

Comparable actions of omalizumab on mast cells and basophils

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Clinical & Experimental Allergy

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Cite this as: E. Serrano-Candelas, R. Martínez-Aranguren, A. Valero, J. Bartra, G. Gastaminza, M. J. Goikoetxea, M. Martín, M. Ferrer, *Clinical & Experimental Allergy*, 2016 (46) 92–102.

Summary

Background Omalizumab (OmAb) has recently been approved for the treatment of diseases other than allergic asthma, including chronic urticaria. The exploration of the use of OmAb in chronic urticaria was based on the presence of IgE autoantibodies against autoantigens such as anti-IgE, anti-FcεRI, and IgE antibodies against thyroid peroxidase in certain patients with chronic urticaria. OmAb recognizes and sequesters free IgE to prevent its interaction with FcεRI. However, OmAb is equally and rapidly effective against autoimmune and non-autoimmune urticaria, suggesting the possible involvement of additional mechanisms of IgE.

Objectives We sought to investigate the *in vitro* mechanism of action of OmAb in mast cells and basophils.

Methods Both LAD2 human mast cell line, previously sensitized with IgE, and *ex vivo* basophils were incubated with OmAb at different doses, analysing its effect on IgE-dependent events (e.g., degranulation, phosphorylation-mediated signalling, and eicosanoid release).

Results We found that OmAb dissociates pre-bound IgE from mast cells and basophils, resulting in a reduction of proximal phosphorylation-mediated signalling events (Syk, PLCγ, and LAT) and in a decrease in degranulation and leukotriene synthesis.

Conclusion Our data prove the existence of common mechanisms of action of OmAb in mast cells and basophils that would explain its effectiveness and rapid effect in chronic urticaria and provide a basis for its use in other diseases mediated by these cells.

Keywords basophils, chronic urticaria, FcεRI, IgE, mast cells, omalizumab

Submitted 3 July 2015; revised 17 October 2015; accepted 21 October 2015

Introduction

Omalizumab (OmAb) is a recombinant humanized monoclonal antibody that binds to the C-epsilon-3 locus of the Fc (constant fragment) portion of free immunoglobulin E (IgE) [1–3]. Anti-IgE OmAb recognizes an epitope that is masked when IgE is bound to its high-affinity receptor, Fc epsilon receptor I (FcεRI). OmAb was synthesized with the aim of sequestering free IgE to reduce its binding capacity, thereby reducing mediator release and allergic inflammation [4]. OmAb rapidly reduced IgE levels and, consequently, also lowered FcεRI levels in mast cells [5] and basophils [6], thus resulting in a

reduction of the amount of positive allergen skin prick tests in patients suffering from allergic rhinitis.

OmAb is currently used in allergic conditions other than asthma, including chronic urticaria and food allergies [3]. Sera from a subset of patients with chronic spontaneous urticaria (CSU) were demonstrated to activate normal basophils [7] and mast cells [8]. These patients had IgG against the alpha subunit of the IgE receptor or IgE itself [7–10]. The administration of OmAb proved to be effective in a proof of concept study carried out with a subset of 12 patients with CSU and with antibodies against FcεRI [11]. Furthermore, after obtaining findings suggestive of IgE autoantibodies against thyroid peroxidase (antithyroid peroxidase Abs) [12], the first large multi-centre, double-blinded,

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and placebo-controlled study that was carried out with OmAb for CSU, included patients with IgE antithyroid antibodies, definitely supported the use of OmAb in cases of CSU [13].

Unexpectedly, a small clinical trial also proved that OmAb was equally effective for the treatment of patients with non-autoimmune CSU [14]. Moreover, in a subset of patients treated with OmAb, effectiveness was reached within the first 24 to 48 h. These results cannot be explained by the previously accepted mechanism of action of OmAb sequestering free IgE.

Eggel et al. recently reported on the ability of non-immunoglobulin-based IgE inhibitor E2_79, a designed ankyrin repeat protein (DARPin), and to a lesser extent of that of OmAb, to dissociate pre-bound IgE from its receptor in *ex vivo* basophils [15]. Our research further explores the effect of physiological doses of OmAb on this action, delving into its functional and signalling consequences in mast cells and comparing them to that of basophils. We also aimed to test our hypothesis that this newly described activity of OmAb does not depend on its immunoglobulin nature, using human IgG as a control.

