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Bianca Balbino, ... , Pierre Bruhns, Laurent L. Reber

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Omalizumab is an anti-IgE monoclonal antibody (mAb) approved for the treatment of severe asthma and chronic spontaneous urticaria. Use of Omalizumab is associated with reported side effects, ranging from local skin inflammation at the injection site to systemic anaphylaxis. To date, the mechanisms through which Omalizumab induces adverse reactions are still unknown. Here, we demonstrated that immune complexes formed between Omalizumab and IgE can induce both skin inflammation and anaphylaxis through engagement of IgG receptors (Fc γ Rs) in Fc γ R-humanized mice. We further developed an Fc-engineered mutant version of Omalizumab, and demonstrated that this mAb is equally potent as Omalizumab at blocking IgE-mediated allergic reactions, but does not induce Fc γ R-dependent adverse reactions. Overall, our data indicate that Omalizumab can induce skin inflammation and anaphylaxis by engaging Fc γ Rs, and demonstrate that Fc-engineered versions of the mAb could be used to reduce such adverse reactions.

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The anti-IgE mAb Omalizumab induces adverse reactions by engaging Fcγ receptors

Authors

Bianca Balbino^{1,2}, Pauline Herviou¹, Ophélie Godon¹, Julien Stackowicz^{1,2}, Odile Richard-Le Goff¹, Bruno Iannascoli¹, Delphine Sterlin^{1,3}, Sébastien Brûlé⁴, Gael A. Millot⁵, Faith M. Harris⁶, Vera A. Voronina⁶, Kari C. Nadeau^{7,8}, Lynn E. Macdonald⁶, Andrew J. Murphy⁶, Pierre Bruhns^{1,*} and Laurent L. Reber^{1,9,*}

Affiliations

¹Unit of Antibodies in Therapy and Pathology, Institut Pasteur, UMR1222 INSERM, 75015 Paris, France. ²Sorbonne Université, Paris, France. ³Assistance Publique–Hôpitaux de Paris, La Pitié-Salpêtrière, Département d’Immunologie, France, Paris. ⁴Plateforme de biophysique moléculaire, Institut Pasteur, UMR 3528 CNRS, Paris 75015, France. ⁵Hub de Bioinformatique et Biostatistique – Département Biologie Computationnelle, Institut Pasteur, USR 3756 CNRS, Paris, France. ⁶Regeneron Pharmaceuticals Inc., Tarrytown, NY 10591, USA. ⁷Sean N. Parker Center for Allergy and Asthma Research, Stanford University, Stanford, California, USA. ⁸Division of Pulmonary and Critical Care, Department of Medicine, Stanford University, California, USA. ⁹Center for Physiopathology of Toulouse-Purpan (CPTP), UMR 1043, University of Toulouse, INSERM, CNRS, Toulouse, France. *These authors contributed equally to this work.

23 **Corresponding authors**

24

25 Laurent L. Reber, Ph.D

26 ATIP-Avenir team “Asthma, Allergy & Immunotherapy”

27 Center for Physiopathology Toulouse-Purpan

28 CHU Purpan – BP 3028

29 31024 Toulouse Cedex 3, France

30 Phone: +33-5-6274-4529

31 e-mail: laurent.reber@inserm.fr

32

33 Pierre Bruhns, Ph.D

34 Unit of Antibodies in Therapy and Pathology

35 Department of Immunology

36 Institut Pasteur

37 25 rue du Docteur Roux

38 Paris, 75015, France

39 Phone: +33-1-4568-8629

40 e-mail: bruhns@pasteur.fr

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42 **Running title**

43 Xolair induces FcγR-dependent inflammation

Abstract

Omalizumab is an anti-IgE monoclonal antibody (mAb) approved for the treatment of severe asthma and chronic spontaneous urticaria. Use of Omalizumab is associated with reported side effects, ranging from local skin inflammation at the injection site to systemic anaphylaxis. To date, the mechanisms through which Omalizumab induces adverse reactions are still unknown. Here, we demonstrated that immune complexes formed between Omalizumab and IgE can induce both skin inflammation and anaphylaxis through engagement of IgG receptors (FcγRs) in FcγR-humanized mice. We further developed an Fc-engineered mutant version of Omalizumab, and demonstrated that this mAb is equally potent as Omalizumab at blocking IgE-mediated allergic reactions, but does not induce FcγR-dependent adverse reactions. Overall, our data indicate that Omalizumab can induce skin inflammation and anaphylaxis by engaging FcγRs, and demonstrate that Fc-engineered versions of the mAb could be used to reduce such adverse reactions.

