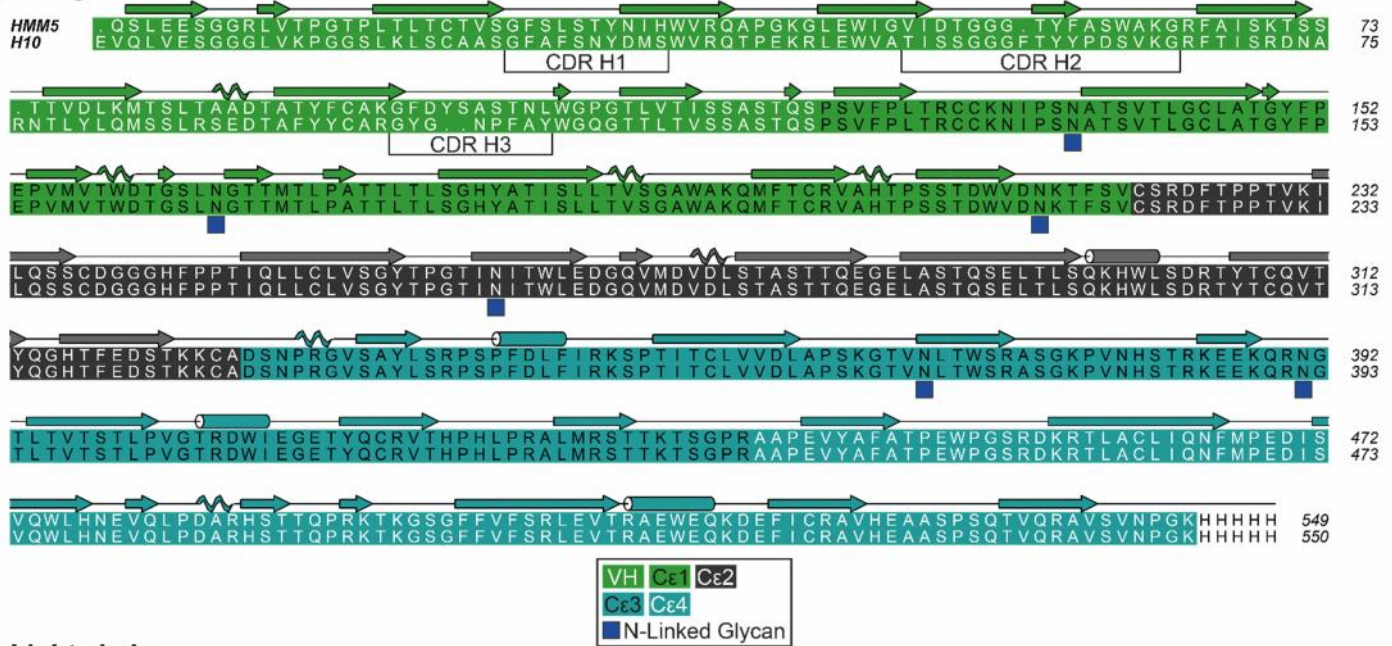
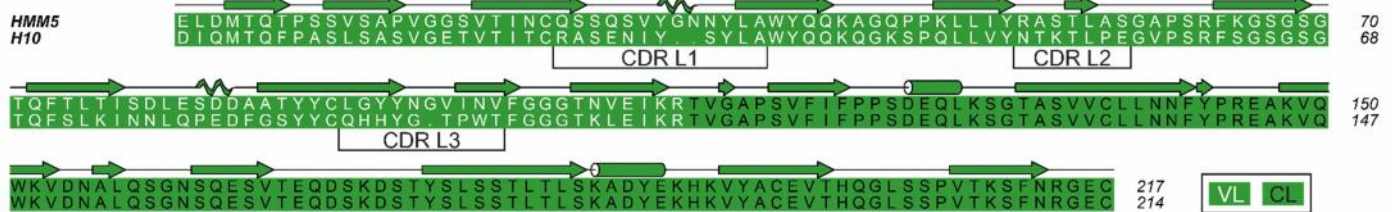
Heavy chain**Light chain**

Figure S1. Sequences of the heavy and light chains of the IgE molecules HMM5 and H10.