In order to perform such study, we first assessed the ability of OmAb to decrease the level of pre-bound IgE from mast cells and compared this to its ability in the case of basophils. Thereafter, we measured mast cell and basophil activation after the administration of the OmAb treatment by means of several techniques, as well as after an anti-IgE challenge to study the mediator release and synthesis, along with the signalling cascade.

Understanding the mechanism of action of OmAb in mast cells and basophils will potentially enable us to find additional applications for this drug.

Materials and methods

Cells and reagents

The LAD2 human mast cell line was provided by Drs. A. Kirshenbaum and D.D. Metcalfe (National Institutes of Health, Bethesda, MD, USA) and grown in StemPro-34 serum-free medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with StemPro-34 Nutrient, L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 µg/mL), and 100 ng/mL recombinant stem cell factor (SCF) (Amgen, Thousand Oaks, CA, USA). The basophil experiments were performed using the buffy coat from blood pertaining to non-atopic donors. The donor blood was processed within the first hour after its collection and was subsequently centrifuged at 500 g for 10 min, removing the serum. The buffy coat was then collected and centrifuged again to remove any remaining serum. The cells were re-sus-

pended in Tyrode's buffer: HEPES calcium buffer (20 mM HEPES, 133 mM NaCl, 5 mM KCl, 7 mM CaCl₂, 3.5 mM MgCl₂, 1 mg/mL bovine serum albumin [Sigma-Aldrich, St. Louis, MO, USA], and 1 µL/mL heparin [5000 UI/mL], pH 7.4).

Omalizumab was obtained from Novartis (Basel, Switzerland). Rabbit anti-PLCγ Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-Syk was obtained from BioLegend (San Diego, CA, USA). Antiphosphotyrosine (pTyr) monoclonal and anti-ERK Abs were obtained from Zymed Laboratories (Invitrogen). Antilinker for activation of T cells (LAT), anti-pLAT_{Y191}, anti-pSyk_{Y352}, anti-pPLCγ_{Y783}, and anti-pERK_{T202/Y204} Abs were purchased from Cell Signaling Technology (Danvers, MA, USA). Biotinylated human IgE (IgE^B) was obtained from Abbiotec (San Diego, CA, USA). Anti-mouse and anti-rabbit IgG peroxidase Abs were obtained from DAKO (Carpinteria, CA, USA) and Bio-Rad (Hercules, CA, USA), respectively. Anti-β-actin peroxidase monoclonal antibody (clone AC-15) and streptavidin (STV) were purchased from Sigma (Sigma, St. Louis, MO, USA). A FITC-conjugated anti-FcεRI antibody, phycoerythrin (PE)-conjugated STV, Fluorescein isothiocyanate (FITC)- and PE-conjugated anti-CD63 antibodies, PE- and Allophycocyanin (APC)- or PE-conjugated Annexin V, 7-Aminoactinomycin D (7AAD), purified mouse anti-IgE antibody, and Pharm lysing buffer were purchased from BD Biosciences (San Jose, CA, USA). Whole-molecule human IgG was obtained from Jackson ImmunoResearch (West Grove, PA, USA). PerCP-Cy5.5-conjugated anti-human HLA-DR, APC-conjugated anti-human FcεRI, and PE-Cy7-conjugated anti-human CD123 antibodies were purchased from eBioscience (San Diego, CA, USA), and FITC-conjugated anti-human IgE antibody was purchased from Invitrogen.

Cell activation

Mast cell activation was performed following the procedures described elsewhere [16]. Briefly, LAD2 cells were sensitized overnight with IgE^B (100 ng/mL) in culture media. The following day, the cells were washed three times in Tyrode's buffer to eliminate unbound IgE, were subsequently incubated with OmAb or hIgG at different concentrations (high-dose experiments: 2, 1, 0.5, and 0.25 mg/mL; or low-dose experiments: 300, 100 and 30 µg/mL) in culture media and for different periods of time, and were then stimulated with streptavidin for varying periods of time. Supernatants were collected for CysLT measurement, and the cells were used for WB analysis or FACS staining.