Introduction

IgE antibodies (Abs) are key mediators of allergic diseases (1-3). Upon exposure to an allergen in allergic patients, such allergen is recognized by IgE bound to their high-affinity receptor FcεRI on the surface of mast cells and basophils, which promotes the immediate activation of these cells, and the release of inflammatory mediators such as histamine, responsible for allergic symptoms (3).

Omalizumab (Xolair®) is a recombinant humanized IgG1 mAb directed against IgE (4). Omalizumab binds to the Cε3 domain of free IgE, and thereby impairs binding of IgE to both FcεRI and the low-affinity IgE receptor CD23 (FcεRII) (5-7). Omalizumab does not recognize IgE already bound to FcεRI or CD23, and therefore cannot induce cell activation by crosslinking of IgE receptors (5, 7). Omalizumab is approved for the treatment of severe asthma (8) and chronic spontaneous urticaria (9). It also shows promises for the treatment of other allergic diseases, including food allergy (10).

However, treatment with Omalizumab is associated with adverse reactions, ranging from skin inflammation at the injection site to anaphylaxis in ~0.1-0.2% of patients (11-13). The mechanism of these side effects is still unknown. Notably, Omalizumab does not induce the formation of anti-drug Abs, and most cases of anaphylaxis occur within the first three injections of the drug (11-13).

80 We hypothesized that the formation of immune complexes (ICs) between
81 Omalizumab and IgE could be responsible for some of the adverse reactions observed with
82 this therapeutic mAb. Using mice humanized for all IgG receptors (FcγRs), we demonstrate
83 here that Omalizumab/IgE ICs can induce both skin inflammation at the site of injection of
84 the drug, as well as systemic anaphylaxis through engagement of FcγRs. Finally, we
85 developed an Fc-engineered version of Omalizumab which blocks IgE-mediated allergic
86 reactions without inducing FcγR-dependent adverse reactions.

87

Results and Discussion

We first co-incubated Omalizumab and human IgE (termed IgE herein) in vitro to form ICs, and assessed the molecular mass of these ICs by size exclusion chromatography coupled to static light scattering (SEC-SLS). As reported previously (14, 15), these ICs were of limited size, mainly consisting of trimeric structures (**Supplemental Figure 1**). It was initially suggested that such small ICs have a low potential to engage FcγRs (15). However, we found that these ICs potently bind all activating human FcγRs (FcγRI, IIA, IIIA and IIIB), but not the inhibitory FcγRIIB that has the lowest affinity for human IgG1 among FcγRs (16) (**Figure 1A**). As expected, we also observed that Omalizumab binds human complement component C1q in a dose-dependent manner (**Figure 1B**).

As neutrophils were reported to contribute to IgG-mediated inflammation and anaphylaxis (17), we next evaluated whether Omalizumab/IgE ICs can activate neutrophils through engagement of FcγRs. We purified neutrophils from healthy donors and incubated these cells with Omalizumab/IgE ICs. We found that such ICs induce marked upregulation of CD66b and downregulation of CD62L on the surface of neutrophils, which are considered hallmarks of neutrophil activation (18, 19) (**Figure 1, C and D**). The ICs also induced downregulation of FcγRII (CD32) (**Figure 1E**). As human neutrophils express FcγRIIA and not FcγRIIB (20), and Omalizumab/IgE ICs do not bind FcγRIIB (**Figure 1A**), our results indicate that the ICs induce active engagement of FcγRIIA in neutrophils.

To further confirm the role of FcγRs in neutrophil activation, we performed similar experiments with neutrophils purified from hFcγR^{KI} mice (in which all mouse FcγRs have been replaced with human FcγRs) (20, 21) or FcγR^{null} mice (deficient for all FcγRs) (**Figure 1F**). Omalizumab/IgE ICs induced a downregulation of CD62L in neutrophils from hFcγR^{KI} mice, but not in neutrophils from FcγR^{null} mice (**Figure 1F**), demonstrating that Omalizumab/IgE can activate neutrophils through engagement of human FcγRs.