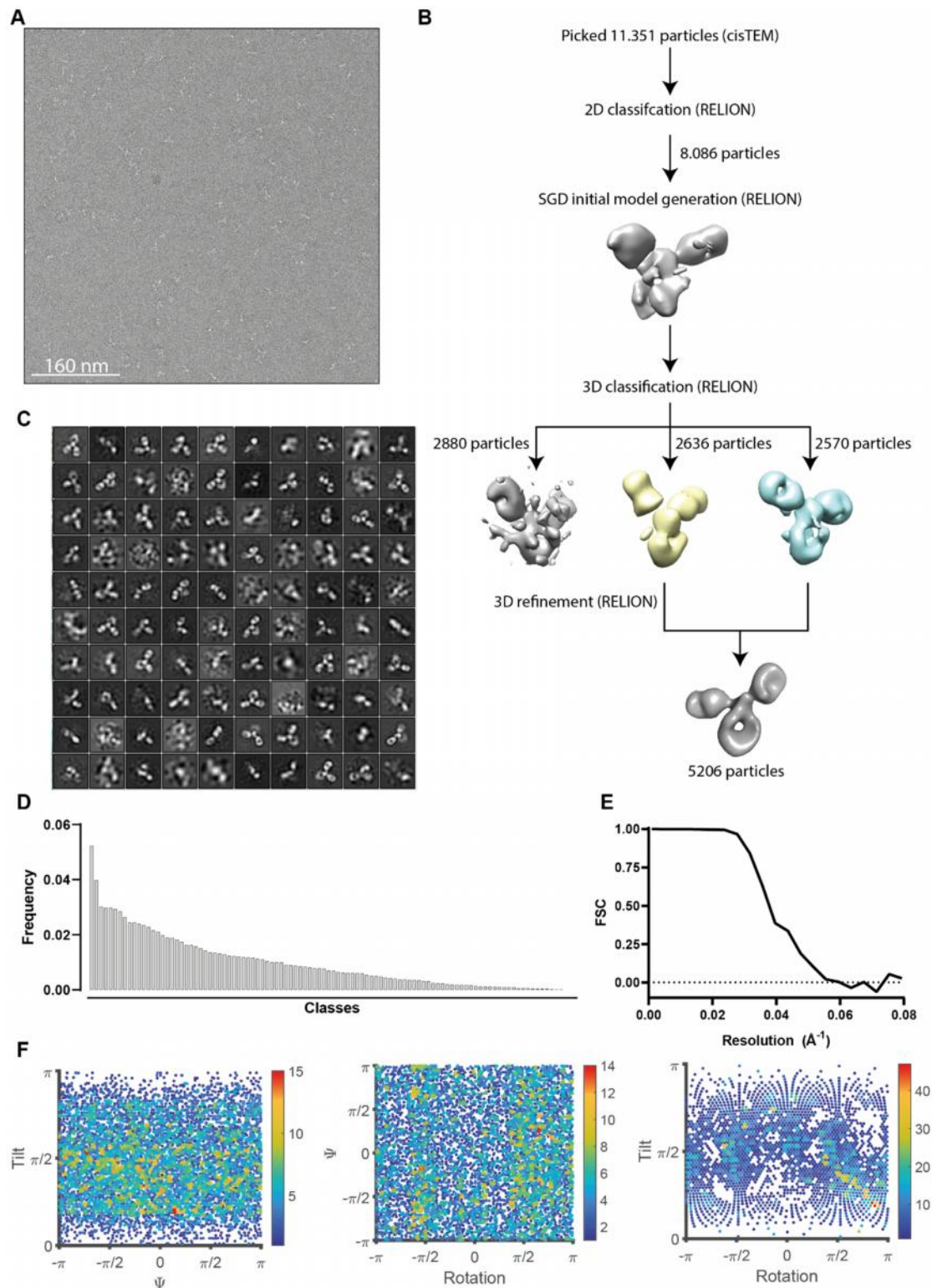


Figure S2. Single particle EM processing of IgE HMM5. **A.** A representative micrograph of IgE HMM5. **B.** Processing flow-chart showing the details and number of particles used for each step of the single particle analysis. **C.** All 2D class averages of IgE HMM5. **D.** Occupancy plot of the 2D classification showing the distribution of particles in different classes. **E.** Fourier shell correlation between the two half-sets from the final 3D refinement in RELION. **F.** Distribution of particle orientations of the particles in the final 3D refinement in RELION plotted as 2D heat-maps against pairs of Euler angles.

