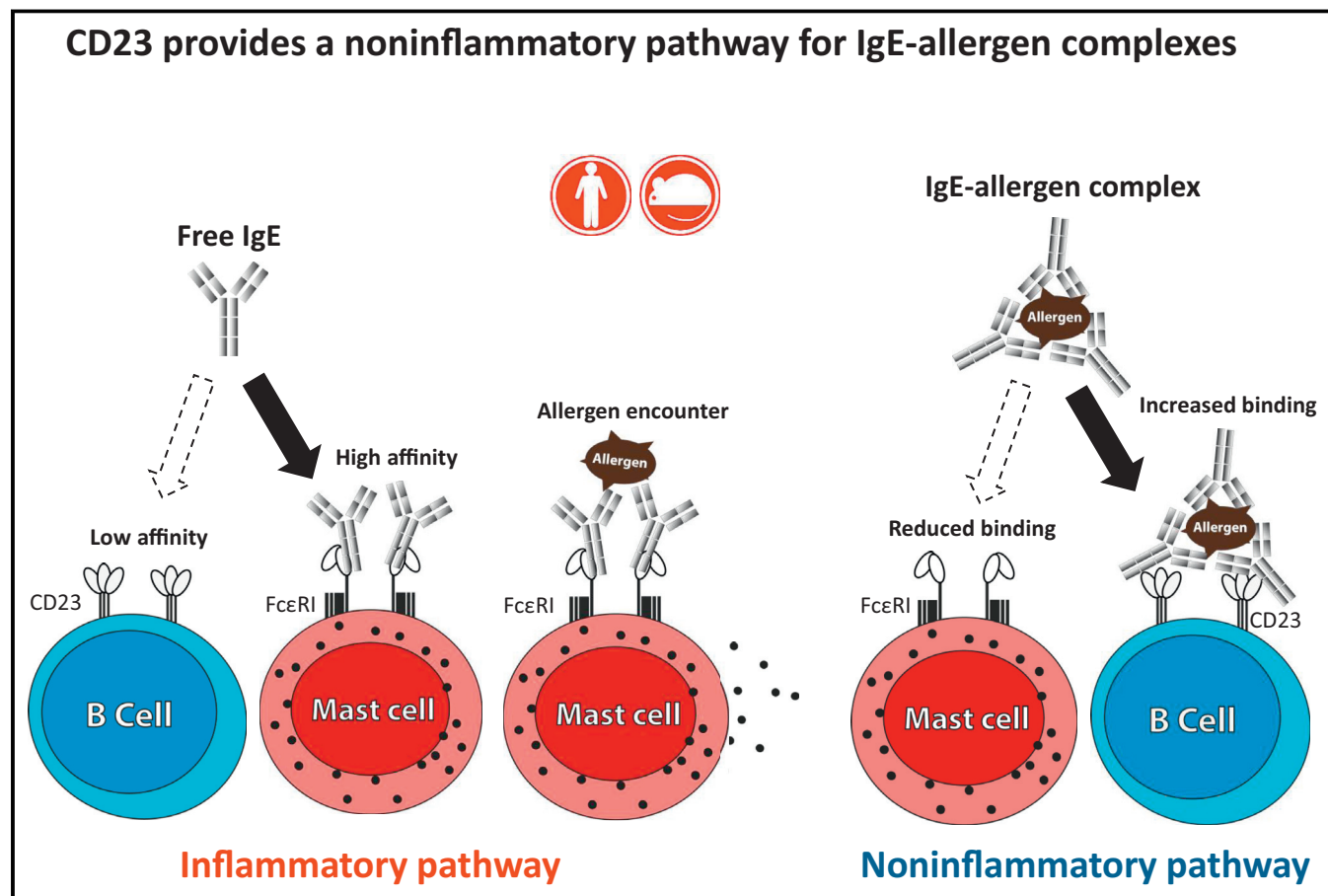


CD23 provides a noninflammatory pathway for IgE-allergen complexes



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GRAPHICAL ABSTRACT



Background: Type I hypersensitivity is mediated by allergen-specific IgE, which sensitizes the high-affinity IgE receptor FcεRI on mast cells and basophils and drives

allergic inflammation upon secondary allergen contact. CD23/FcεRII, the low-affinity receptor for IgE, is constitutively expressed on B cells and has been shown to

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regulate immune responses. Simultaneous binding of IgE to FcεRI and CD23 is blocked by reciprocal allosteric inhibition, suggesting that the 2 receptors exert distinct roles in IgE handling.

Objective: We aimed to study how free IgE versus precomplexed IgE-allergen immune complexes (IgE-ICs) target the 2 IgE receptors FcεRI and CD23, and we investigated the functional implications of the 2 pathways.

Methods: We performed binding and activation assays with human cells *in vitro* and IgE pharmacokinetics and anaphylaxis experiments *in vivo*.

Results: We demonstrate that FcεRI preferentially binds free IgE and CD23 preferentially binds IgE-ICs. We further show that those different binding properties directly translate to distinct biological functions: free IgE initiated allergic inflammation through FcεRI on allergic effector cells, while IgE-ICs were noninflammatory because of reduced FcεRI binding and enhanced CD23-dependent serum clearance.

Conclusion: We propose that IgE-ICs are noninflammatory through reduced engagement by FcεRI but increased targeting of the CD23 pathway. (J Allergy Clin Immunol 2020;145:301-11.)

Key words: IgE sensitization, IgE-allergen complex, CD23, FcεRI, IgE clearance, inflammation

Allergic subjects respond with inflammation to typically harmless environmental substances, such as dust mites, pollen, or animal dander, and the number of affected persons is increasing worldwide.¹⁻⁴ Allergic responses arise through generation of allergen-specific IgE, which binds with high affinity to the FcεRI receptor expressed by mast cells or basophils, a process referred to as IgE sensitization.^{3,5} Allergen-specific IgE is then displayed on the cell surface, and an allergic response is triggered when secondary allergen exposure induces cross-linking of FcεRI, resulting in degranulation of mast cells and basophils and causing release of preformed and *de novo*-synthesized inflammatory mediators.^{6,7}

The low-affinity IgE receptor CD23 (FcεRII) is expressed in a variety of cells, but its function in the context of allergic responses is not yet fully understood.^{8,9} CD23 is constitutively expressed in B cells and has been shown to negatively regulate IgE levels.¹⁰⁻¹⁷ Furthermore, it has been shown that CD23 facilitates induction of antibody and T-cell responses.¹⁸⁻²² IgE binding to its 2 primary receptors, FcεRI and CD23, is uniquely regulated by dynamic IgE structure-receptor relationships, whereas direct interaction between the 2 receptors through IgE molecules is prohibited by reciprocal allosteric inhibition.²³⁻²⁷ This cross-inhibition is thought to be regulated by conformational plasticity of the IgE antibody, which can adopt 2 conformational states. Within the IgE constant region, Cε3 and Cε4 can adopt an “open” conformation when bound to FcεRI and a “closed” conformation while binding CD23.²⁷⁻³⁰ However, the functional implications of IgE regulation by FcεRI and CD23, especially in the presence of allergen, are less clear.

The reasons why allergens, a quite broadly defined group of proteins, elicit IgE responses and are highly reactive in sensitized effector cells are still not known. However, many shared patterns and characteristics of allergens have been described.³¹ It has been shown that the number, affinity, and proximity of IgE-binding sites

Abbreviations used

APC: Allophycocyanin
CD23KO: CD23 knockout mice on BALB/c background
FITC: Fluorescein isothiocyanate
IgE-IC: IgE-allergen immune complex

determine optimal properties of allergens to induce degranulation of effector cells.^{32,33} Recently, we have shown that allergens displayed on virus-like particles do not induce mast cell activation, showing that structurally modifying allergens can lead to a loss of FcεRI response.³⁴ In regard to CD23 interaction, IgE clonality, avidity, and affinity have been shown to impact IgE-allergen binding and antigen presentation.^{35,36}

Both IgE receptors seem to have distinct functions in the context of allergy; however, the mechanisms that regulate the anaphylactic potential of allergens by leading to differential targeting of IgE receptors are still unclear.

Here we investigated FcεRI versus CD23 binding and the functional implications of those interactions. We define 2 modes of allergen-IgE-receptor binding: (1) allergen binding to cells that have previously bound IgE with FcεRI or CD23 we refer to as “IgE sensitization” and (2) allergen binding to IgE-forming immune complexes that subsequently bind to IgE receptors we term “IgE-allergen immune complexes” (IgE-ICs). We show that the 2 modes determine the targeting of FcεRI or CD23 and functionally dictate the induction of allergic inflammation.

