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

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42 **Running title**

43 Xolair induces FcγR-dependent inflammation

44 **Abstract**

45

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

58 **Introduction**

59

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

65

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

73

74 However, treatment with Omalizumab is associated with adverse reactions,
75 ranging from skin inflammation at the injection site to anaphylaxis in ~0.1-0.2% of patients
76 (11-13). The mechanism of these side effects is still unknown. Notably, Omalizumab does
77 not induce the formation of anti-drug Abs, and most cases of anaphylaxis occur within the
78 first three injections of the drug (11-13).

79

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

88 **Results and Discussion**

89

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

99

100 As neutrophils were reported to contribute to IgG-mediated inflammation and
101 anaphylaxis (17), we next evaluated whether Omalizumab/IgE ICs can activate neutrophils
102 through engagement of FcγRs. We purified neutrophils from healthy donors and incubated
103 these cells with Omalizumab/IgE ICs. We found that such ICs induce marked upregulation
104 of CD66b and downregulation of CD62L on the surface of neutrophils, which are
105 considered hallmarks of neutrophil activation (18, 19) (**Figure 1, C and D**). The ICs also
106 induced downregulation of FcγRII (CD32) (**Figure 1E**). As human neutrophils express
107 FcγRIIA and not FcγRIIB (20), and Omalizumab/IgE ICs do not bind FcγRIIB (**Figure**
108 **1A**), our results indicate that the ICs induce active engagement of FcγRIIA in neutrophils.

109

110 To further confirm the role of FcγRs in neutrophil activation, we performed similar
111 experiments with neutrophils purified from hFcγR^{KI} mice (in which all mouse FcγRs have
112 been replaced with human FcγRs) (20, 21) or FcγR^{null} mice (deficient for all FcγRs) (**Figure**
113 **1F**). Omalizumab/IgE ICs induced a downregulation of CD62L in neutrophils from
114 hFcγR^{KI} mice, but not in neutrophils from FcγR^{null} mice (**Figure 1F**), demonstrating that
115 Omalizumab/IgE can activate neutrophils through engagement of human FcγRs.
116
117 The most frequent side effect observed with Omalizumab is skin inflammation (13).
118 We hypothesized that such local inflammation could be a consequence of FcγRs
119 engagement. To assess this, we injected Omalizumab/IgE ICs subcutaneously into hairless
120 (to avoid shaving-induced skin inflammation) nude hFcγR^{KI} mice and nude FcγR^{null} mice,
121 and assessed skin inflammation after 2 h by bioluminescence imaging of myeloperoxidase
122 (MPO) activity (20, 22). We observed a strong MPO activity at the site of ICs injection in
123 hFcγR^{KI} mice (**Figure 2, A and B**). By contrast, MPO activity was markedly reduced upon
124 injection of IgE alone or Omalizumab alone, or injection of ICs in FcγR^{null} mice. Thus, our
125 results indicate that Omalizumab/IgE ICs can induce skin inflammation through
126 engagement of hFcγRs.
127
128 The most dramatic side effect reported for Omalizumab is anaphylaxis (12, 13). We
129 thus assessed whether Omalizumab/IgE ICs can induce anaphylaxis in hFcγR^{KI} mice.
130 Intravenous injection of ICs induced significant hypothermia (the main readout of
131 anaphylaxis in mice (23)) in hFcγR^{KI} mice (**Figure 2C**). Importantly, hypothermia was not
132 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 ($N_{297}A$ 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

156 produced a non-mutated version of this mAb ('WT anti-IgE'). Both the WT and NA anti-
157 IgE mainly formed trimers when incubated with IgE in vitro (**Supplemental Figure 4, A-**
158 **D**), which is consistent with the data we obtained using commercial Omalizumab
159 (**Supplemental Figure 1**). As expected, ICs made of IgE and the WT anti-IgE could bind
160 all activating FcγRs, while binding to FcγRs was markedly reduced with ICs made of IgE
161 and the NA anti-IgE (**Figure 3A**). Indeed, IgE/NA anti-IgE ICs could only bind to FcγRI,
162 which is consistent with a previous report showing that the $N_{297}A$ mutation does not
163 abrogate binding to this high-affinity FcγR (28). In addition, WT anti-IgE could bind
164 human C1q (**Supplemental Figure 4E**), but we detected no binding to C1q with the NA
165 anti-IgE (**Supplemental Figure 4E**). Finally, we observed activation of human neutrophils
166 with ICs made of IgE and the WT anti-IgE, but markedly reduced activation with ICs made
167 of IgE and the NA anti-IgE (**Figure 3, B-D**).
168
169 FcRn/β2m heterodimers extend the half-life of IgG by reducing lysosomal
170 degradation in endothelial cells (29). To assess the half-life of our anti-IgE mAbs in vivo,
171 we generated $hFc\gamma R^{KI}hFcRn^{KI}h\beta 2m^{KI}$ mice which recapitulate binding of IgG to all human
172 FcγRs and to the human FcRn-β2m complex (**Supplemental Figure 5**). We injected WT
173 or NA anti-IgE into $hFc\gamma R^{KI}hFcRn^{KI}h\beta 2m^{KI}$ mice, and observed similar mAbs levels in
174 sera collected at different time-points (**Figure 3E**). We obtained similar results when
175 comparing the half-life of commercial Omalizumab and the Fc-engineered NA anti-IgE
176 **(Supplemental Figure 6)**. Altogether, these results demonstrate that the $N_{297}A$ mutation
177 does not affect the half-life of the anti-IgE mAb in vivo.
178