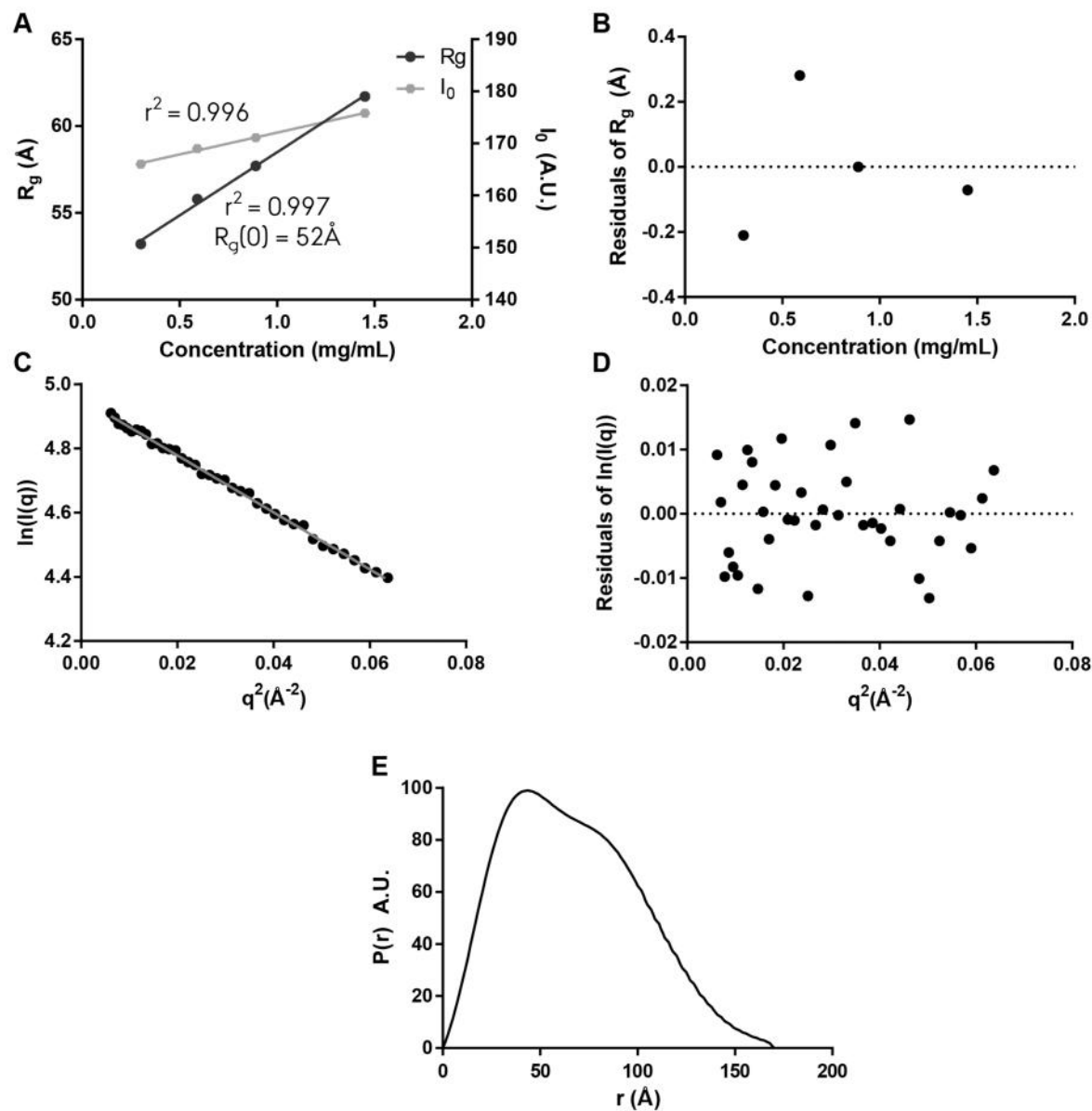


Figure S3. SAXS analysis of intact IgE HMM5. **A.** R_g and concentration scaled I_0 determined by Guinier analysis are plotted as a function of IgE concentration, showing a clear concentration dependence of both parameters. The fit of a linear regression of the R_g and I_0 is indicated in the plot as well as the R_g determined by extrapolation to zero concentration. **B.** The residuals of the linear regression of R_g . **C.** Guinier analysis of the scattering curve obtained by extrapolation to infinite dilution. **D.** The residuals of the linear Guinier fit. **E.** Pair distribution function of IgE, revealing a D_{max} of 17 nm.

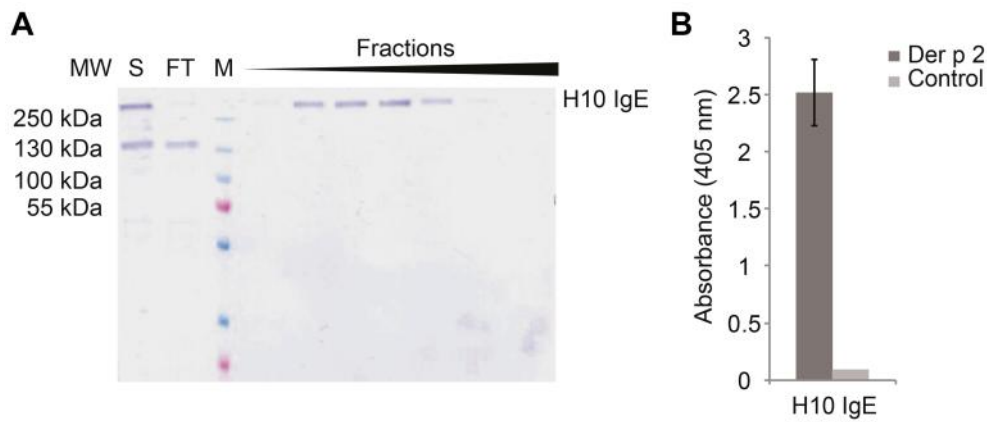


Figure S4. Purification and characterization of H10 IgE **A.** Non-reducing SDS-PAGE analysis demonstrating how the H10 IgE purification using anti-IgE affinity chromatography after the initial kappa chain specific purification removed remaining IgE half-molecules. MW: Molecular weight of molecular weight marker, S: Input cell supernatant, FT: Flowthrough, M: Molecular weight marker. **B.** The H10 IgE exhibits a pronounced immunoreactivity against the major house dust mite allergen Der p 2 in ELISA compared to control with omitted antigen.

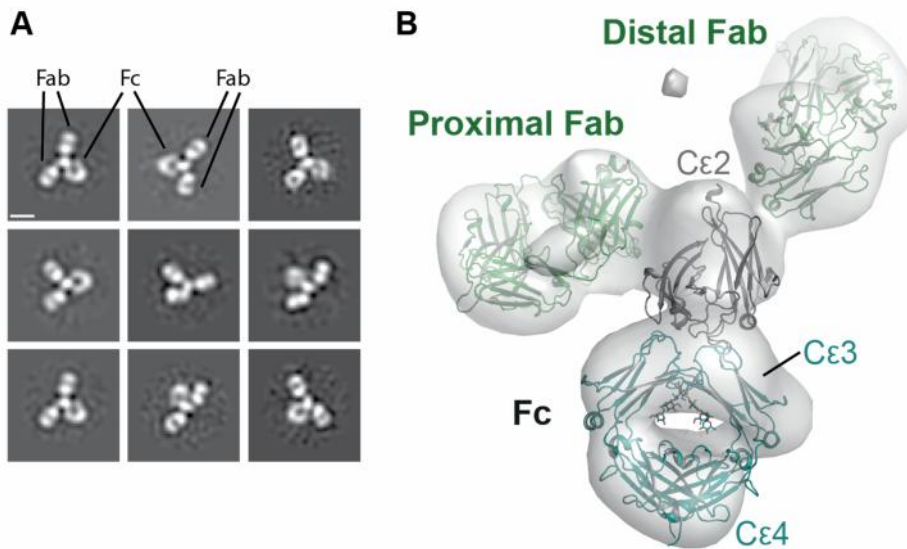


Figure S5. Additional negative stain EM analysis of intact IgE H10. **A.** The 9 most populated 2D class averages observed in single particle negative stain EM analysis of the IgE H10. The Fc and Fabs are clearly resolved. The white scale bar corresponds to 50 Å. **B.** The negative stain EM 3D reconstruction of intact H10 IgE. There is clear density for both of the Fabs and the Fc. The IgE H10 adopts the same conformation as the IgE HMM5. The Fc is fitted with IgE Fc fragments Cε3-4 and Cε2 (RCSB entry 2WQR), and the Fabs are fitted with an IgG Fab (RCSB entry 4X7S).