The most frequent side effect observed with Omalizumab is skin inflammation (13). We hypothesized that such local inflammation could be a consequence of FcγRs engagement. To assess this, we injected Omalizumab/IgE ICs subcutaneously into hairless (to avoid shaving-induced skin inflammation) nude hFcγR^{KI} mice and nude FcγR^{null} mice, and assessed skin inflammation after 2 h by bioluminescence imaging of myeloperoxidase (MPO) activity (20, 22). We observed a strong MPO activity at the site of ICs injection in hFcγR^{KI} mice (**Figure 2, A and B**). By contrast, MPO activity was markedly reduced upon injection of IgE alone or Omalizumab alone, or injection of ICs in FcγR^{null} mice. Thus, our results indicate that Omalizumab/IgE ICs can induce skin inflammation through engagement of hFcγRs.

The most dramatic side effect reported for Omalizumab is anaphylaxis (12, 13). We thus assessed whether Omalizumab/IgE ICs can induce anaphylaxis in hFcγR^{KI} mice. Intravenous injection of ICs induced significant hypothermia (the main readout of anaphylaxis in mice (23)) in hFcγR^{KI} mice (**Figure 2C**). Importantly, hypothermia was not observed upon ICs injection in hFcγR^{null} mice (**Figure 2C**), or injection of IgE or

133 Omalizumab alone in hFcγR^{KI} mice (**Supplemental Figure 2**), demonstrating that the ICs
134 induce systemic anaphylaxis through engagement of human FcγRs. Previous work
135 indicates that hFcγRIIA contributes to experimental anaphylaxis in humanized mice (21,
136 24, 25). We did not observe anaphylaxis in mFcγR^{null}hFcγRIIA^{Tg} mice (which express only
137 hFcγRIIA (21)), indicating that hFcγRIIA is not sufficient to trigger Omalizumab/IgE-
138 mediated anaphylaxis (**Supplemental Figure 3A**). By contrast, anaphylaxis was markedly
139 reduced in hFcγR^{KI} mice pre-treated with a blocking mAb against hFcγRIII (**Supplemental**
140 **Figure 3B**). This suggests that hFcγRIII plays an important role in Omalizumab/IgE-
141 mediated anaphylaxis. However, these results have to be interpreted carefully as we cannot
142 exclude that pre-treatment with the anti-hFcγRIII mAb induces engagement of the receptor
143 to some extent, thereby “desensitizing” cells expressing hFcγRIII.

144
145 Since Omalizumab also binds complement component C1q (**Figure 1B**), we assessed
146 the potential contribution of C1q to ICs-induced anaphylaxis. We found that anaphylaxis
147 is markedly reduced in hFcγR^{KI}C1q^{-/-} mice, which express all hFcγRs but lack mouse C1q
148 (**Figure 2D**). Although further work is required to confirm the implication of human C1q,
149 our data strongly suggest that the complement pathway plays an important role, through
150 C1q engagement, in Omalizumab/IgE-induced anaphylaxis.

151
152 Based on these results, we decided to produce an Fc-engineered form of Omalizumab
153 (using available Omalizumab V_H and V_L sequences (4)) lacking the N-linked glycan
154 attached to asparagine 297 in the Fc portion (N₂₉₇A mutation) to reduce binding to FcγRs
155 and complement (26, 27). We refer to this mAb as ‘NA anti-IgE’. As a control, we also

produced a non-mutated version of this mAb ('WT anti-IgE'). Both the WT and NA anti-IgE mainly formed trimers when incubated with IgE in vitro (**Supplemental Figure 4, A-D**), which is consistent with the data we obtained using commercial Omalizumab (**Supplemental Figure 1**). As expected, ICs made of IgE and the WT anti-IgE could bind all activating *FcγRs*, while binding to *FcγRs* was markedly reduced with ICs made of IgE and the NA anti-IgE (**Figure 3A**). Indeed, IgE/NA anti-IgE ICs could only bind to *FcγRI*, which is consistent with a previous report showing that the N₂₉₇A mutation does not abrogate binding to this high-affinity *FcγR* (28). In addition, WT anti-IgE could bind human C1q (**Supplemental Figure 4E**), but we detected no binding to C1q with the NA anti-IgE (**Supplemental Figure 4E**). Finally, we observed activation of human neutrophils with ICs made of IgE and the WT anti-IgE, but markedly reduced activation with ICs made of IgE and the NA anti-IgE (**Figure 3, B-D**).