179 We also verified that the $N_{297}A$ mutation does not affect the ability of the anti-IgE
180 mAb to block IgE. Both the WT and NA anti-IgE recognized IgE with the same affinity
181 (**Supplemental Figure 7A**), and were equally potent at blocking binding of IgE to human
182 mast cells (**Supplemental Figure 7B**). Moreover, we showed that pre-treatment of
183 $hFc\varepsilon RI^{Tg}$ mice (which express the human IgE receptor hFcεRI (30)) with either
184 Omalizumab or the NA anti-IgE can block IgE-mediated anaphylaxis (**Figure 4A**).
185 Altogether, our results demonstrate that the Fc-engineered NA anti-IgE is equally potent
186 as Omalizumab at blocking IgE-mediated allergic reactions.
187
188 We then compared skin inflammation induced by IgE/Omalizumab or IgE/NA anti-
189 IgE ICs in $hFc\gamma R^{KI}$ mice. Injection of IgE/Omalizumab ICs induced marked MPO activity
190 in the skin (**Figure 4, B and C**). This was reduced to levels observed with injection of IgE
191 alone in $hFc\gamma R^{KI}$ mice injected with IgE/NA anti-IgE ICs (**Figure 4, B and C**). Finally, we
192 compared the ability of ICs made of IgE and Omalizumab or the NA anti-IgE to induce
193 anaphylaxis in $hFc\gamma R^{KI}$ mice. We observed anaphylaxis in mice injected with
194 IgE/Omalizumab ICs but not in mice injected with IgE/NA anti-IgE ICs (**Figure 4D**).
195
196 In summary, our findings demonstrate that Omalizumab forms ICs with IgE which
197 can activate neutrophils, and induce skin inflammation and systemic anaphylaxis through
198 human FcγRs in FcR-humanized mice. Such findings could explain some of the side effects
199 which have been described in patients treated with Omalizumab (12, 13). One must be
200 careful when extrapolating these findings obtained in humanized mice to humans, as very
201 few data have been reported supporting the existence of FcγR-mediated anaphylaxis in

202 humans. However, one recent report provides evidence of an IgG-induced, FcγR-
203 dependent neutrophil activation pathway in anaphylaxis to neuromuscular-blocking agents
204 (NMBAs) in humans (31), which reinforces the potential clinical relevance of our findings.
205 The Fc-engineered anti-IgE mAb we developed is equally potent as Omalizumab at
206 blocking IgE-mediated allergic reactions but does not induce FcγR-mediated
207 inflammation. It could thus potentially be used in patients with very high levels of IgE,
208 and/or in patients with a history of anaphylaxis or other adverse reactions to Omalizumab.
209 Finally, we envision that IC-mediated engagement of FcγRs could be a more general
210 mechanism of therapeutic mAbs-mediated adverse reactions.
211
212
213
214

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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245 France.