Basophil suspensions (50 µL) were incubated with 50 µL of OmAb or hIgG at different concentrations (high-dose experiments: 2, 1 and 0.5 mg/mL; or low-

dose experiments: 300, 100 and 30 µg/mL) for 3, 12 or 24 h. After washing them three times with stimulation buffer (Tyrode's buffer containing 2 ng/mL IL-3), the cells were stimulated with 1 µg/mL anti-human IgE or stimulation buffer alone for 40 min at 37°C. The reaction was stopped with 100 µL of stopping buffer (20 mM HEPES, 133 mM NaCl, 5 mM KCl, and 0.27 mM EDTA), and the tubes were centrifuged at 4°C at 900 g for 5 min. The cells were then labelled for FACS staining, and the supernatants were used for CysLT measurements.

FACS staining

Mast cells were activated following the aforementioned protocol. Surface-bound biotinylated IgE and FcεRI were analysed by direct staining with STV-PE or anti-FcεRI-FITC, respectively, for 30 min at 4°C. For the detection of CD63, IgE^B-sensitized cells were stimulated by incubation with 200 ng/mL STV for 30 min and, after being washed, were subsequently stained with anti-CD63-FITC. The cell apoptosis analysis was performed by incubation with Annexin-APC and 7AAD for 15 min as described elsewhere [17].

Basophil-bound CD63, FcεRI, and IgE were determined by incubation with 20 µL of an HLA-DR-PerCP-Cy5.5, CD123-PE-Cy7, anti-IgE-FITC, anti-CD63-PE, and anti-FcεRI-APC cocktail for 30 min at 4°C. Erythrocytes were lysed with 2 mL of 10× pharm lysing buffer (Becton Dickinson, Shannon, County Clare, Ireland) for 10 min in the dark. Cell lysis was stopped by centrifugation at 900 g at 4°C for 5 min. The cells were then washed and re-suspended with 500 µL of stopping buffer to perform the cytometric analysis. Cell apoptosis was evaluated by incubation of Annexin-PE and 7AAD for 15 min with cells that had been previously labelled with HLA-DR-PerCP-Cy5.5 and CD123-PE-Cy7. Erythrocytes were lysed following the aforementioned procedure.

The cells were then analysed using a FACSCalibur or a FASCCanto II flow cytometer (FACScan; BD Biosciences, Mountain View, CA, USA) and FlowJo Tree Star software. In all cases, dead cells were gated out based on the forward (FSC) and side scattering (SSC) profile.

Immunoblotting

IgE^B-sensitized cells (5×10^5 cells/sample) were incubated with OmAb or hIgG as described above. After undergoing extensive washing, the cells were stimulated with 200 ng/mL STV for the indicated times (0, 1 or 5 min). Whole-cell lysates were obtained as described elsewhere [18], separated by SDS-PAGE and

electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The blots were probed with the indicated Abs. In all blots, proteins were visualized with the aid of an ECL reagent (Advanta, CA, USA) and quantified using the Quantity One software (Bio-Rad).

Cysteinyl leukotriene (CysLT) measurements

Mast cells were activated following the aforementioned protocol. The IgE^B-sensitized cells (2×10^4 /sample) were subsequently incubated with OmAb or hIgG for the indicated times, washed and seeded in 96-well flat-bottom microtiter assay plates and stimulated with 200 ng/mL STV for 30 min at 37°C. The supernatants were used to measure total CysLT levels using the Cysteinyl Leukotriene Express Competitive EIA Kit (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's protocol. The analysis was performed in triplicate.

The supernatants (125 µL) from the basophil activation assays described above were used in the CysLT Cellular Antigen Stimulation Test (CAST ELISA, Bühlmann Laboratories, Switzerland) according to the manufacturer's instructions.

Statistical data analysis

All results are expressed as means $\pm$ standard deviations of the mean value (SD). One-way ANOVA and Bonferroni post hoc tests were used to determine significant differences (*P*-value) using the GraphPad software. Asterisks indicate the following *p* values: **P* value ≤ 0.05 , ***P* ≤ 0.01 , ****P* ≤ 0.001 , and *****P* ≤ 0.0001 .