FcRn/β2m heterodimers extend the half-life of IgG by reducing lysosomal degradation in endothelial cells (29). To assess the half-life of our anti-IgE mAbs in vivo, we generated h*FcγR*^{KI}h*FcRn*^{KI}hβ2m^{KI} mice which recapitulate binding of IgG to all human *FcγRs* and to the human *FcRn*-β2m complex (**Supplemental Figure 5**). We injected WT or NA anti-IgE into h*FcγR*^{KI}h*FcRn*^{KI}hβ2m^{KI} mice, and observed similar mAbs levels in sera collected at different time-points (**Figure 3E**). We obtained similar results when comparing the half-life of commercial Omalizumab and the Fc-engineered NA anti-IgE (**Supplemental Figure 6**). Altogether, these results demonstrate that the N₂₉₇A mutation does not affect the half-life of the anti-IgE mAb in vivo.

We also verified that the N₂₉₇A mutation does not affect the ability of the anti-IgE mAb to block IgE. Both the WT and NA anti-IgE recognized IgE with the same affinity (**Supplemental Figure 7A**), and were equally potent at blocking binding of IgE to human mast cells (**Supplemental Figure 7B**). Moreover, we showed that pre-treatment of hFcεRI^{Tg} mice (which express the human IgE receptor hFcεRI (30)) with either Omalizumab or the NA anti-IgE can block IgE-mediated anaphylaxis (**Figure 4A**). Altogether, our results demonstrate that the Fc-engineered NA anti-IgE is equally potent as Omalizumab at blocking IgE-mediated allergic reactions.

We then compared skin inflammation induced by IgE/Omalizumab or IgE/NA anti-IgE ICs in hFcγR^{KI} mice. Injection of IgE/Omalizumab ICs induced marked MPO activity in the skin (**Figure 4, B and C**). This was reduced to levels observed with injection of IgE alone in hFcγR^{KI} mice injected with IgE/NA anti-IgE ICs (**Figure 4, B and C**). Finally, we compared the ability of ICs made of IgE and Omalizumab or the NA anti-IgE to induce anaphylaxis in hFcγR^{KI} mice. We observed anaphylaxis in mice injected with IgE/Omalizumab ICs but not in mice injected with IgE/NA anti-IgE ICs (**Figure 4D**).

In summary, our findings demonstrate that Omalizumab forms ICs with IgE which can activate neutrophils, and induce skin inflammation and systemic anaphylaxis through human FcγRs in FcR-humanized mice. Such findings could explain some of the side effects which have been described in patients treated with Omalizumab (12, 13). One must be careful when extrapolating these findings obtained in humanized mice to humans, as very few data have been reported supporting the existence of FcγR-mediated anaphylaxis in

humans. However, one recent report provides evidence of an IgG-induced, FcγR-dependent neutrophil activation pathway in anaphylaxis to neuromuscular-blocking agents (NMBAs) in humans (31), which reinforces the potential clinical relevance of our findings. The Fc-engineered anti-IgE mAb we developed is equally potent as Omalizumab at blocking IgE-mediated allergic reactions but does not induce FcγR-mediated inflammation. It could thus potentially be used in patients with very high levels of IgE, and/or in patients with a history of anaphylaxis or other adverse reactions to Omalizumab. Finally, we envision that IC-mediated engagement of FcγRs could be a more general mechanism of therapeutic mAbs-mediated adverse reactions.

Materials and Methods

See the supplemental Methods for the description of all experimental procedures and statistical analyses.

Study approval. All animal care and experimentation were conducted in compliance with the guidelines and specific approval of the Animal Ethics committee CETEA (Institut Pasteur, Paris, France) registered under #2013-0103, and by the French Ministry of Research under agreement #00513.02.

Author contributions

Experimental design, B.B, P.B and L.L.R; Conducting experiments, B.B, P.H, O.G, J.S and L.L.R; Acquiring data B.B, P.H, O.G, J.S, O.R.G, B.I, D.S, S.B and L.L.R; Providing mice: F.M.H, V.A.V, L.E.M and A.J.M; Providing reagents: K.C.N; Statistical analysis: G.A.M; Formal analysis, B.B and L.L.R; Writing (original draft), B.B and L.L.R; Writing (review and editing), all authors.