METHODS

Human B cells and mast cells

Buffy coats were purchased from the blood donation center (Bern, Switzerland). This study is approved by the local ethics committee. PBMCs were isolated by using density gradient centrifugation on Ficoll-Paque (GE Healthcare, Chalfont St Giles, United Kingdom). B cells were isolated from PBMCs by using negative selection (STEMCELL Technologies, Grenoble, France). B cells were cultured in 48-well plates at 1×10^6 cells/mL in RPMI 1640 (Gibco, Grand Island, NY) containing 10% FBS (Thermo Fisher Scientific, Cramlington, United Kingdom), 40 μg/mL transferrin (Calbiochem, Darmstadt, Germany), 50 μmol/L mercaptoethanol (Sigma, St Louis, Mo), 1 mmol/L sodium pyruvate (Sigma), 4 μg/mL bovine insulin (Sigma), 2 mmol/L L-glutamine (Gibco), and 1:100 Minimum Essential Medium nonessential amino acids (Gibco). B cells were activated with 20 ng/mL IL-4 (PeproTech, Rocky Hill, NJ) and 1 μg/mL anti-CD40 antibody (Thermo Fisher Scientific) overnight. Mast cell progenitors were isolated from PBMCs by using CD133 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and *in vitro* matured over 8 weeks, as previously described.^{34,37} Cells were cultured in Gibco StemPro (Thermo Fisher Scientific) containing supplements, 100 ng/mL Gibco stem cell factor (Thermo Fisher Scientific), and 40 ng/mL IL-6 (BioLegend, San Diego, Calif). Additionally, medium contained 10 ng/mL IL-3 (BioLegend) for the first 3 weeks, and after 6 weeks, medium was supplemented with 10 ng/mL IL-4 (PeproTech) and 10% FCS.

Mice

Wild-type BALB/c mice were purchased from Envigo, Huntingdon, United Kingdom. CD23 knockout mice on BALB/c background (CD23KO mice)³⁸ were kindly provided by Professor J. Ravetch (Rockefeller University, New York, NY). All animals were treated for experimentation according to protocols approved by the Swiss Federal Veterinary Office, were used for experiments at the age of 6 weeks, and were kept at the DBMR Animal Facility (Bern, Switzerland).

IgE–Fel d 1 immune complexes

Recombinant Fel d 1 and Fel d 1–specific IgE and IgG mAbs were produced, as previously described.^{34,39,40} Fel d 1 was used in its heterodimeric form, which is required to activate mast cells with one IgE clone and is necessary to form IgE–ICs. The antibodies have been genetically engineered to be expressed as chimeric human/mouse or mouse antibodies and were produced in CHO cells (Evitria AG, Zurich, Switzerland) and purified by affinity chromatography using protein L or protein G Sepharose columns (GE Healthcare), respectively. All mouse experiments were performed with the mouse IgE clone F127, whereas all human experiments were performed with chimeric human murine IgE clone F127. IgE and Fel d 1 were preincubated for 15 minutes at 37°C to generate IgE–Fel d 1 complexes. For *in vitro* binding and CD63 activation experiments, Fel d 1 concentrations were constant at 6.25 nmol/L, whereas IgE was titrated from 0.39 to 100 nmol/L (data are displayed as IgE/Fel d 1 ratio). For calcium flux and *in vivo* experiments, complexes were always formed at a 1:1 molecular ratio.

Binding assays

Titrated doses of IgE were added to mast cells or B cells for 30 minutes at 4°C, washed, and incubated with 6.25 nmol/L Fel d 1 for 30 minutes at 4°C to assess IgE binding. This binding protocol is referred to as “IgE sens + Fel d 1.” Alternatively, titrated doses of IgE were directly added in complex with 6.25 nmol/L Fel d 1. This binding protocol is referred to as “IgE–Fel d 1.” IgE binding was then evaluated by using anti-IgE staining for 30 minutes at 4°C, followed by analysis with flow cytometry. Fel d 1 binding was assessed by using anti–Fel d 1 staining for 30 minutes at 4°C, followed by analysis with flow cytometry.

Activation assays

Activation assays for stem cell–derived human mast cells, flow cytometry–based assessment of the activation marker CD63, and calcium flux protocols were performed, as previously described by our group, using the same reagents.³⁴ β -Hexosaminidase release assays from Fig E1, D, in this article’s Online Repository at www.jacionline.org were performed with mouse bone marrow–derived mast cells by using the isolation protocol, buffer, and reagents, as previously described by our group.⁴¹ The notable difference for activation assays in this study compared with previous studies is that IgE was either added, followed by a washing step with medium, and subsequently challenged with Fel d 1 (IgE sens + Fel d 1) or IgE and Fel d 1 were added as a complex (IgE–Fel d 1).

Passive systemic anaphylaxis

For passive systemic anaphylaxis experiments, 21.5 μ g of IgE F127/100 μ L of PBS was administered per BALB/c or CD23KO mouse for sensitization with free IgE by means of intravenous injection 1 day before allergen challenge. The next day, 5 μ g of Fel d 1/100 μ L PBS per mouse was injected as an allergen challenge. Alternatively, IgE–ICs were injected as a mix of 21.5 μ g of IgE plus 5 μ g of Fel d 1 per 100 μ L of PBS. For Fig E2, D, in this article’s Online Repository at www.jacionline.org, 10 μ g of IgE and 2.5 μ g of Fel d 1 were used. To assess anaphylaxis, baseline body temperature was measured with the MiniTemp rectal probe for mice (Vetronic Services, Abbotskerswell, United Kingdom). Rectal temperature was measured at 10-minute intervals for 1 hour.

Flow cytometry and staining antibodies

For activation assays, mast cells were stained with fluorescein isothiocyanate (FITC)–labeled anti-CD63 clone H5C6 (BD Biosciences, Franklin Lakes, NJ) antibodies. Marker expressions were assessed with the allophycocyanin (APC)–labeled anti-human c-kit clone 104D2 (BioLegend) and the phycoerythrin-labeled anti-human Fc ϵ RI clone AER-37 (BioLegend), and IgE binding was assessed with the APC-labeled anti-IgE clone MB10-5C4 (Miltenyi Biotec). Mast cell binding of Fel d 1 was assessed by staining with the FITC-labeled monoclonal mouse anti–Fel d 1 IgG recognizing the

nonoverlapping epitope G078.³⁹ Murine basophils were marked with the APC-labeled anti-mouse CD49b clone HM α 2 (BioLegend) and the phycoerythrin-labeled anti-mouse IgE clone RME-1 (BioLegend), whereas B cells were marked with the peridinin-chlorophyll-protein complex–Cy5.5–labeled anti-mouse CD19 clone eBio1D3 (Thermo Fisher Scientific). Contamination with mast cells was excluded by staining with FITC-labeled anti-mouse CD117 clone 2B8 (BD Biosciences). Flow cytometry was performed with the Guava easyCyte (Merck Millipore, Darmstadt, Germany) or BD FACSCanto (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, Ore).

ELISA

For determination of serum IgE titers, 96-well Nunc MaxiSorp ELISA plates (Thermo Fisher Scientific, Waltham, Mass) were coated with 2 μ g/mL of the rat anti-mouse anti-IgE clone R35-72 (BD Biosciences) in PBS at 4°C overnight. After blocking with PBS/0.15% casein solution for 2 hours, plates were washed 5 times with PBS/0.05% Tween. Serum dilutions were added to the plates and incubated for 2 hours at room temperature. Plates were then washed 8 times with PBS. Thereafter, horseradish peroxidase–labeled goat anti-mouse IgE (Bio-Rad Laboratories, Hercules, Calif) antibodies were incubated at room temperature for 1 hour. ELISAs were developed with tetramethylbenzidine and H₂O₂ and stopped with 1 mol/L sulfuric acid. ODs were measured at 450 nm. Half-maximal antibody titers are defined as the reciprocal of the dilution leading to half of the OD measured at saturation.

Blue native PAGE

IgE–Fel d 1 complex analysis with blue native PAGE was adapted from previously established protocols.⁴² The reagents used for stacking gel (4%), as well as 15% resolving gels made from acrylamide/Bis, tetramethylethylenediamine, and ammonium persulfate were all from Bio-Rad Laboratories. Cathode buffers contained 7.5 mmol/L imidazole (Merck-Millipore, Burlington, Mass) and 50 mmol/L tricine (Santa Cruz Biotechnology, Santa Cruz, Calif) with either 0.02% Coomassie Brilliant Blue G-250 (Sigma) or 0.002%. Anode buffer contained 25 mmol/L imidazole, and loading buffer contained 50 mmol/L imidazole and 50 mmol/L NaCl (Sigma) and 5% glycerol (Sigma). Running buffer contained 50 mmol/L imidazole (Merck-Millipore) and 50 mmol/L NaCl. Gels were destained with a 10% acetic acid and 30% ethanol solution.