246

247 **Competing interests**

248

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

254

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256

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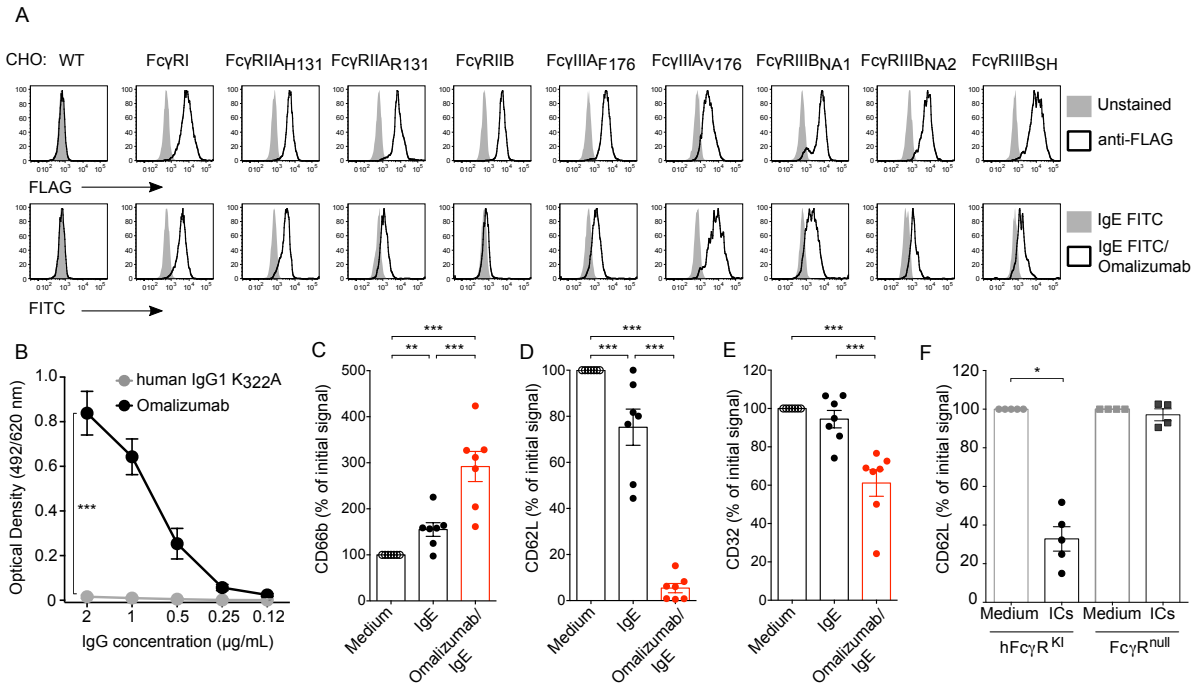

Figure 1. Omalizumab/IgE immune complexes (ICs) bind FcγRs and activate neutrophils. (**A**) Binding of pre-formed IgE/Omalizumab ICs to FcγRs in CHO cells stably transfected with each one of the human FcγRs (16). Upper histograms show binding of an anti-FLAG mAb as a control for FcγR expression. Lower 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 ($K_{322}A$) 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 form $hFc\gamma R^{KI}$ or $Fc\gamma R^{null}$ 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 ($Fc\gamma R^{null}$; total *n*=4/group) or three ($hFc\gamma R^{KI}$; 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.