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Competing interests

B.B, P.B and L.L.R are inventors on a patent related to this work (PCT/EP2019/059414). L.E.M and A.M. are employees of Regeneron Pharmaceuticals, Inc. hold stock in the company, and are inventors on patents and patent applications related to the mice used for this work. P.B. is a paid consultant for Regeneron Pharmaceuticals. The authors declare no additional competing financial interests.

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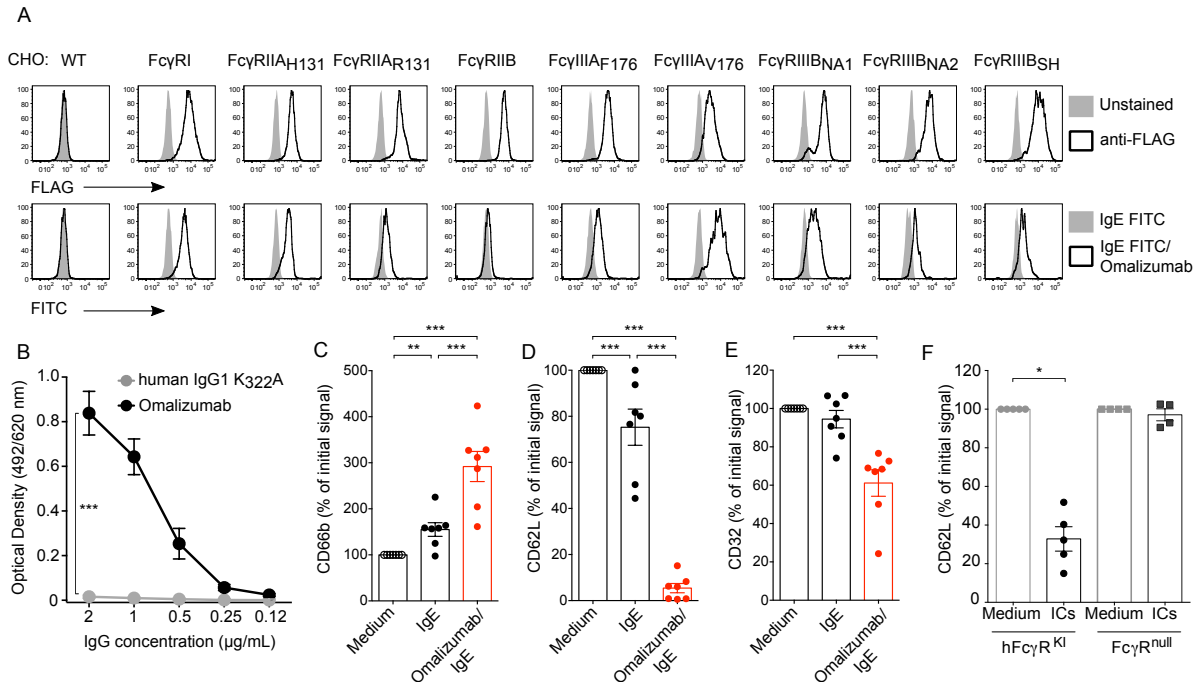


Figure 1. Omalizumab/IgE immune complexes (ICs) bind FcγRs and activate neutrophils. (A) Binding of pre-formed IgE/Omalizumab ICs to FcγRs in CHO cells stably transfected with each one of the human FcγRs (16). Upper histograms show binding of an anti-FLAG mAb as a control for FcγR expression. Lower histograms show binding of ICs or IgE FITC alone. Data are representative of three independent experiments. (B) Binding of Omalizumab to human C1q assessed by ELISA. An irrelevant IgG1 mutated in its Fc portion at position 322 (K322A) to preclude binding to C1q was used as a negative control. Results in B show means ± SD from data pooled from two independent experiments (total of $n=4$ replicates). (C-E) Expression of CD66b (C), CD62L (D) and CD32 (E) on purified CD45⁺CD15⁺ human neutrophils after 1 h incubation with Omalizumab/IgE immobilized ICs, IgE or medium alone. Results in C-E show values from neutrophils from individual donors normalized against cells stimulated with medium alone; bars indicate means ± SEM of $n=7$ total values per group pooled from three independent experiments. (F) CD62L expression on CD11b⁺Ly6G⁺ neutrophils purified from hFcγR^{KI} or FcγR^{null} mice after 1 h incubation with ICs or medium. Results in F show values from individual mice with bars indicating means ± SEM pooled from two (FcγR^{null}; total $n=4$ /group) or three (hFcγR^{KI}; total $n=5$ /group) independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ using one-way analysis of variance (ANOVA) in B, contrast linear model in C, D and E and Welch test in F. For further details on the statistical analysis, please refer to Table S1.