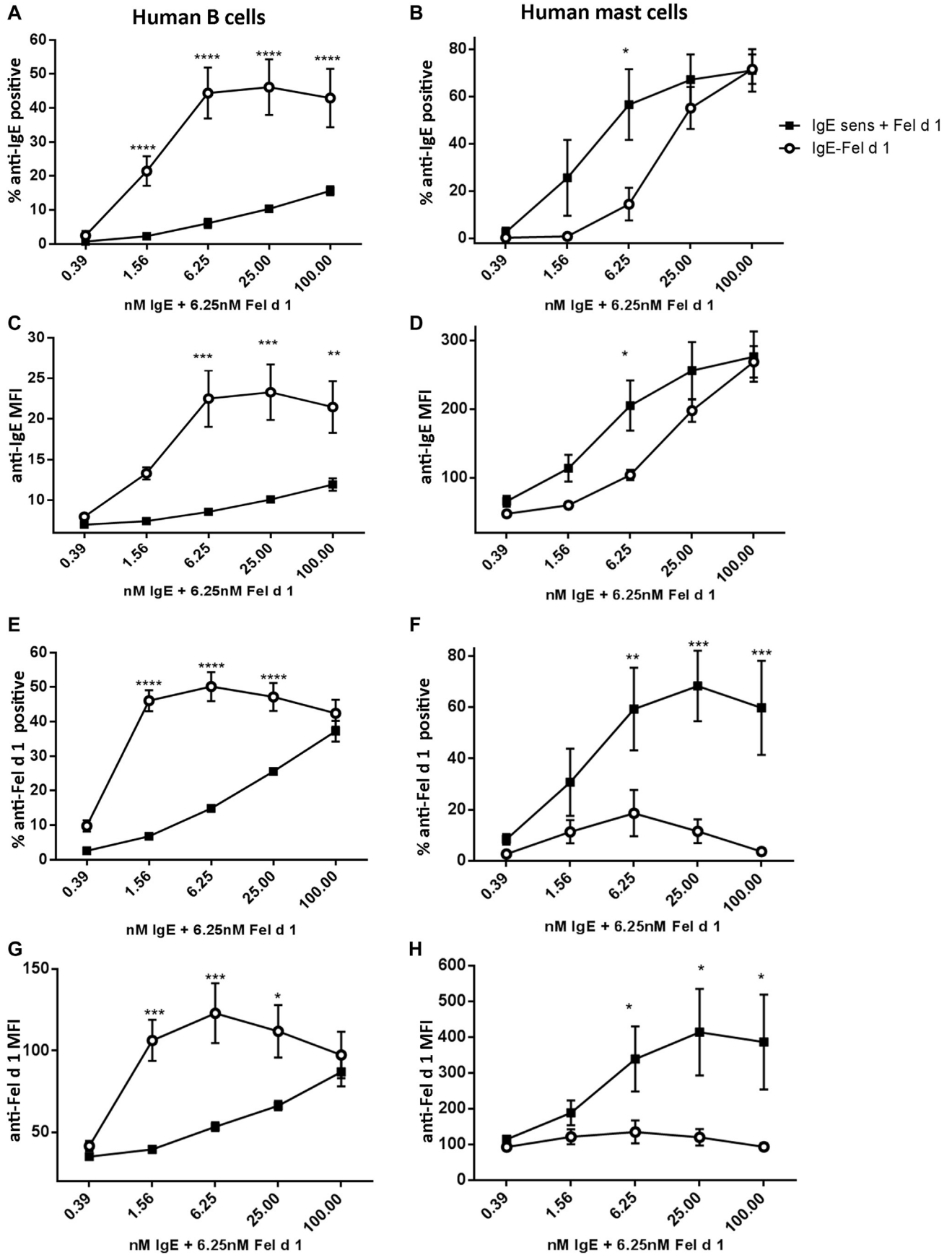
Statistical analysis

Statistical tests were performed with GraphPad Prism 6.0 software (GraphPad Software, La Jolla, Calif). For all experiments, α values of .05 and statistical significance are shown. Two groups were analyzed by using the 2-tailed Student *t* tests. All data in graphs are displayed as mean $\pm$ SEM; multiple concentrations and time points were analyzed by 2-way ANOVA, followed by Tukey testing.

RESULTS

IgE–Fel d 1 complexation reduces IgE and Fel d 1 binding to Fc ϵ RI but enhances CD23 binding

To study the sensitization versus IgE–IC models in terms of binding to Fc ϵ RI and CD23, we compared binding of free IgE with IgE–Fel d 1 complexes to CD23 on human B cells or to Fc ϵ RI on human mast cells. We decided to perform IgE titrations to cover the whole range of IgE binding at different IgE/Fel d 1 ratios. To prevent activation and internalization of IgE receptors, we performed all binding at 4°C, even though results at 37°C showed similar results (data not shown). Mast cells or B cells were sensitized with free IgE, washed, and in a second step challenged with Fel d 1 or the cells were directly incubated with IgE–Fel d 1



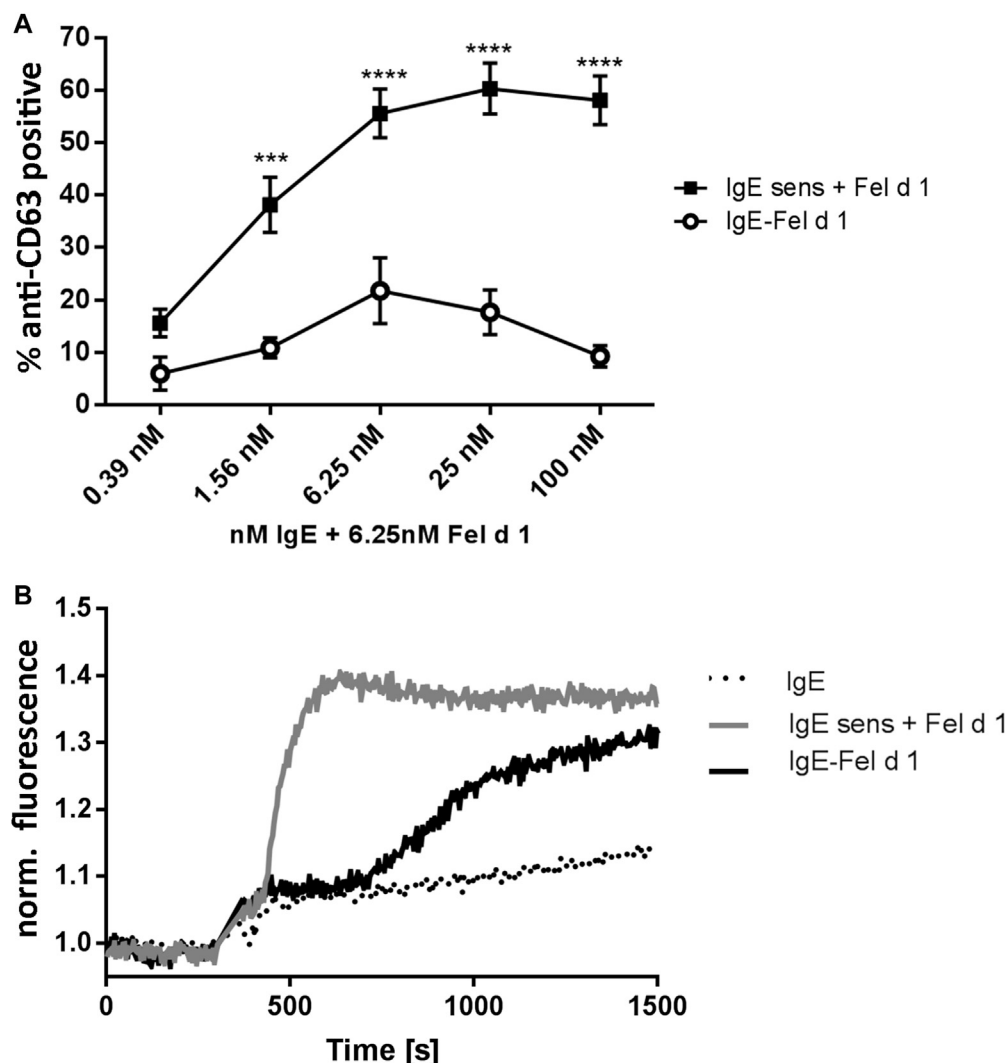


FIG 2. Complexation of IgE and allergen reduces degranulation of human mast cells *in vitro*. **A**, Mast cells were incubated with titrated doses of IgE, washed, and challenged with Fel d 1 (IgE sens + Fel d 1). Alternatively, IgE was preincubated with Fel d 1 and added to mast cells (IgE-Fel d 1). CD63 upregulation was assessed by using flow cytometry on anti-CD63 staining. Mean $\pm$ SEM CD63⁺ cells from 4 individual donors are displayed. Regular 2-way ANOVA followed by the Tukey test was performed for statistical analysis. *** $P \leq .001$ and **** $P \leq .0001$. **B**, Mast cells were labeled with Fluo-4-AM. Cells were either preincubated with IgE and challenged with Fel d 1 or directly challenged with IgE-Fel d 1 complexes. Shown is a representative calcium flux experiment.

complexes at 4°C. For assessing IgE binding, the cells were stained with anti-IgE antibodies for 30 minutes at 4°C. As seen in Fig 1, A-D, when the IgE/Fel d 1 ratios were 1 or less, free IgE bound better to mast cells than in the form of IgE-Fel d 1 complexes. In B cells the opposite was the case as binding of

free IgE was lower than with IgE in complex with Fel d 1. Representative histograms of IgE binding are shown in Fig E1, C and D.