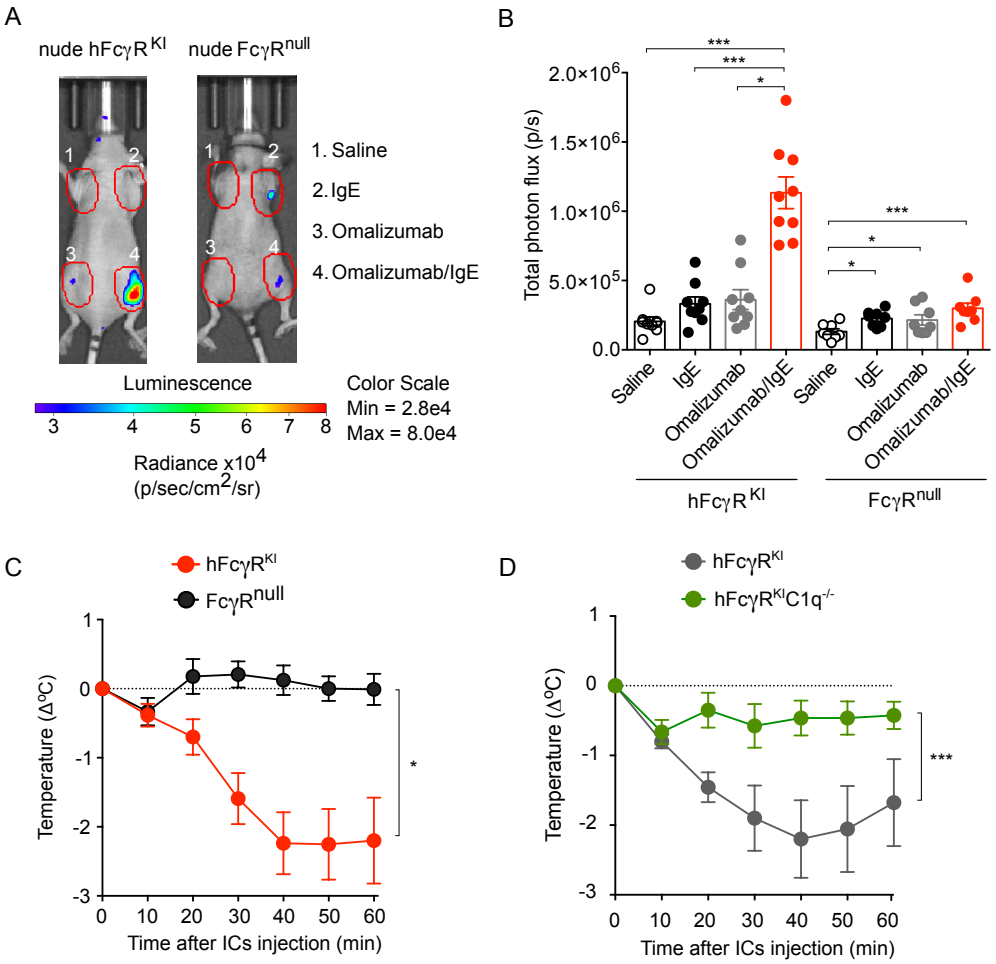

Figure 2. Omalizumab/IgE ICs induce skin inflammation and anaphylaxis through engagement of FcγRs in FcγR-humanized mice. Representative bioluminescent images (**A**) and quantification (**B**) of MPO activity 2 h after subcutaneous injection of IgE/Omalizumab ICs in nude hFcγR^{KI} mice (n=9) or nude FcγR^{null} mice (n=8). Regions of interest outlined in red in **A** surround sites of injection. Data in **B** are means ± SEM pooled from two independent experiments. (**C**-**D**) Changes in body temperature (Δ°C [mean ± SEM]) after intravenous injection of IgE/Omalizumab ICs into hFcγR^{KI} mice (n=13) or FcγR^{null} mice (n=9) (**C**), or hFcγR^{KI} mice (n=9) or hFcγR^{KI} C1q$^{-/-}$ mice (n=8). 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.