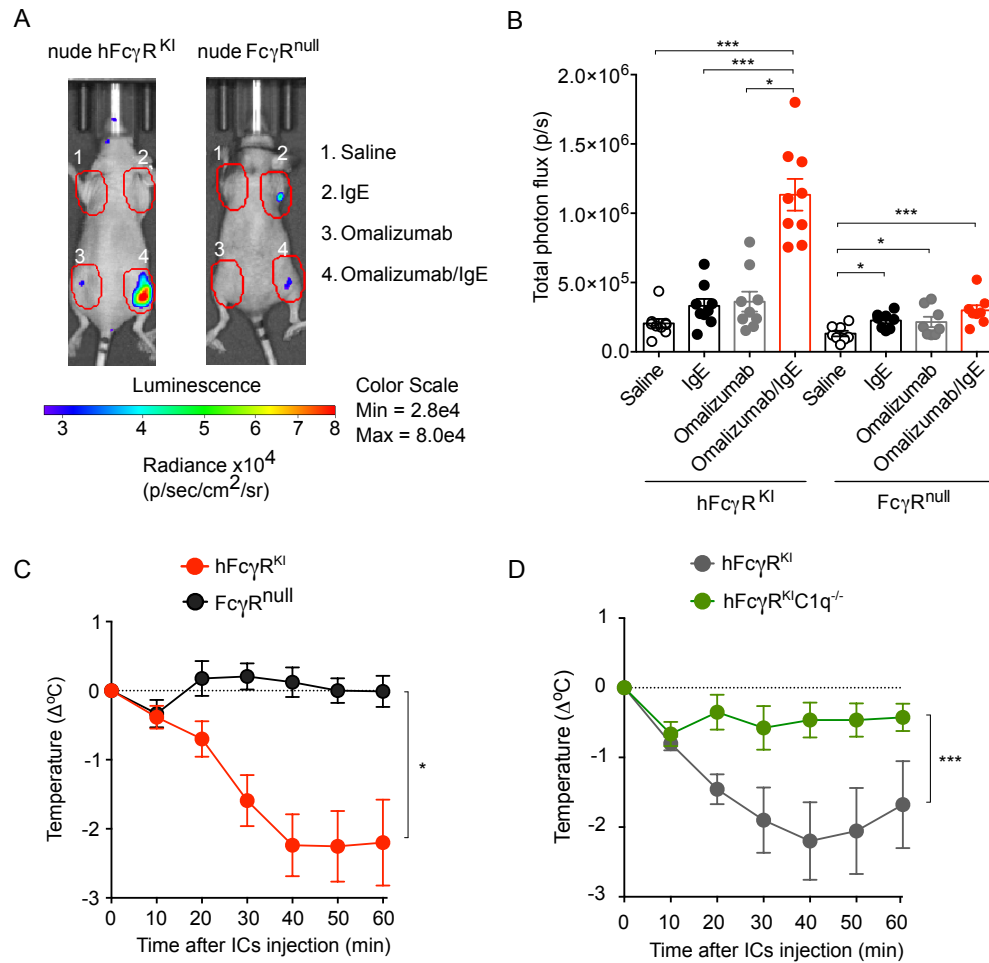


Figure 2. Omalizumab/IgE ICs induce skin inflammation and anaphylaxis through engagement of $Fc\gamma$ Rs in $Fc\gamma$ R-humanized mice. Representative bioluminescent images (A) and quantification (B) of MPO activity 2 h after subcutaneous injection of IgE/Omalizumab ICs in nude hFc γ R^{KI} mice ($n=9$) or nude Fc γ R^{null} mice ($n=8$). Regions of interest outlined in red in A surround sites of injection. Data in B are means $\pm$ SEM pooled from two independent experiments. (C-D) Changes in body temperature ($\Delta^\circ\text{C}$ [mean $\pm$ SEM]) after intravenous injection of IgE/Omalizumab ICs into hFc γ R^{KI} mice ($n=13$) or Fc γ R^{null} mice ($n=9$) (C), or hFc γ R^{KI} mice ($n=9$) or hFc γ R^{KI} C1q^{-/-} mice ($n=8$) (D). Data are pooled from three (C) or two (D) independent experiments. *, $P < 0.05$; ***, $P < 0.001$ by Contrast test in linear model (B and C) or ANOVA (D). For further details on the statistical analysis, please refer to Table S1.