We next assessed the 2 modes regarding allergen binding by staining the cells with anti-Fel d 1 antibodies for 30 minutes at

FIG 1. Complexation of IgE and allergen reduces FcεRI binding but enhances CD23 binding. Human B cells or mast cells were incubated with titrated doses of IgE, washed, and incubated with Fel d 1 (IgE sens + Fel d 1). Alternatively, IgE was preincubated with Fel d 1 and directly added to human B cells or human mast cells (IgE-Fel d 1). **A-D**, IgE binding was evaluated by using flow cytometry on anti-IgE staining. Shown are mean $\pm$ SEM percentages of IgE⁺ human B cells (Fig 1, A) or IgE⁺ human mast cells (Fig 1, B), as well as mean $\pm$ SEM anti-IgE mean fluorescence intensity (MFI) of human B cells (Fig 1, C) and human mast cells (Fig 1, D) on incubation with IgE sens + Fel d 1 or IgE-Fel d 1 binding assay protocols (4 individual donors). **E-H**, Fel d 1 binding was evaluated by using flow cytometry on anti-Fel d 1 staining. Shown are mean $\pm$ SEM percentages of Fel d 1-positive human B cells (Fig 1, E) or human mast cells (Fig 1, F) and mean $\pm$ SEM anti-Fel d 1 MFI of B cells (Fig 1, G) and mast cells (Fig 1, H) on incubation with IgE sens + Fel d 1 or IgE-Fel d 1 binding assay protocols (4 individual donors). * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$, and **** $P \leq .0001$.

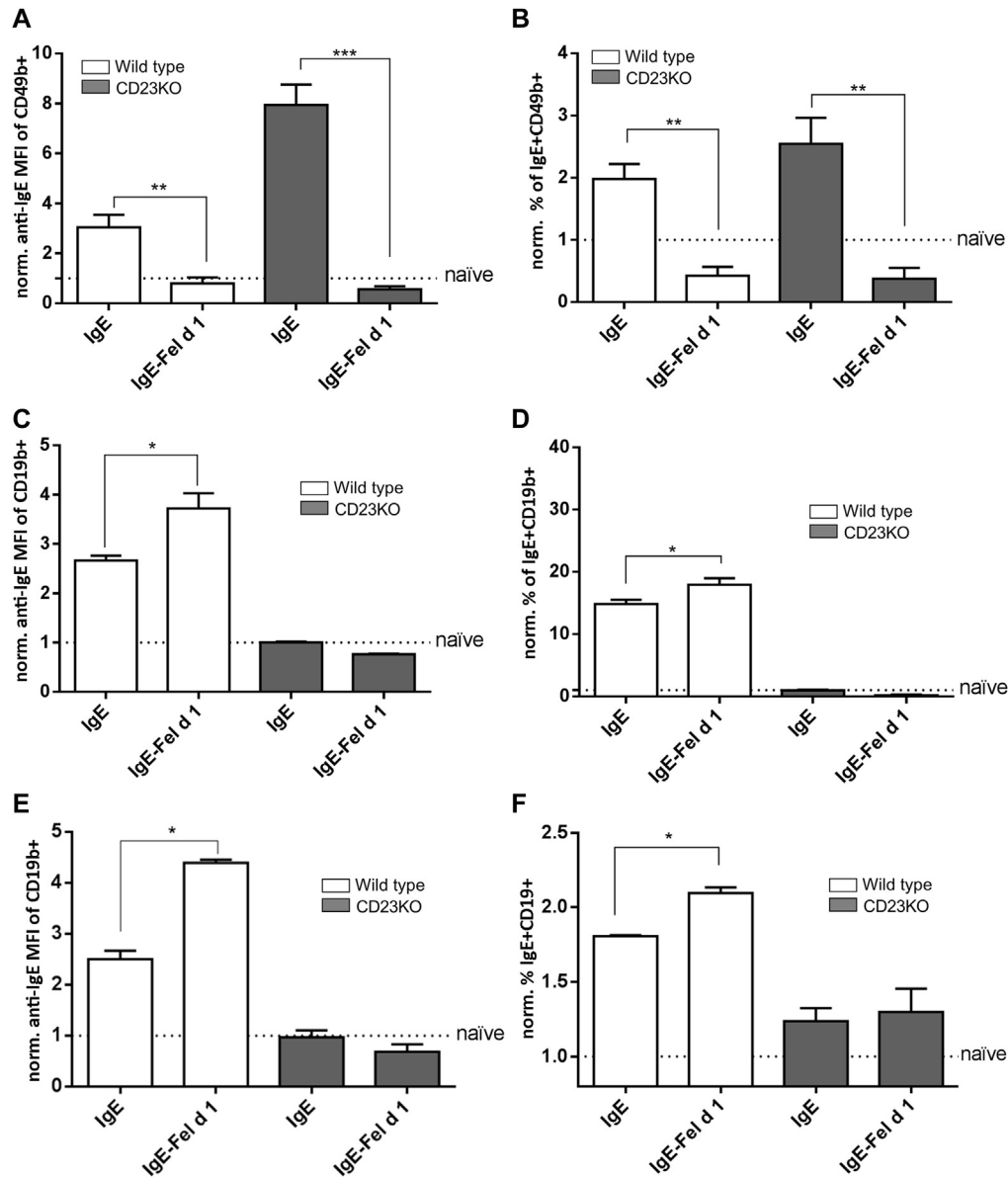


FIG 3. IgE-Fel d 1 complexes do not sensitize basophils but target B cells in mice. Murine IgE⁺ basophils (CD117⁺CD19⁺CD49b⁺IgE⁺) and murine B cells (CD117⁺CD49b⁺CD19⁺IgE⁺) were assessed by using flow cytometry for 3 hours on intravenous IgE or IgE-Fel d 1 injection in wild-type or CD23KO mice. All data are expressed relative to those for naive mice. **A**, Mean $\pm$ SEM of anti-IgE mean fluorescence intensity (MFI) of CD49b⁺ cells (n = 6 per group). **B**, Mean $\pm$ SEM of percentages of IgE⁺CD49b⁺ basophils (n = 6 per group). **C**, Mean $\pm$ SEM of IgE MFI of CD19⁺ blood B cells (n = 6 per group). **D**, Mean $\pm$ SEM percentages of IgE⁺CD19⁺ blood B cells (n = 6 per group). **E**, Mean $\pm$ SEM of IgE MFI of IgE⁺CD19⁺ splenic B cells (n = 6 per group). **F**, Mean $\pm$ SEM percentages of IgE⁺CD19⁺ splenic B cells (n = 6 per group). * $P \leq .05$, ** $P \leq .01$, and *** $P \leq .001$.

4°C (Fig 1, E-H). Again, Fel d 1 bound well to IgE-sensitized mast cells, whereas IgE-Fel d 1 complexes only showed poor binding. In contrast, B cells sensitized with IgE bound less Fel d 1 than when incubated with IgE-Fel d 1 complexes.

In summary, only free IgE binds well to mast cells, whereas complexed IgE preferentially binds to B cells and shows reduced mast cell binding. Because we did not observe binding of antigen to mast cells in a precomplexed form at an IgE/Fel d 1 ratio of greater than 1 but observed IgE binding to some extent, we speculated that IgE binding occurs because of the presence of free IgE. This was confirmed by using blue native PAGE, where we

showed that optimal complex size was achieved at an equimolar IgE/Fel d 1 ratio while some free IgE and Fel d 1 were still present (see Fig E3 in this article's Online Repository at www.jacionline.org).

Complexation of IgE and Fel d 1 results in less degranulation of human mast cells *in vitro*

We next investigated whether differential binding of free IgE or IgE-Fel d 1 complexes has functional implications for the induction of *in vitro* degranulation of human mast cells. To