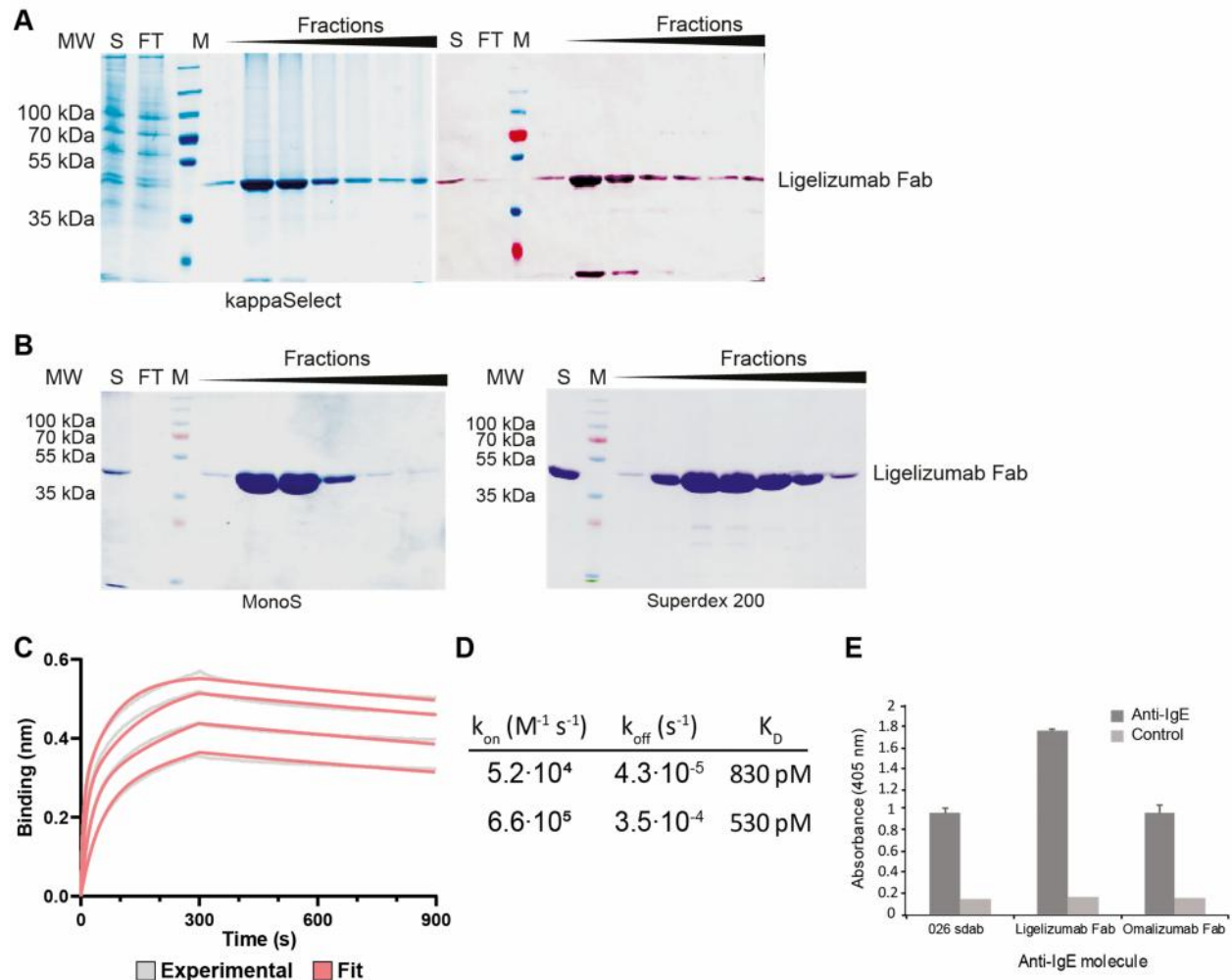


Figure S6. Characterisation of the ligelizumab Fab. **A.** Non-reducing SDS-PAGE (left) and immunoblot (right) analysis of the purification of ligelizumab Fab using kappa chain-specific affinity chromatography. MW: Molecular weight of marker, S: Input cell supernatant, FT: Flowthrough, M: Molecular weight marker. **B.** Non-reducing SDS-PAGE analysis of the purification of ligelizumab Fab using Mono S and size exclusion chromatography. Abbreviations are the same as for A. **C.** The raw sensorgrams (grey) of the BLI experiment assessing the interaction between IgE Fc and Ligelizumab Fab are shown together with the fits (red) obtained using a 2:1 heterogeneous ligand model. The ligelizumab Fab was present in the binding step at concentrations of 250, 125, 62.5, and 31.25 nM. **D.** The apparent kinetic parameters derived for the two binding sites obtained from fitting the data in panel C. **E.** Comparative analysis of IgE binding to immobilized ligelizumab Fab, omalizumab Fab and 026 sdab in ELISA documented a pronounced reactivity of the ligelizumab Fab.