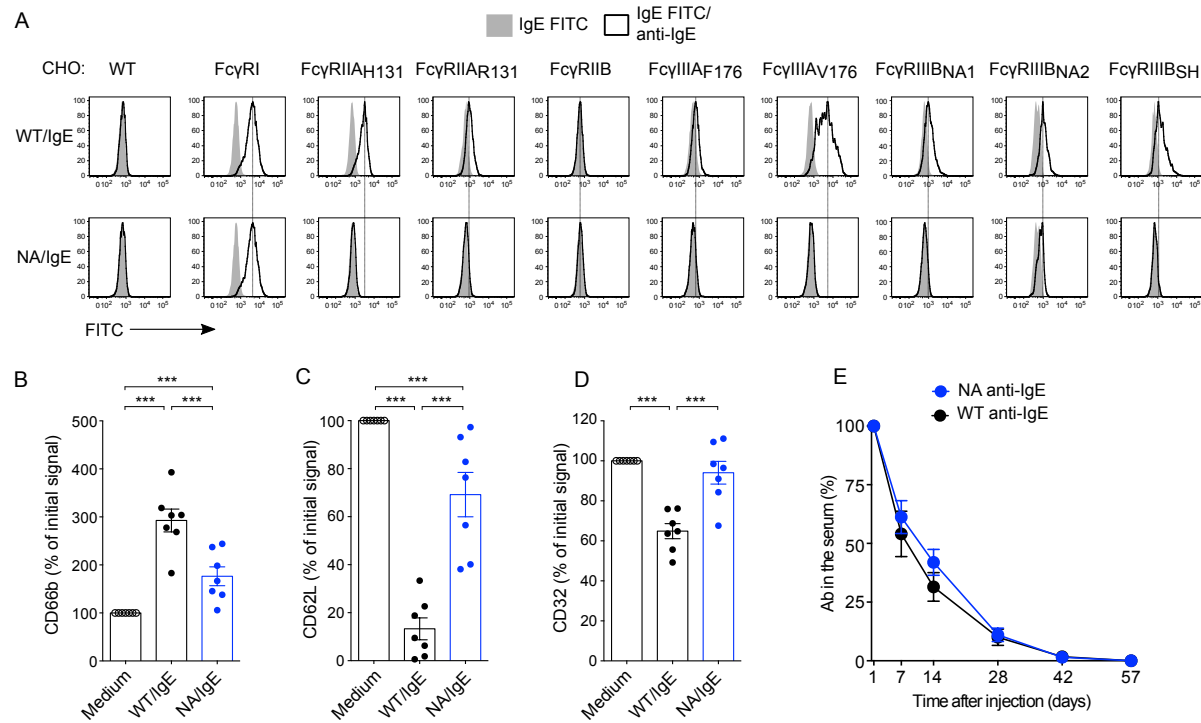
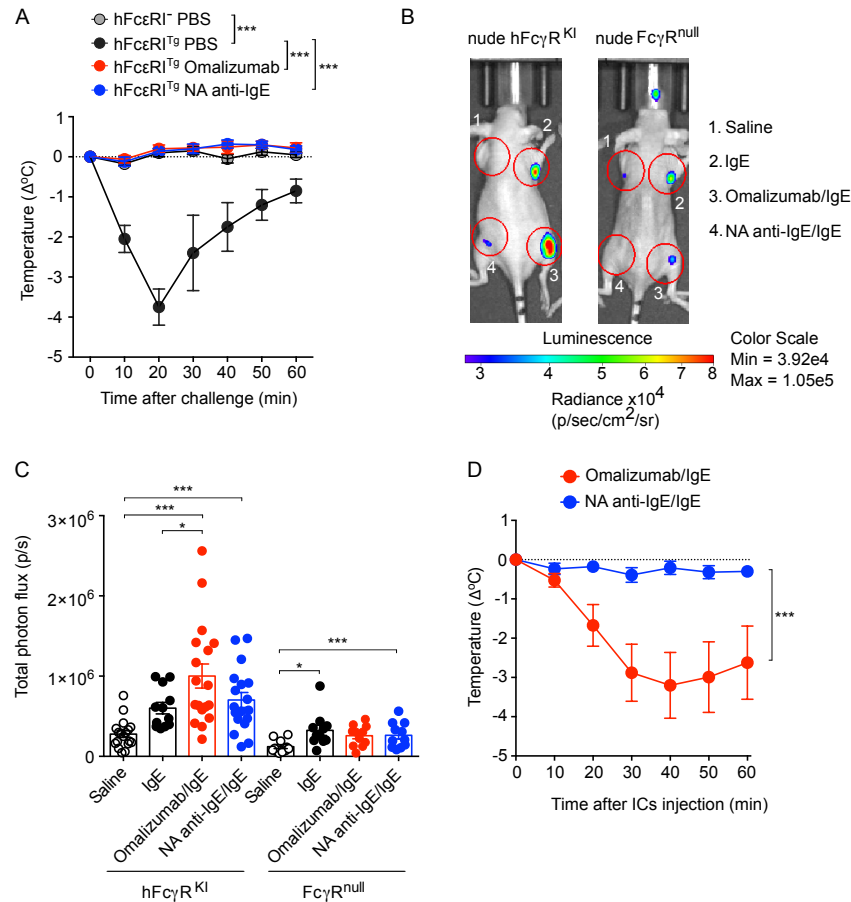