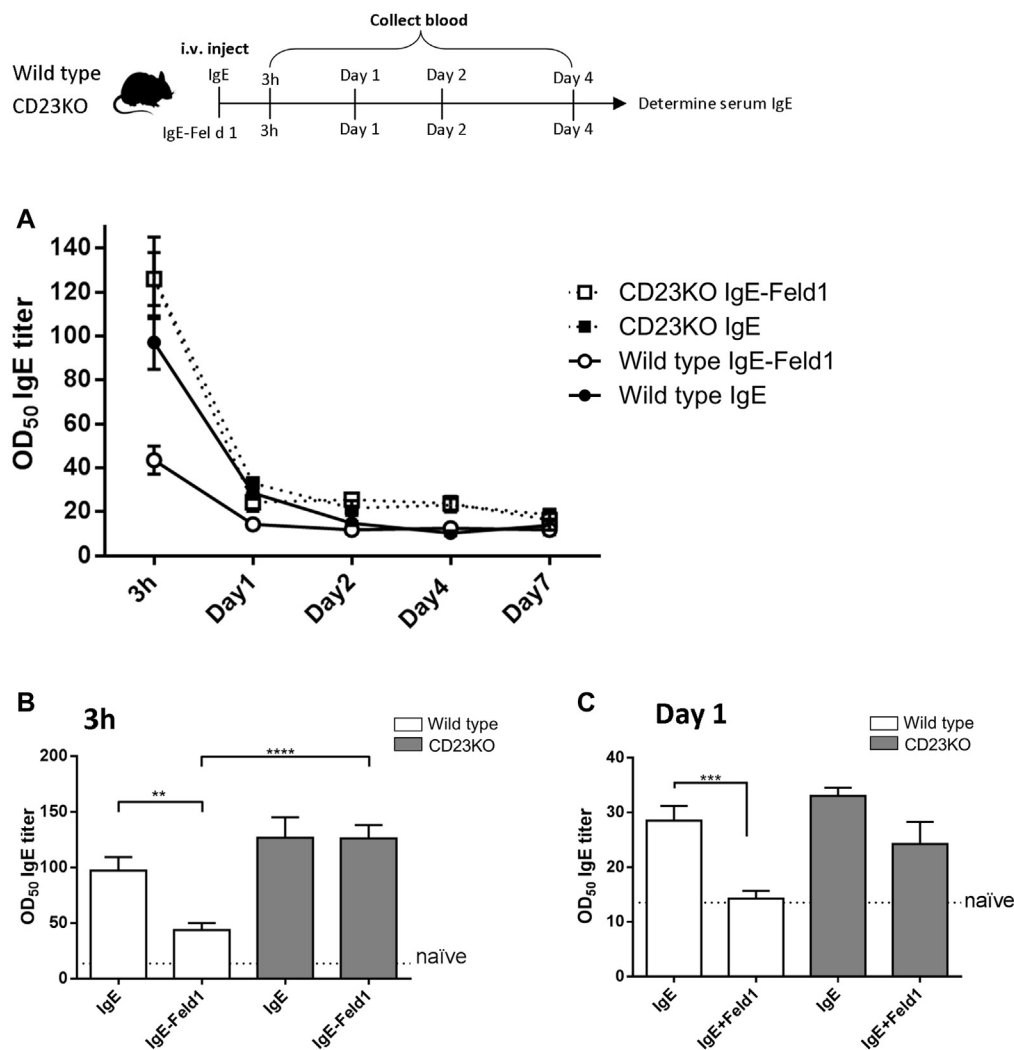


FIG 4. IgE-Fel d 1 complexes are cleared from serum in a CD23-dependent fashion. Wild-type and CD23KO mice were intravenously (*i.v.*) injected with IgE or IgE-Fel d 1, and serum IgE levels were examined by using ELISA. Shown are mean $\pm$ SEM IgE OD₅₀ titers at various time points. **A**, IgE titer kinetic after injection of IgE or IgE-Fel d 1 complexes. **B**, Mean $\pm$ SEM IgE OD₅₀ titer in wild-type and CD23KO mice after 3 hours. **C**, Mean $\pm$ SEM IgE OD₅₀ titer in wild-type and CD23KO mice after day 1. In **B** and **C**, dotted lines represent the mean IgE OD₅₀ titer in naive wild type and CD23KO mice. ** $P \leq .01$, *** $P \leq .001$, and **** $P \leq .0001$.

this end, human mast cells were sensitized with free IgE, washed, and challenged with Fel d 1 or directly challenged with IgE-Fel d 1 complexes. The cells were then incubated for 30 minutes at 37°C and thereafter stained with anti-CD63, a marker of degranulation, for 15 minutes at room temperature. As seen in Fig 2, A, Fel d 1 complexed with IgE did not induce high mast cell activation compared with free IgE, followed by Fel d 1 challenge. As shown in Fig 2, B, IgE-Fel d 1 complexes at a ratio of 1:1 showed lower induction of calcium flux compared with sensitized and challenged mast cells. Hence the same amount of IgE and allergen can induce greater mast cell activation in a 2-step sensitization and challenge encounter than when challenge occurs in a complexed form.

To exclude that IgE-Fel d 1 complexes are not activating mast cells in a delayed fashion, we tracked CD63 upregulation after stimulation with IgE-Fel d 1 complexes at a ratio of 1:1. As

shown in Fig E1, C, activation levels of mast cells never exceeded 20% of activation and decreased after 30 minutes.

To further assess the activation capacity of IgE compared with IgE-Fel d 1 complexes on mast cells, we performed a β -hexosaminidase assay using murine bone marrow-derived mast cells. As shown in Fig E1, D, mast cell activation occurred only in case of IgE sensitization and allergen challenge and not when IgE-Fel d 1 complexes were added to the cells, thus confirming our previous data on human mast cells.

IgE-Fel d 1 complexes do not sensitize basophils but display enhanced targeting of B cells in mice

After showing that IgE-Fel d 1 complexes display lower Fc ϵ RI binding and mast cell degranulation *in vitro*, we next sought to investigate the target cells of IgE in mice injected with free IgE or IgE-Fel d 1 complexes *in vivo*. Therefore

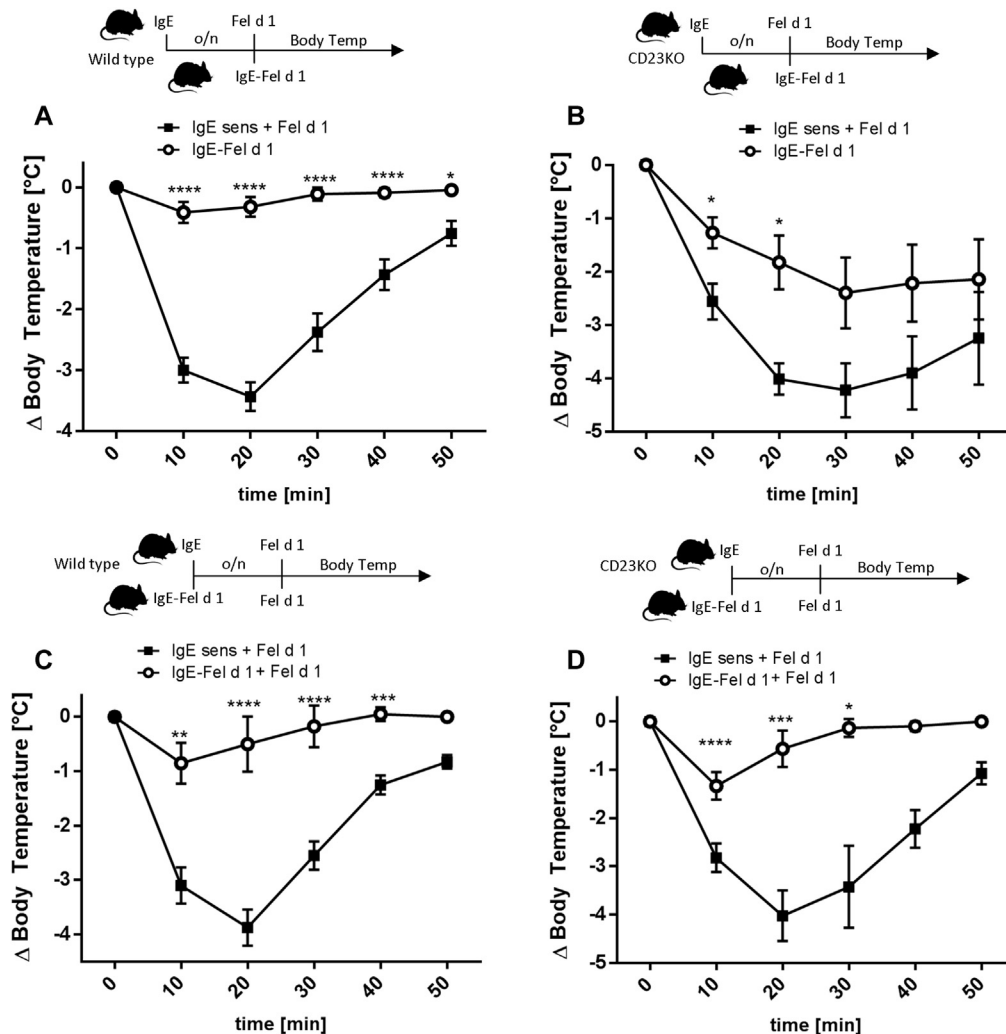


FIG 5. IgE–Fel d 1 complexes do not cause systemic anaphylaxis in the presence of CD23. **A** and **B**, IgE was administered to wild-type or CD23KO mice for sensitization by means of intravenous injection 1 day before allergen challenge with Fel d 1 (IgE sens + Fel d 1). Alternatively, mice were directly challenged with IgE–Fel d 1. Anaphylactic reactions to Fel d 1 were assessed by measuring rectal body temperature at 10-minute intervals. Shown are mean $\pm$ SEM changes in body temperature ($n = 10$ per group) in wild-type mice (Fig 5, **A**) or CD23KO mice (Fig 5, **B**). **C** and **D**, One day before Fel d 1 challenge, free IgE (IgE sens + Fel d 1) or IgE–Fel d 1 complexes (IgE–Fel d 1 sens + Fel d 1) were injected intravenously. On Fel d 1 challenge, anaphylaxis was assessed by measuring rectal body temperature at 10-minute intervals. Shown are mean $\pm$ SEM changes in body temperature in wild-type mice (Fig 5, **C**) or CD23KO mice (Fig 5, **D**; $n = 5$ per group). * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$, and **** $P \leq .0001$.

BALB/c wild-type or CD23KO mice were injected intravenously with IgE or IgE–Fel d 1, and 3 hours after injection, mice were bled and IgE binding to basophils and B cells was investigated. As shown in Fig 3, A–D, and Fig E4 in this article's Online Repository at www.jacionline.org, on injection of free IgE, the mean fluorescence intensity of IgE on basophils was drastically greater than that in wild-type and CD23KO mice injected with IgE–Fel d 1 complexes. In contrast, B cells from wild-type but not CD23KO mice had greater levels of IgE binding when IgE–Fel d 1 complexes were injected compared with free IgE (Fig 3, C and D). Hence we confirmed our results obtained with human cells *in vitro* in that IgE complexation results in less IgE targeting of basophils but enhances B-cell binding *in vivo*, suggesting that the mice are not sensitized to Fel d 1 by IgE–Fel d 1 injection.