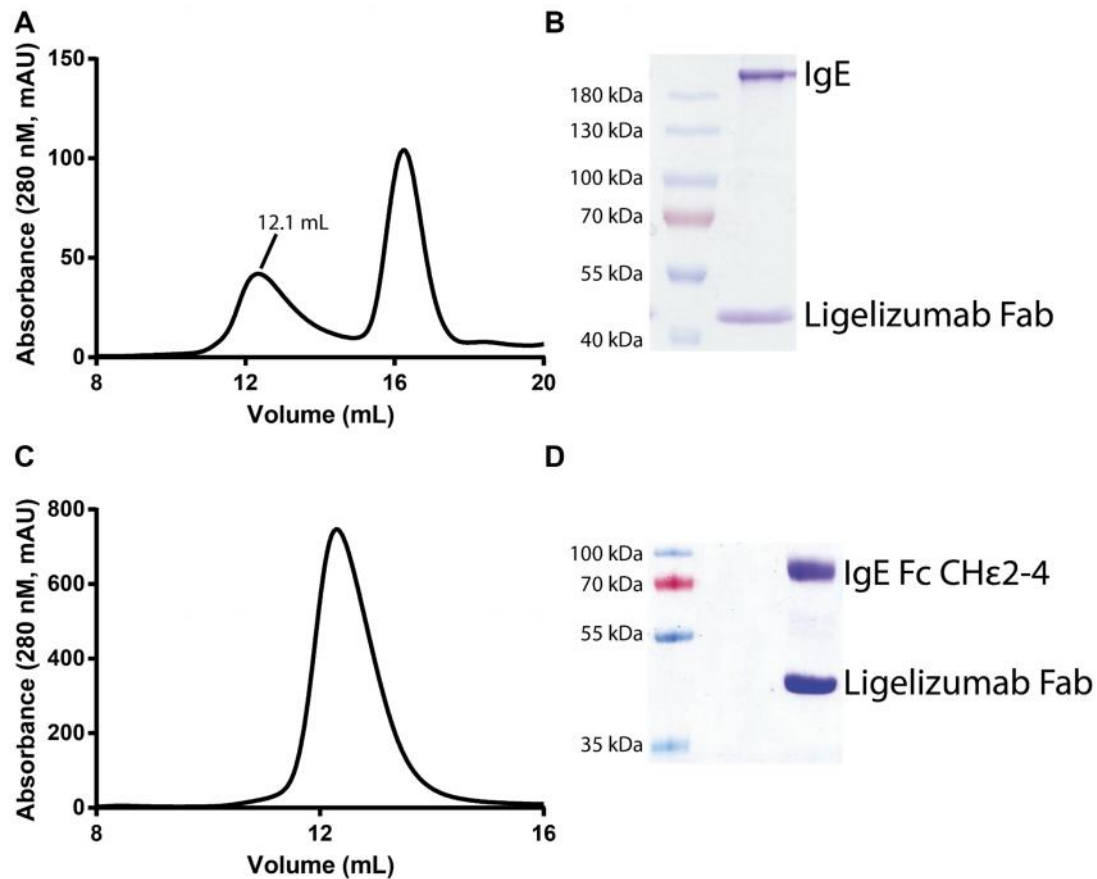


Figure S7. Complex formation between ligelizumab Fab and IgE HMM5 or IgE Fc.
A. SEC elution profile of IgE HMM5 in complex with ligelizumab Fab. **B.** SDS-PAGE analysis of the two top fraction from the peak at 12.1 mL in panel A. **C.** SEC elution profile of the IgE Fc – ligelizumab Fab complex. **D.** SDS-PAGE analysis of the two top fractions from the SEC in panel C.