Figure 3. Fc-engineered anti-IgE antibodies display markedly reduced FcγR-binding and neutrophil activation. (A) Binding of ICs made of FITC-IgE and WT anti-IgE or Fc-engineered N297A ('NA') anti-IgE. Data are representative of three independent experiments. (B-C) Expression of CD66b (B), CD62L (C) and CD32 (D) on purified CD45⁺CD15⁺ human neutrophils after 1 h incubation with IgE/WT anti-IgE or IgE/NA anti-IgE ICs or medium alone. Results in B-D show values from neutrophils from individual donors normalized against cells stimulated with medium alone. Bars indicate means ± SEM pooled from three independent experiments (total $n=7$ /group). (E) 100 μg of WT or NA anti-IgE was injected intraperitoneally (i.p.) into hFcγR^{K1}hFcRn^{K1}hβ2m^{K1} mice, and serum was collected at different time-points. Levels of anti-IgE mAbs were measured by ELISA. Data are indicated as means ± SEM pooled from two independent experiments ($n=13$ /group). ***, $P < 0.001$ by contrast linear model in B, C and D and ANOVA in E. For further details on the statistical analysis, please refer to Table S1.



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Figure 4. Fc-engineered anti-IgE antibodies block IgE-mediated anaphylaxis but do not induce Fc γ R-dependent inflammation. (A) Changes in body temperature ($\Delta^{\circ}\text{C}$ [mean $\pm$ SEM]) after intravenous (i.v.) challenge with 500 μg of nitrophenyl-coupled BSA (NP-BSA) in hFc ϵ RI^{Tg} mice pre-treated i.v. with 700 μg Omalizumab, NA anti-IgE or PBS 30 min before sensitization with anti-NP IgE (10 μg). Data in A are pooled from two independent experiments (total $n=4-6/\text{group}$). hFc ϵ RI⁻ mice were used as a control. (B and C) Representative bioluminescent images (B) and quantification (C) of MPO activity 2 h after subcutaneous injection of IgE/Omalizumab or IgE/NA anti-IgE ICs in nude hFc γ R^{KI} or nude Fc γ R^{null} mice. Regions of interest outlined in red surround the site of injection. Bars in C indicate means $\pm$ SEM pooled from five (nude hFc γ R^{KI}, total $n=18$) or four (nude Fc γ R^{null}, total $n=11$) independent experiments. The color bar represents bioluminescent signal in radiance (p/sec/cm²/sr). (D) Changes in body temperature ($\Delta^{\circ}\text{C}$ [mean $\pm$ SEM]) after i.v. injection of IgE/Omalizumab ($n=10$) or IgE/NA anti-IgE ($n=11$) into hFc γ R^{KI} mice. Data in D are pooled from two independent experiments. *, $P < 0.05$; ***, $P < 0.001$ by Contrast test linear model (A and C) or 2-way repeated-measures (ANOVA) (D). For further details on the statistical analysis, please refer to Table S1.