Because it was shown that B cells rapidly transport IgE–antigen complexes into the spleen,¹⁸ we further investigated splenic B cells 3 hours after IgE or IgE–Fel d 1 injection. We again observed greater binding of IgE to B cells injected with IgE–Fel d 1 complexes than with free IgE (Fig 3, E and F). For CD23KO mice, no significant difference in IgE binding was observed on injection of IgE–ICs compared with free IgE.

IgE–Fel d 1 complexes are cleared from the serum in CD23-dependent fashion

We speculated that IgE–Fel d 1 complexes increasingly target CD23, which leads to serum clearance of IgE–Fel d 1 in a CD23-dependent fashion. Hence we compared clearance of IgE and IgE–Fel d 1 on intravenous injection in wild-type and

CD23KO mice. As shown in Fig 4, A, IgE–Fel d 1 complexes were much more quickly cleared in wild-type mice, whereas CD23KO mice did not efficiently clear IgE and IgE–Fel d 1 complexes from the serum. The difference in clearing IgE–Fel d 1 complexes and IgE in wild-type compared with CD23KO mice was most striking 3 hours after injection (Fig 4, B) but still visible after 24 hours (Fig 4, C). Most importantly, in CD23KO mice no difference in clearance of IgE compared with IgE–Fel d 1 complexes was observed throughout the time course of the experiment. However, even though CD23KO mice were less efficient than wild-type mice in IgE clearance, serum levels still decreased over time and returned to naive IgE levels after 7 days to levels similar to those seen in wild-type mice (see Fig E2, A–C). Hence, in the absence of CD23, clearance of noncomplexed IgE was not completely abolished, but CD23 seems to exert a very specific role in quickly clearing IgE-ICs.

IgE–Fel d 1 complexes do not cause systemic anaphylaxis in the presence of CD23

To assess whether sensitization and anaphylaxis are reduced with IgE–allergen complexes *in vivo*, we either passively sensitized wild-type mice or CD23KO mice with murine Fel d 1–specific IgE followed by Fel d 1 challenge the next day or we directly challenged the mice with IgE–Fel d 1 complexes without previous IgE sensitization. We then assessed systemic anaphylaxis by measuring rectal body temperature at 10-minute intervals. As shown in Fig 5, A, overnight sensitization and Fel d 1 challenge induced systemic anaphylaxis, whereas IgE–Fel d 1 complexes did not induce any reaction. When CD23KO mice were injected with IgE–Fel d 1, they showed fewer anaphylactic reactions than when sensitized overnight and challenged with allergen but clearly more anaphylaxis than wild-type mice (Fig 5, B). At lower IgE and Fel d 1 concentrations (10 μ g of IgE and 2.5 μ g of Fel d 1), the difference between overnight sensitization and direct IgE–Fel d 1 challenge was also more prominent in CD23KO mice (see Fig E2, D).

To assess whether IgE–Fel d 1 complexes fail to sensitize basophils for successive allergen challenge, we intravenously injected either IgE–Fel d 1 complexes or IgE and challenged the mice with Fel d 1 the next day. As shown in Fig 5, C and D, for wild-type and CD23KO mice, injection of IgE–Fel d 1 did not sensitize both wild-type and CD23KO mice to further Fel d 1 challenge, whereas only IgE-sensitized mice show signs of systemic anaphylaxis. These results are in line with the basophil IgE-loading data shown in Fig 3. Altogether, our data suggest that sensitization to an allergen is reduced when the allergen is in complex with IgE.

DISCUSSION

Even though studies have shown beneficial properties of IgE, such as protection against cancer, venoms, and worms,^{43–45} IgE is most commonly perceived as a harmful factor in the context of allergic disease. It has long been established that allergic reactions require a 2-step process to be initiated. Allergen-specific IgE must sensitize allergic effector cells, such as mast cells and basophils expressing Fc ϵ RI, before a second encounter of allergen can trigger an allergic reaction. This pathway is

facilitated by the uniquely high-affinity and long-lived interaction of IgE with Fc ϵ RI. We show here that IgE complexed with an allergen does not bind unoccupied Fc ϵ RI receptor well and therefore displays a reduced ability to sensitize mast cells and basophils.

This is a very unconventional mechanism because antigen-antibody complexation typically enhances cellular binding, as for example, with IgG antibodies, which bind poorly to Fc γ receptors without antigen complexation.⁴⁶ Here we show that for IgE, this type of classical interaction occurs through the low-affinity IgE receptor CD23 because complexation of IgE with allergen enhances its targeting. We have previously shown that IgE-immune complexes cause CD23 capping on the surfaces of B cells.²¹ This surface aggregation can further enhance formation of CD23-IgE-allergen complexes by bringing multiple IgE-binding sites close together and hence potentiate the capacity of IgE and allergen uptake. It must be noted that in mice injected with IgE or IgE–Fel d 1, the difference in IgE binding was not as striking as that with human B cells *in vitro*. Because CD23 has 2 binding sites on IgE, it is very likely that CD23 surface density on B cells determines free IgE binding. In general, CD23 expression in mouse B cells is greater in frequency and density than on human B cells, which might partially explain the enhanced binding of free IgE that we observed *in vivo* on mouse B cells compared with isolated human B cells. The exact binding kinetics of IgE and IgE–Fel d 1 complexes still require more detailed investigations. Membrane CD23 is thought to undergo oligomerization, which could affect IgE binding.⁴⁷ The mechanism and extent of this oligomerization is still not clearly understood but complicates the biochemical studying of protein-protein interactions. Additionally, CD23 has a complicated array of interaction partners that could affect membrane distribution or stabilization.⁸ Such a mechanism of membrane distribution regulating receptor activity was recently described for Fc ϵ RI.⁴⁸

Furthermore, the physicochemical properties of IgE complexes require more detailed investigations. As expected, our native PAGE experiments show that optimal complex size is achieved at equimolar IgE/Fel d 1 ratio. However, free Fel d 1 was still present at an equimolar IgE/Fel d 1 ratio, which could explain the remaining mast cell activation *in vitro* and systemic anaphylaxis *in vivo* observed in CD23KO mice.

A potential structural explanation of the here reported distinct binding of IgE to CD23 and Fc ϵ RI might be the unique conformational attributes of IgE. In comparison with other antibody classes, IgE contains one extra constant domain, C ϵ 4, and regulates the interaction with its 2 receptors by bending into distinct conformations. It has been shown that IgE bound to Fc ϵ RI shows a different conformational state in comparison with IgE bound to CD23.²⁷ Specifically, Fc ϵ RI binds to the open and CD23 binds to the closed conformation of the C ϵ 3–C ϵ 4 domain, making simultaneous IgE binding to the 2 receptors mutually exclusive. Our findings that IgE–allergen complexes preferably bind to CD23 and show strongly reduced binding to Fc ϵ RI could potentially be explained as well by a change of IgE conformation on allergen binding. Conversely, the limiting factor might also be Fc ϵ RI, selecting for free IgE by a lack of flexibility. Hence the binding regulation of IgE to its 2 receptors still requires more in-depth investigations.

Nevertheless, our data on serum clearance of IgE in mice clearly show that CD23 is responsible for clearing IgE–Fel d 1

complexes in accelerated fashion compared with free IgE. Free IgE is also cleared more quickly from the serum in the presence of CD23, which is in line with a previous study showing that CD23 regulates serum kinetics.¹⁷ Our data also fit to a study that has shown rapid transport of IgE-ICs into the spleen,¹⁸ a phenomenon that we also observed with IgE–Fel d 1 injections. It has to be noted here that CD23 can be present not only in membrane form but also in soluble form (soluble CD23)⁴⁹; it might be possible that soluble CD23 also contributes to serum IgE clearance or transport. Furthermore, we did not observe IgE on any blood cells other than B cells or basophils; however, because it has been shown that CD23 can be expressed on epithelial cells,⁵⁰ we do not exclude an involvement of other cell types in IgE-IC clearance.

In terms of the functional consequences, more studies are also required to further understand the mechanism identified here. Obviously, the more IgE binds to B cells, the less it can sensitize allergic effector cells, such as basophils and mast cells, and induce allergic inflammation. We interpret the phenomenon that IgE–allergen complexes do not bind to FcεRI as a mechanism of negative feedback. A high presence of allergen could lead to complexation of allergen-specific IgE in the serum. Because basophils are short lived, complexation of IgE with an allergen could prevent sensitization of free FcεRI on new basophils. On the other hand, CD23, which has been known as a negative regulator of IgE synthesis,¹⁴ would be increasingly targeted by the complexes. Hence, when more IgE binds to B cells, synthesis of new IgE by these B cells might be increasingly suppressed.

To explain the noninflammatory properties of IgE-ICs, it could also be hypothesized that IgE-ICs activate CD23-dependent signaling pathways. However, CD23 signaling and factor release were only really shown in macrophages, where CD23 cross-linking was shown to trigger proinflammatory factors, such as nitric oxide.⁵¹ Moreover, it was also shown that CD23-dependent signaling is different in B cells and monocyte-related cells.⁵² To our knowledge, B cells were never shown to release any mediators on CD23 cross-linking. Therefore we and others believe that CD23-expressing B cells likely act as carriers of IgE-immune complexes, which regulate IgE-dependent T-cell activation.^{18,19,21}

On the other hand, CD23 could also be understood as a decoy receptor for IgE-immune complexes, taking IgE away from the serum and from allergic effector cells. The outcome would still be that CD23 counteracts the allergic pathway to prevent sensitization.