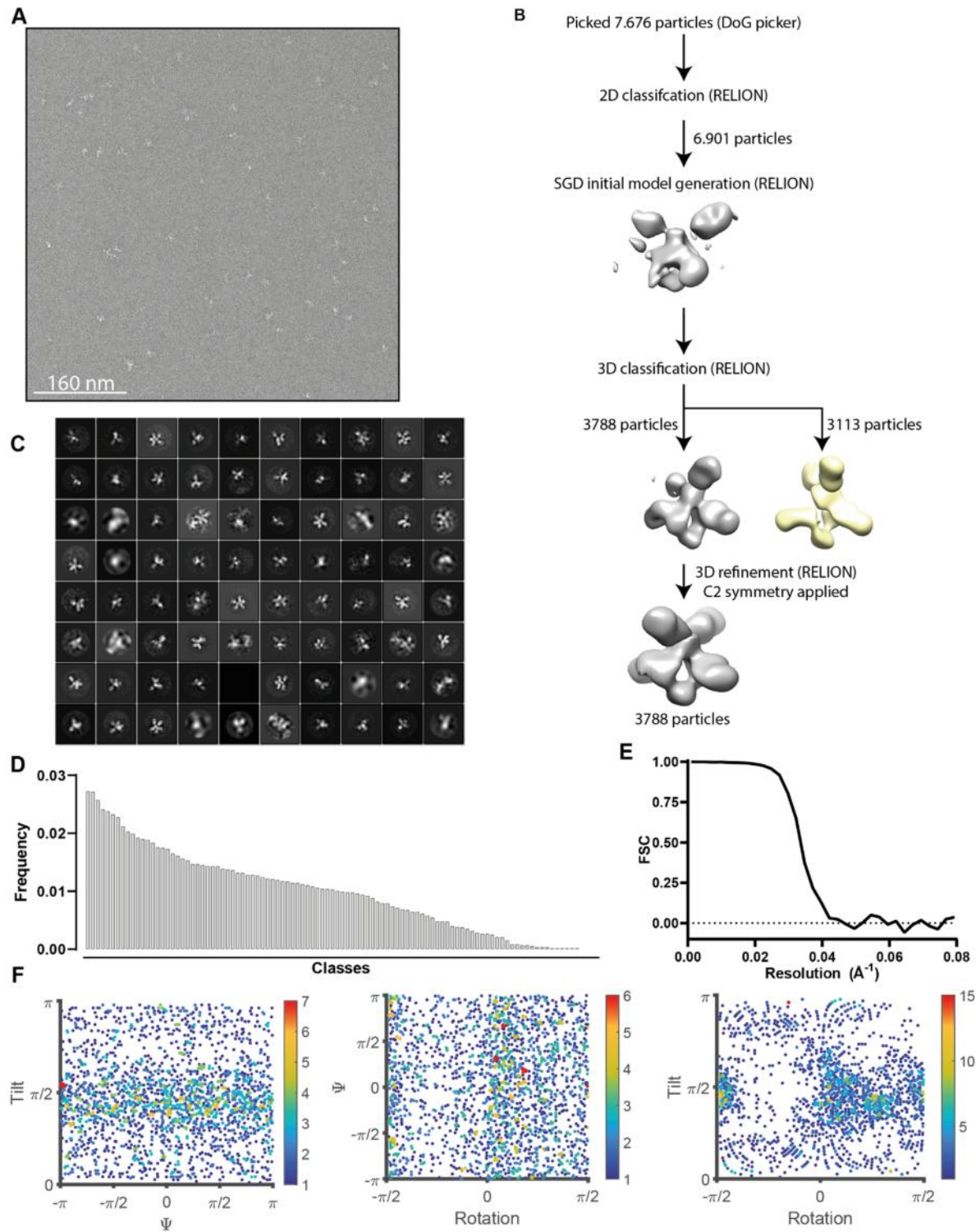


Figure S8. Single particle EM processing of IgE HMM5 in complex with ligelizumab Fab. **A.** A representative micrograph of IgE HMM5 in complex with ligelizumab Fab. **B.** Processing flow-chart showing the details and number of particles used for each step of the single particle analysis. **C.** All 2D class averages of the IgE HMM5:ligelizumab Fab complex. **D.** Occupancy plot of the 2D classification showing the distribution of particles in different classes. **E.** Fourier shell correlation between the two half-sets from the final 3D refinement in RELION. **F.** Distribution of particle orientations of the particles in the final 3D refinement in RELION plotted as 2D heat-maps against pairs of Euler angles. Due to the 2-fold symmetry the rotation axis is shown from $-\pi/2$ to $\pi/2$.

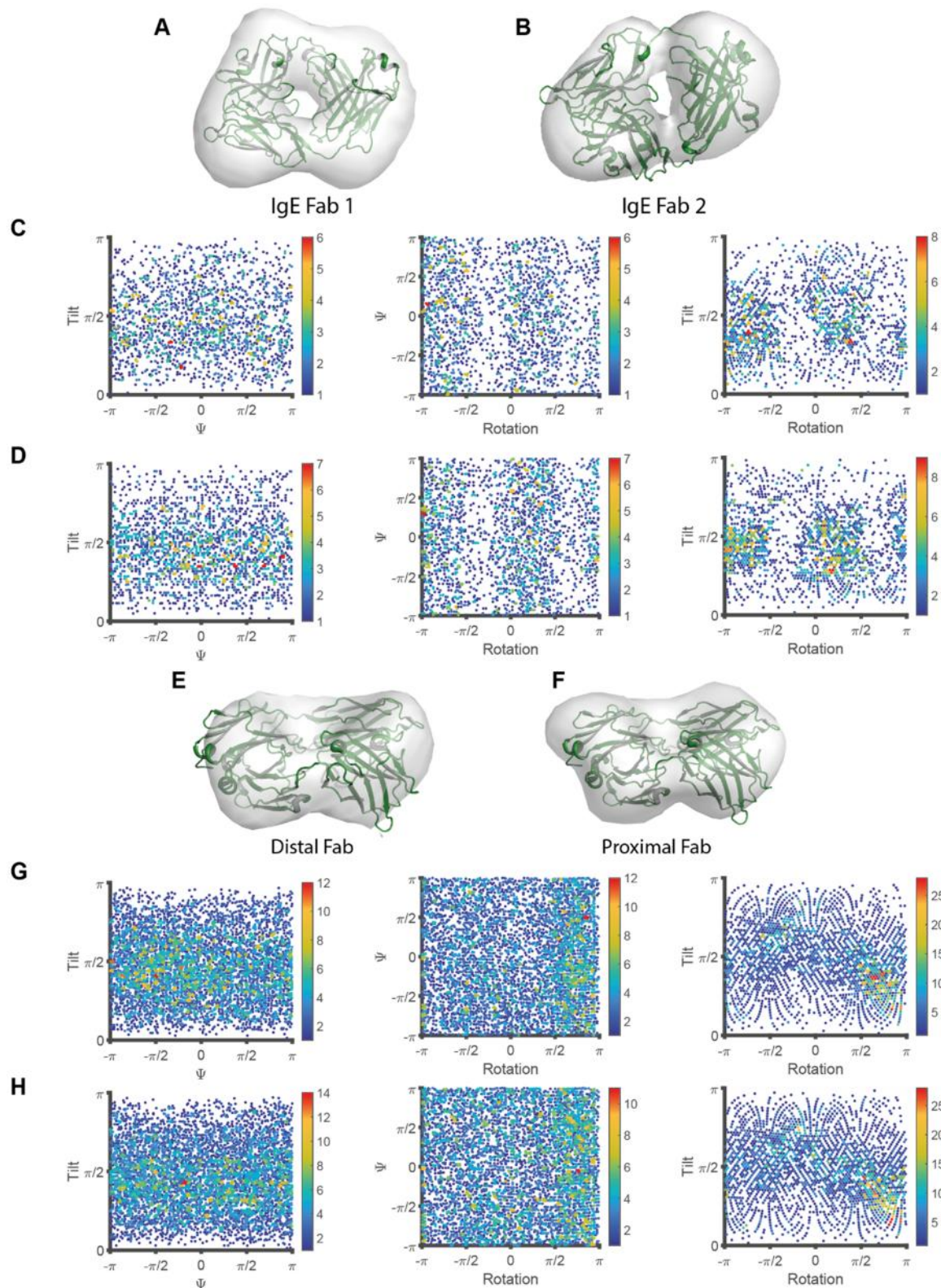


Figure S9. Focused classification of the IgE Fabs of the IgE HMM5:ligelizumab Fab complex and unbound IgE HMM5. **A-B.** Reconstruction of the Fabs within the mask used for the focused classification. **C-D.** Distribution of particle orientations after focused classification, plotted as a 2D heat-map against pairs of Euler angles. **E.** Reconstruction of the distal Fab within the mask used for the focused classification. **F.** Same as panel E but for the proximal Fab. **G-H.** Same as panels A-B but for the distal and proximal Fab of IgE HMM5, respectively.

