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

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**Running title**

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 (FceRII) (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).
We hypothesized that the formation of immune complexes (ICs) between Omalizumab and IgE could be responsible for some of the adverse reactions observed with this therapeutic mAb. Using mice humanized for all IgG receptors (FcγRs), we demonstrate here that Omalizumab/IgE ICs can induce both skin inflammation at the site of injection of the drug, as well as systemic anaphylaxis through engagement of FcγRs. Finally, we developed an Fc-engineered version of Omalizumab which blocks IgE-mediated allergic reactions without inducing FcγR-dependent adverse reactions.
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
Omalizumab alone in hFcγR^K1^ mice (Supplemental Figure 2), demonstrating that the ICs induce systemic anaphylaxis through engagement of human FcγRs. Previous work indicates that hFcγRIIA contributes to experimental anaphylaxis in humanized mice (21, 24, 25). We did not observe anaphylaxis in mFcγR^null^hFcγRIIA^Tg^ mice (which express only hFcγRIIA (21)), indicating that hFcγRIIA is not sufficient to trigger Omalizumab/IgE-mediated anaphylaxis (Supplemental Figure 3A). By contrast, anaphylaxis was markedly reduced in hFcγR^K1^ mice pre-treated with a blocking mAb against hFcγRIII (Supplemental Figure 3B). This suggests that hFcγRIII plays an important role in Omalizumab/IgE-mediated anaphylaxis. However, these results have to be interpreted carefully as we cannot exclude that pre-treatment with the anti-hFcγRIII mAb induces engagement of the receptor to some extent, thereby “desensitizing” cells expressing hFcγRIII.

Since Omalizumab also binds complement component C1q (Figure 1B), we assessed the potential contribution of C1q to ICs-induced anaphylaxis. We found that anaphylaxis is markedly reduced in hFcγR^K1^C1q^−/−^ mice, which express all hFcγRs but lack mouse C1q (Figure 2D). Although further work is required to confirm the implication of human C1q, our data strongly suggest that the complement pathway plays an important role, through C1q engagement, in Omalizumab/IgE-induced anaphylaxis.

Based on these results, we decided to produce an Fc-engineered form of Omalizumab (using available Omalizumab V_H and V_L sequences (4)) lacking the N-linked glycan attached to asparagine 297 in the Fc portion (N_{297}A mutation) to reduce binding to FcγRs 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 hFcγRIIaβ2mKI 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 hFcγRIIaβ2mKI 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 hFceRI^Tg mice (which express the human IgE receptor hFceRI (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
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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


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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.

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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 hFcγRκI or FcγRnull 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 (FcγRnull; total n=4/group) or three (hFcγRκI; 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<sup>KI</sup> mice (n=9) or nude FcγR<sup>null</sup> 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<sup>KI</sup> mice (n=13) or FcγR<sup>null</sup> mice (n=9) (C), or hFcγR<sup>KI</sup> mice (n=9) or hFcγR<sup>KI</sup>C1q<sup>−/−</sup> 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^K/KhFcRn^K/Khβ2m^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 hFceRI 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). hFceRI 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/cm²/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.