We showed that in CD23KO mice the clearance of IgE-ICs was significantly delayed compared with that in wild-type mice. Even though IgE-ICs were also less potent at causing an anaphylactic reaction in CD23KO mice, the reaction was strong in comparison with that in wild-type mice. Therefore IgE clearance by CD23 is an important component of the noninflammatory properties of IgE-ICs *in vivo*. Moreover, when mice injected with IgE-ICs were reinjected with Fel d 1 the next day, less anaphylaxis was observed. In wild-type mice this clearly fits the lower number of IgE⁺ basophils observed after IgE-IC injection the next day. After 1 day, CD23KO mice also are resistant to further Fel d 1 challenge. This is likely due to the fact that anaphylaxis already occurred the previous day and that clearance of IgE can also be mediated in the absence of CD23.

Our findings have a variety of potential clinical applications. Allergen-specific immunotherapy is the only disease-modifying

treatment available for allergic disorders.^{53–56} For this therapy, multiple injections of increasing doses of allergen result in allergen desensitization over time. Interestingly, a phenomenon observed in successful allergen-specific immunotherapy is an increase in serum IgE levels. Hence it is plausible that higher levels of IgE and allergen lead to IgE-IC formation, which could prevent ongoing sensitization of basophils and mast cells and lead to allergen tolerance. Novel therapeutics could be designed that specifically induce the complexation of IgE. The anti-IgE mAb omalizumab, which is already on the market and used in clinics, actually acts through such a mechanism (ie, it leads to the formation of IgE–anti-IgE immune complexes).⁵⁷ However, omalizumab requires high injection doses to reach clinical benefits. It could be speculated that this limited effect might be explained by the fact that omalizumab also inhibits IgE binding to CD23.⁵⁸ If CD23 is physiologically the noninflammatory IgE pathway, an anti-IgE antibody designed in a way that it reduces FcεRI binding but enhances CD23 targeting could potentially be more effective.

Altogether, we describe a mechanism that regulates IgE targeting to its receptors and controls allergic inflammation. In complex with an antigen, IgE loses its ability to sensitize unoccupied FcεRI while simultaneously increasing CD23 binding, resulting in IgE clearance and resistance to anaphylaxis. Understanding the regulation of IgE receptor targeting described here in further detail might contribute to our understanding of IgE biology and thereby contribute to the development of improved therapeutic alternatives to treat allergic diseases.

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Key messages

- Complexation of IgE and Fel d 1 (IgE–Fel d 1) reduces IgE binding to FcεRI but enhances IgE binding to CD23 in human cells and in mice.
- In contrast to IgE sensitization and Fel d 1 challenge, IgE–Fel d 1 does not trigger degranulation of human mast cells *in vitro* and does not induce systemic anaphylaxis in mice.
- CD23 specifically clears IgE–Fel d 1 complexes from the serum, which might prevent anaphylaxis to IgE–Fel d 1 complexes.

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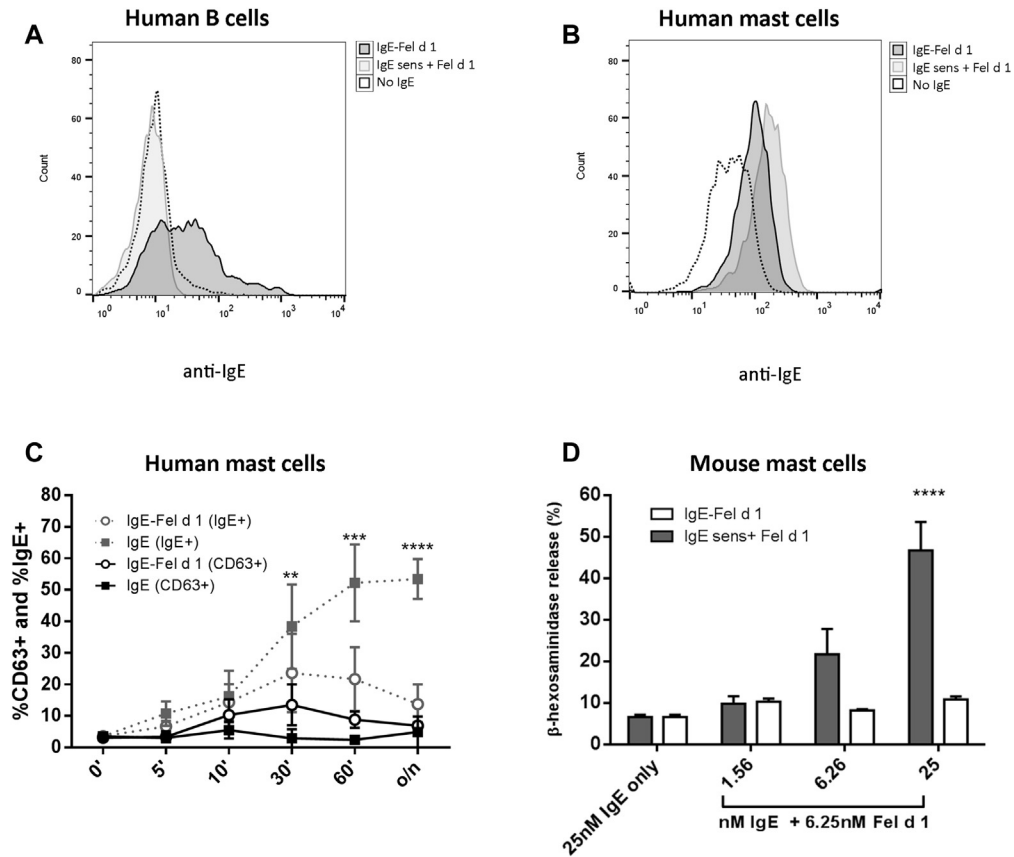


FIG E1. **A** and **B**, Representative histograms of human IgE F127 binding to human B cells (Fig E1, **A**) or mast cells (Fig E1, **B**) at 6.25 nmol/L IgE and 6.25 nmol/L IgE comparing the 2 IgE binding models, as described in Fig 1. **C**, IgE binding and mast cell activation over time. Human mast cells were incubated with either 6.25 nmol/L IgE or IgE–Fel d 1 (6.25 nmol/L:6.25 nmol/L) complexes. Cells were stained at various time points with anti-IgE and anti-CD63. Shown are means $\pm$ SEMs of CD63⁺ and IgE⁺ cells from 3 individual donors. **D**, β -Hexosaminidase release assay using mouse bone marrow–derived mast cells and mouse IgE F127. Cells were sensitized with titrated doses of IgE, followed by Fel d 1 challenge (6.25 nmol/L), or alternatively, cells were directly incubated with precomplexed IgE–Fel d 1. ** $P \leq .01$, *** $P \leq .001$, and **** $P \leq .0001$.