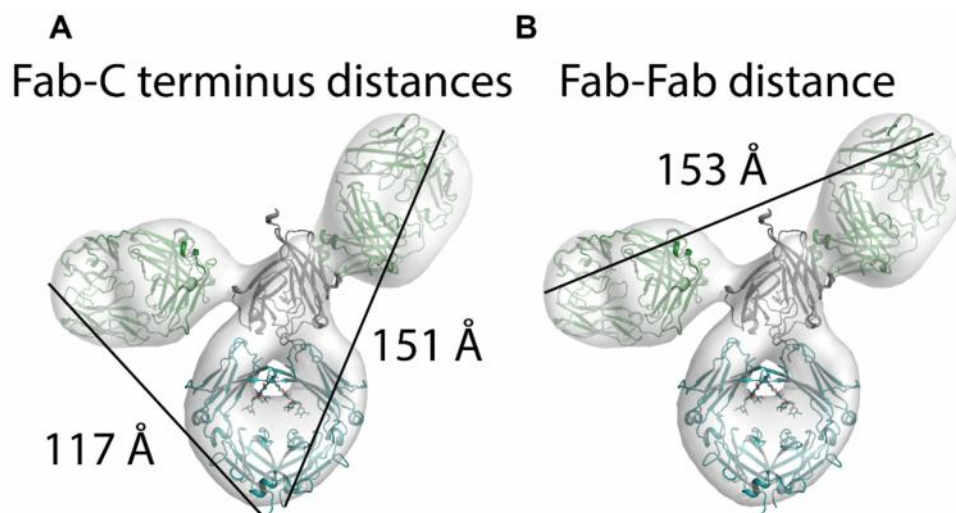


Figure S10. Characteristic geometric features for a docked model of intact IgE HMM5. **A.** The two non-equivalent distances from the Fab antigen binding sites to the C-terminal of IgE are indicated. **B.** The distance between the antigen binding sites of the two IgE Fabs. The separation of the antigen binding site was determined as the distance between two points each representing the centre of mass of the outermost residues in the CDR3 loops in one Fab.

Materials and Methods

Protein production

IgE Fc Cε2-4 was expressed and purified as described in ¹. The full-length IgE HMM5 (Fig. S1), was expressed in insect cells and purified as described in ². Full-length IgE H10 (Fig. S1 & S3) was expressed in HEK293 cells and purified using IMAC affinity chromatography and anti-IgE affinity chromatography.

The DNA of ligelizumab Fab was obtained as a synthetic gene and cloned into the pFastBac Dual expression vector for baculovirus-mediated expression in insect cells. Sf9 insect cells (Life technologies) were grown at 27 °C in serum free medium (HyClone™ SFX-Insect medium containing 16.5 mM glutamine and 10 g/mL gentamycin). Recombinant baculovirus was generated by transfection of Sf9 cells with bacmid DNA isolated from DH10Bac cells transformed with the ligelizumab Fab expression vector. Sf9 cells were inoculated with viral supernatant for 3 days and cryopreserved. These cryo stocks were used to infect Sf9 cells (1.5×10^6 cells/mL) in 500 mL suspension cultures. For protein production, the cells were incubated at 27 °C with agitation at 110 rpm for 120 h. The supernatant was harvested and applied to a 1 mL kappaSelect column (GE Healthcare) equilibrated in PBS. The column was washed with 10 mL PBS and the protein was eluted by a linear gradient from PBS into 100 mM glycine pH 2.0. The 1 mL fractions were directly neutralised with 500 µL 1M TRIS pH 7.5. Ligelizumab Fab was dialyzed against 20 mM MES, 50 mM NaCl, pH 6 and subsequently applied to a 1 mL MonoS column (GE Healthcare) equilibrated in 20 mM MES, 50 mM NaCl, pH 6. The column was washed with 10 mL 20 mM MES, 50 mM NaCl, pH 6, followed by a 30 mL linear gradient from 50-500 mM NaCl. A final polishing step was performed using a 24 ml Superdex 200 20/300 GL column (GE Healthcare) equilibrated in 20 mM HEPES, 50 mM NaCl, pH 7.2.

Characterization of IgE and ligelizumab

For comparative assessment of anti-IgE antibodies, ELISA microtiter plates (Greiner) were coated with purified 026 sdab, omalizumab Fab, and ligelizumab Fab ($50 \mu\text{g ml}^{-1}$) at 4°C overnight. After blocking with 4% milk (w/v) in PBS, human HMM5 hIgE ($10 \mu\text{g/ml}^{-1}$) in 2% milk (w/v) in PBS was incubated for 4 h at RT. Bound IgE was detected using alkaline phosphatase-conjugated goat anti-human IgE (Sigma) 1:10000 and 50 μl of substrate solution (5 mg ml^{-1} 4-nitro-phenylphosphate, AppliChem) at 405 nm.

The binding of H10 IgE to its antigen, the major house dust mite allergen Der p 2, was assessed in ELISA. Purified Der p 2 ($2 \mu\text{g ml}^{-1}$, Indoor Bioscience) was coated on microtiter plates (Greiner) at 4°C overnight and blocked with 4% milk (w/v) in PBS. Thereafter, the H10 IgE was incubated overnight at 4°C . After washing, binding to Der p 2 was detected using alkaline phosphatase-conjugated anti-human IgE antibody (Sigma) diluted 1:10,000 and 50 μl of substrate solution as above.