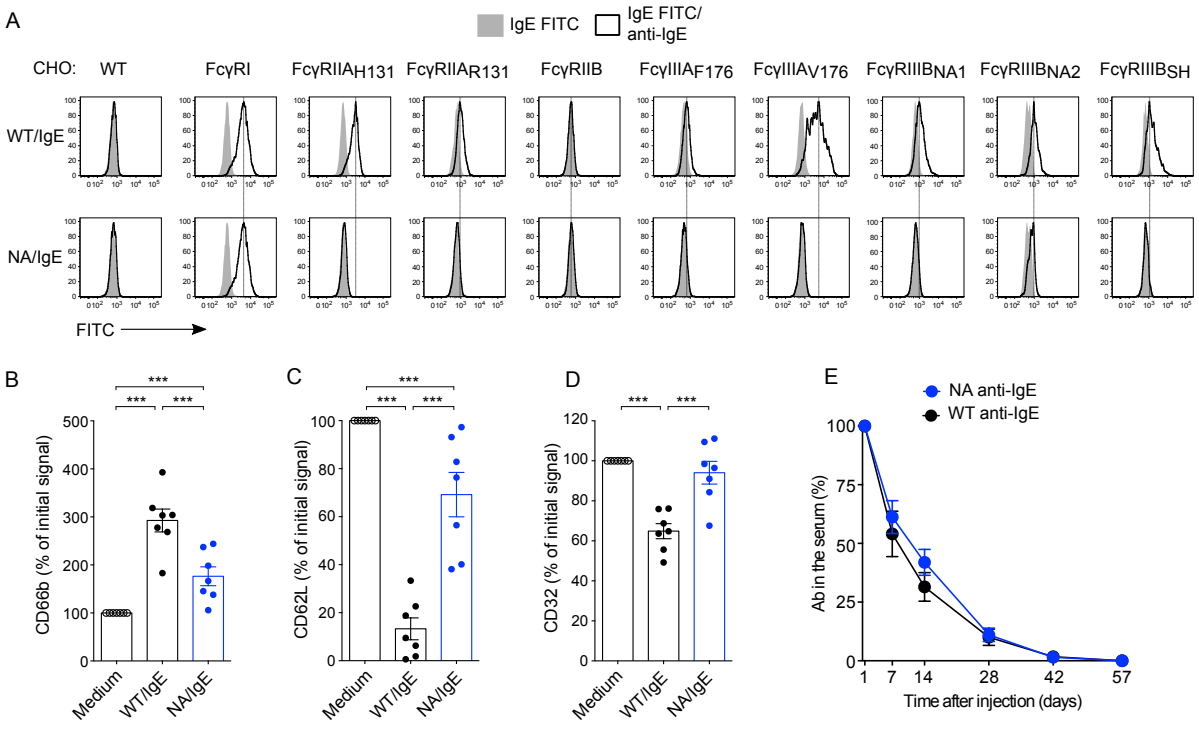

Figure 3. Fc-engineered anti-IgE antibodies display markedly reduced FcγR-binding and neutrophil activation. (**A**) Binding of ICs made of FITC-IgE and WT anti-IgE or Fc-engineered N297A ('NA') anti-IgE. Data are representative of three independent experiments. (**B-C**) Expression of CD66b (**B**), 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 $hFc\gamma R^{KI}hFcRn^{KI}h\beta 2m^{KI}$ 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.

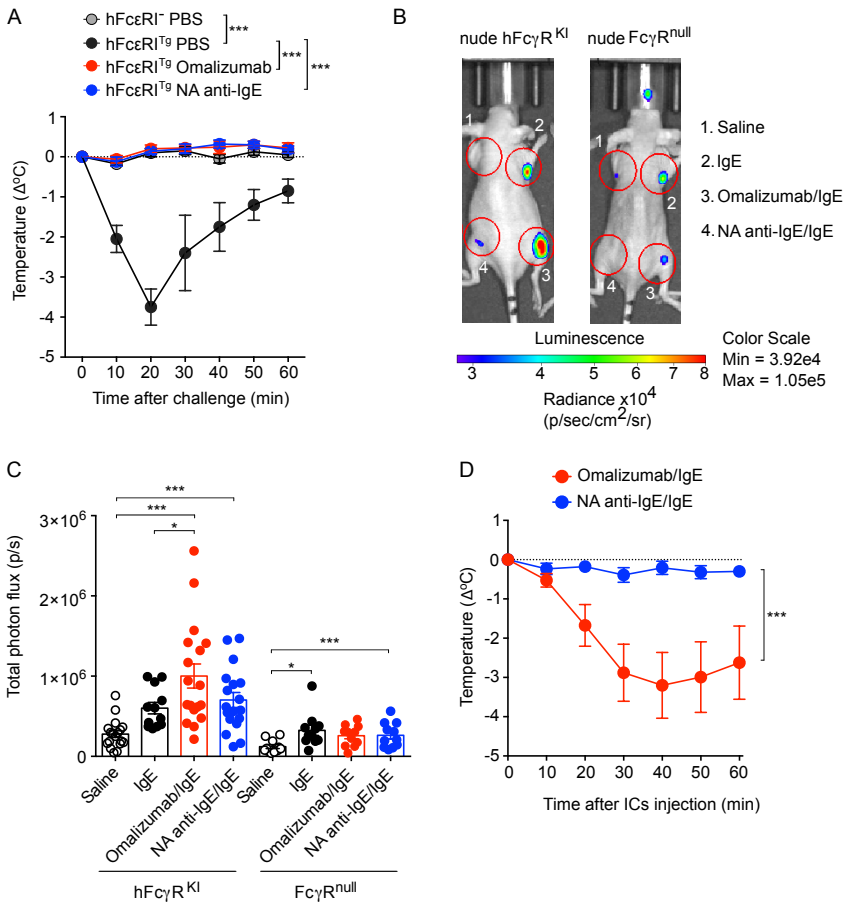

Figure 4. Fc-engineered anti-IgE antibodies block IgE-mediated anaphylaxis but do not induce FcγR-dependent inflammation. (**A**) Changes in body temperature (Δ°C [mean ± SEM]) after intravenous (i.v.) challenge with 500 µg of nitrophenyl-coupled BSA (NP-BSA) in hFcεRI^{Tg} mice pre-treated i.v. with 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/group). hFcεRI^{-} 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 hFcγR^{KI} or nude FcγR^{null} mice. Regions of interest outlined in red surround the site of injection. Bars in **C** indicate means ± SEM pooled from five (nude hFcγR^{KI}, total *n*=18) or four (nude FcγR^{null}, total *n*=11) independent experiments. The color bar represents bioluminescent signal in radiance (p/sec/cm^2/sr). (**D**) Changes in body temperature (Δ°C [mean ± SEM]) after i.v. injection of IgE/Omalizumab (*n*=10) or IgE/NA anti-IgE (*n*=11) into hFcγR^{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.