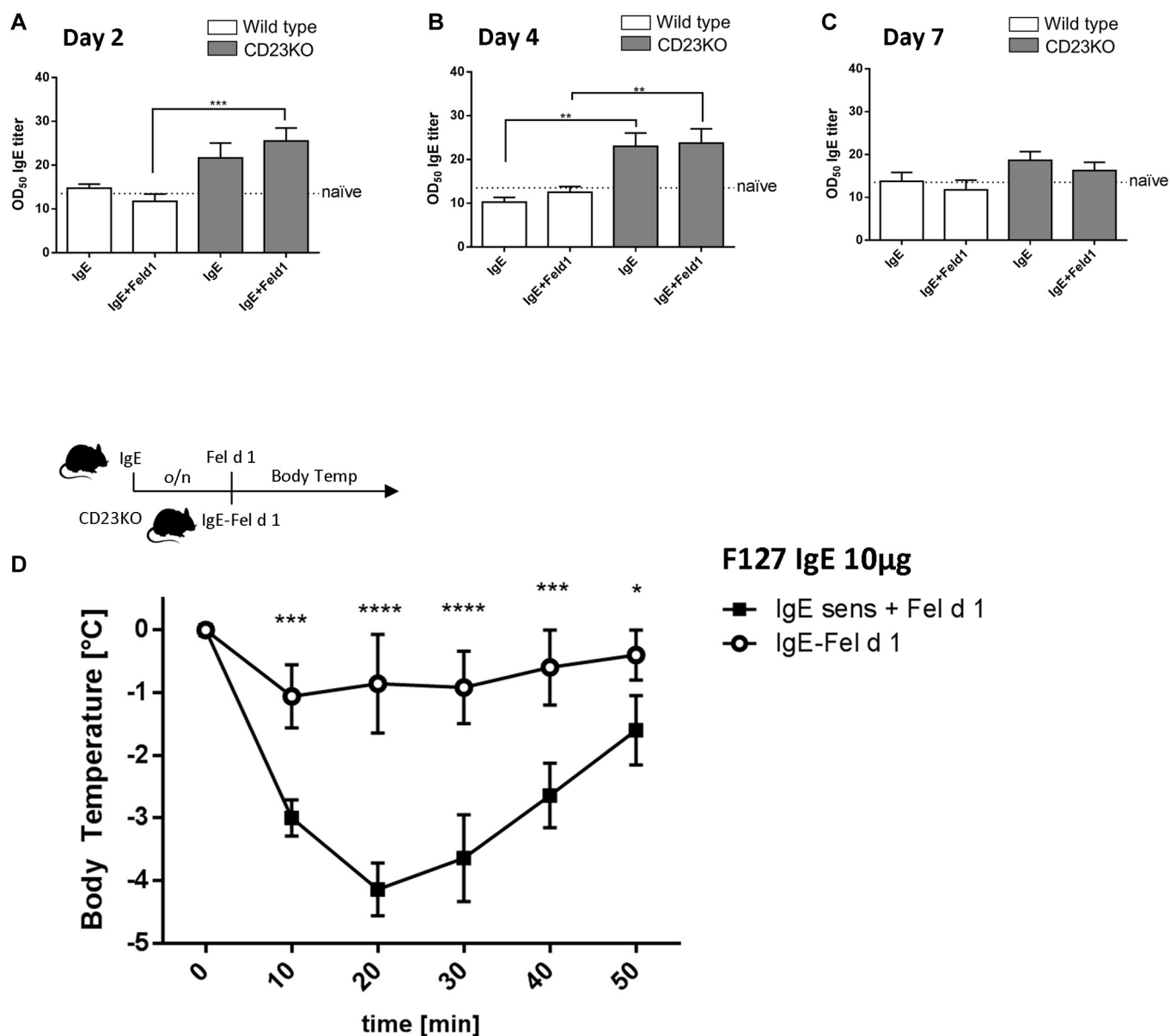


FIG E2. A-C, IgE serum levels on IgE or IgE-Fel d 1 injection (see Fig 4) at days 2, 4, and 7 in wild-type and CD23KO mice. D, Instead of 21.5 μg of IgE plus 5 μg of Fel d 1 (see Fig 5, B), CD23KO mice were injected with 10 μg plus 2.5 μg of Fel d 1. IgE was either injected before allergen challenge with Fel d 1 (*IgE o/n + Fel d 1*) or mice were directly injected with IgE-Fel d 1. Anaphylactic reactions to Fel d 1 were assessed by measuring rectal body temperature at 10-minute intervals. Shown are mean ± SEM changes in body temperature (n = 5). **P* ≤ .05, ***P* ≤ .01, ****P* ≤ .001, and *****P* ≤ .0001.

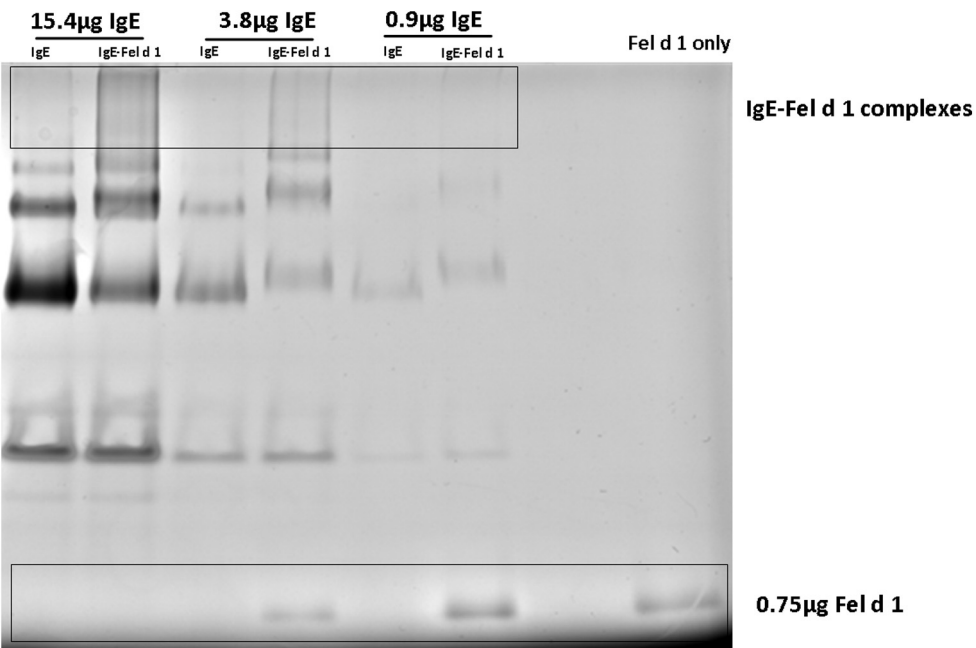


FIG E3. Blue native PAGE gel was loaded with either free IgE or IgE in complex with Fel d 1 (IgE–Fel d 1). Fel d 1 was kept constant at 0.75 µg, whereas IgE was titrated at IgE/Fel d 1 molar ratios of 4:1 (15.4 µg of IgE), 1:1 (3.8 µg of IgE), and 1:4 (0.9 µg of IgE). The last lane shows free Fel d 1, which decreases with increasing IgE concentrations. Smears on the top part of the gel indicate the presence of protein complexes larger than IgE with varying sizes.

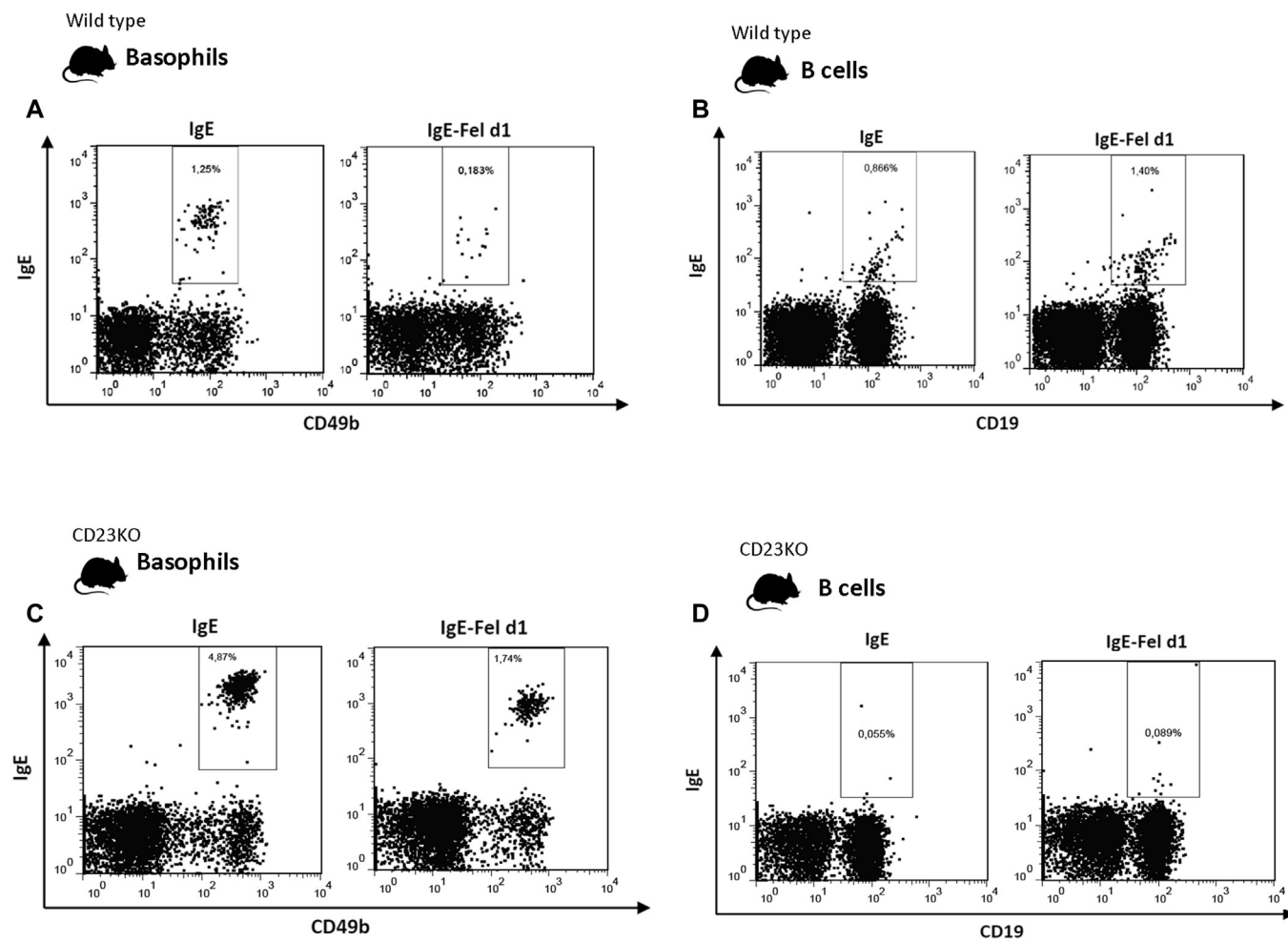


FIG E4. Raw fluorescence-activated cell sorting dot plots from IgE binding to mouse peripheral basophils and B cells. **A**, Wild-type mouse basophils. **B**, Wild-type mouse B cells. **C**, CD23KO mouse basophils. **D**, CD23KO mouse B cells.