Affinity determination using Biolayer Interferometry

BLI measurements (Fig. S5) were performed at 30°C on an Octet RED96 machine (ForteBio) using amine reactive second-generation (AR2G) biosensors (ForteBio). IgE Fc-coated sensors were prepared by dipping the sensors into a solution of IgE Fc at $1 \mu\text{g/ml}$ in 10 mM Na acetate pH 5.0 for 100 s. The reaction was quenched for 300 s in 1 M ethanolamine pH 8.5 and the baseline was obtained after 120 s wash in kinetic buffer (PBS, 0.02% Tween20, 0.1% BSA). Association with ligelizumab Fab (2-fold dilution series from $1 \mu\text{M}$ to 31.25 nM in kinetic buffer) was performed over 300 s and the dissociation phase in the same buffer was monitored for 600 s. For each point in the dilution series, binding of ligelizumab Fab in the absence of IgE Fc was used as a reference to subtract the non-specific interactions with the biosensor. The curves were

fitted with a global 2:1 heterogeneous ligand binding model, assuming two binding sites for Ligelizumab Fab on each IgE Fc using the ForteBio data analysis 7.1 software.

Human material

The study was approved by the Ethics Committee of the Medical Faculty of Philipps University, Marburg, Germany; the donors provided written informed consent to participate in the trial.

Basophil activation tests and mediator release assays

Peripheral EDTA blood from an allergic donor was preincubated with ligelizumab, 026 sdab, or omalizumab (Novartis) at final concentrations ranging from 0 to a maximum of 20 $\mu\text{mol l}^{-1}$, in IL-3-containing stimulation buffer (Bühlmann) for 30 min on a shaker. Samples were stained with anti-CCR3-PE (2.5 $\mu\text{g/ml}$) in a 37 °C water bath for 15 min and anti-IgE-FITC (1.25 $\mu\text{g/ml}$) (both BioLegend) for 20 min at 4 °C. Erythrocytes were lysed for 7 min followed by centrifugation at 500×g for 5 min. Cell pellets were resuspended in 100 μl washing buffer (Bühlmann) and the mean fluorescence intensity (MFI) of IgE on CCR3⁺SSC^{low} basophils was measured by flow cytometry (FACS Calibur, BD Biosciences). Results were analyzed using BD FACSDiva software (BD Biosciences).

In order to analyze surface IgE of RBL-SX38 cells (kindly provided by J.P. Kinet), the cells were sensitized with 1 $\mu\text{g/ml}$ rIgE at 37 °C and 5% CO₂. After washing with PBS, the cells were incubated for 1 h with ligelizumab Fab and 026 sdab in culture medium (0-12.5 $\mu\text{mol l}^{-1}$). The cells were detached with 10 mM EDTA in DPBS supplemented additionally with 0.03 M NaCl, stained with goat anti-IgE FITC (Betyl) for 30 min at 4 °C, and analyzed by flow cytometry (Sony SH800).

Analyses of CD23 binding using the ELIFAB assay

The ELIFAB assay, an ELISA-based cell-free variant of the FAB assay, was performed to determine the capacity of ligelizumab, 026 sdab and omalizumab to prevent binding of IgE:allergen complexes to CD23³. In brief, an allergen-specific indicator serum (Api m 1-specific IgE concentration $>100 \text{ kUA l}^{-1}$) was preincubated with the respective allergen (Api m 1, Latoxan) in the presence of different concentrations of ligelizumab, 026 sdab or omalizumab in order to form IgE:allergen complexes. After complex formation at 37 °C for 1 h, IgE:allergen complexes were transferred to plates coated with CD23 (R&D Systems, Bio-Techne) and incubated for another 1 h at RT. Immobilized CD23-bound IgE:allergen complexes were detected by adding biotin-conjugated anti-human IgE antibody (BD Biosciences), streptavidin-peroxidase (Sigma-Aldrich), and 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Calbiochem, Merck Millipore). All samples were analyzed in duplicates. In addition, to evaluate the ability of ligelizumab, 026 sdab and omalizumab to displace IgE:allergen complexes from immobilized CD23, preformed IgE:allergen complexes bound to CD23 were incubated with either ligelizumab, 026 sdab or omalizumab up to 60 min at a final concentration of $0.25 \text{ } \mu\text{mol l}^{-1}$.

SAXS data collection and modeling

SAXS data collection of IgE was performed in batch mode at the BM29 beamline at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. The data were collected using a PILATUS 1M detector and $\lambda=0.992 \text{ Å}$ in a temperature controlled capillary at 4°C. The sample-to-detector distance was 2.862 m, covering a range of momentum transfer $0.04 < q < 5 \text{ nm}^{-1}$. Four SAXS measurements were performed on IgE in 20 mM HEPES, 50 mM NaCl, pH 7.2 between 0.3 and 1.5 mg/mL. Data analysis was performed using the ATSAS package⁴. The R_g and I_0 increased linearly with concentration, so the data were extrapolated to infinite dilution to remove any interparticle interference effects using ALMERGE. The molecular weight of IgE was estimated using

BSA as a standard with $I(0)=59.5$ and a molecular weight (MW) of 72 kDa and taking into account the BSA monomer-dimer equilibrium ⁵. The pair distribution function was calculated by indirect Fourier Transform using GNOM. DAMMIF was used to generate 20 ab initio models of IgE, which were subsequently aligned and clustered using the DAMAVER suite. Three clusters containing 17, 2, and 1 models were identified by damclust. The major cluster containing the 17 models were averaged and filtered using the DAMAVER suite.

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