Results

OmAb removes surface IgE from mast cells and basophils

High doses of OmAb have proven to be effective in accelerating the dissociation of pre-bound IgE in *ex vivo* basophils [15]. To explore this novel activity in mast cells and its functional consequences, we first analysed surface IgE in sensitized LAD2 cells after removing free IgE and incubating the cells with OmAb or hIgG as a control. Incubation of OmAb with the IgE^B-sensitized LAD2 cells reduced the pre-bound IgE levels in a time- and dose-dependent manner (Fig. 1). hIgG had no effect on cell surface IgE levels, indicating the specificity for OmAb in this experiment. High OmAb levels (2–0.5 mg/mL) were able to dissociate pre-bound IgE as early as 4 h (Fig. 1a–c); however, lower OmAb doses (100–30 µg/mL), closer to the

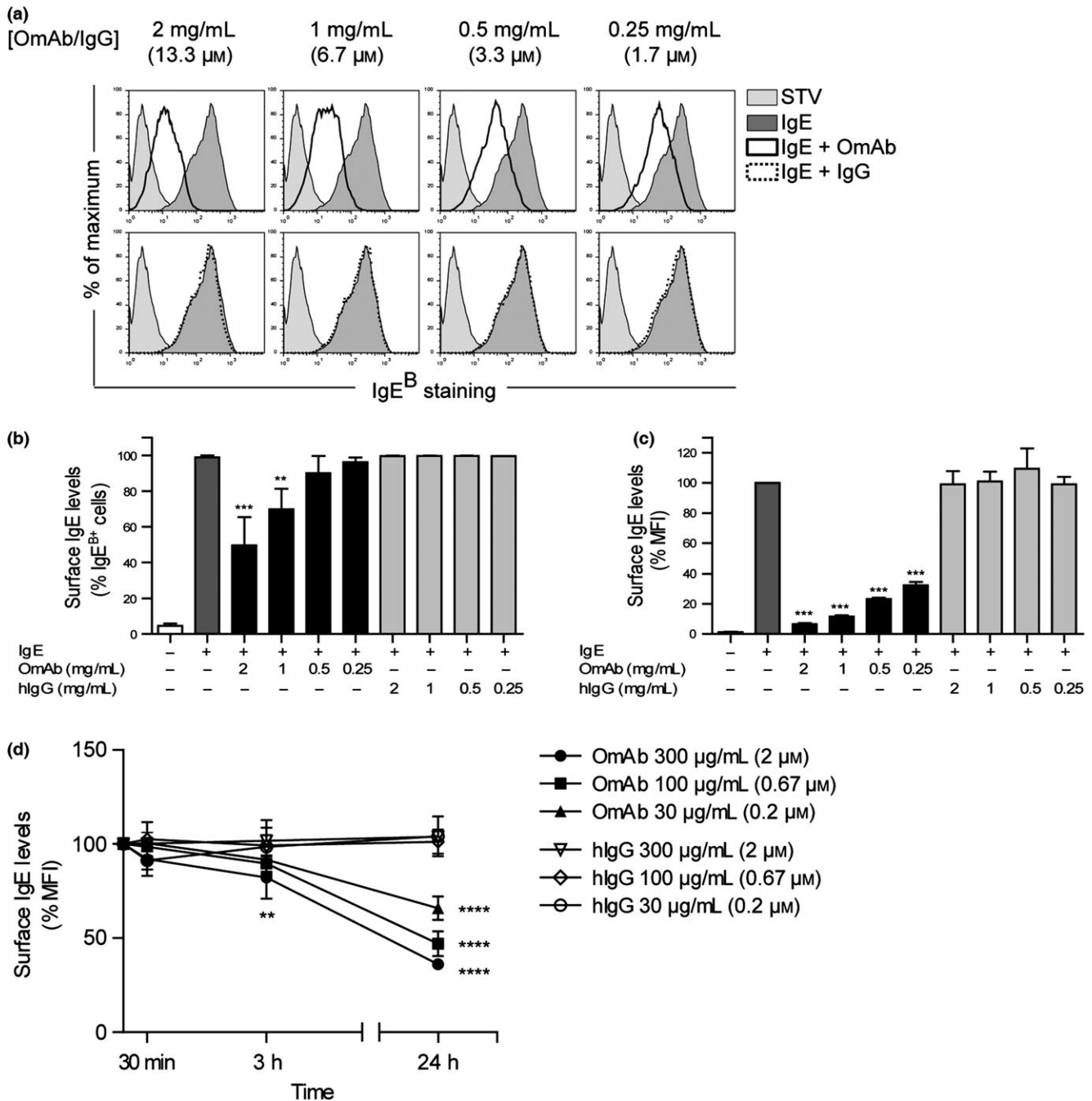


Fig. 1. OmAb removes surface-bound IgE in LAD2 cells. (a) Histograms of surface IgE^B staining of pre-sensitized LAD2 cells after incubation with OmAb for 4 h at different doses (black line). hIgG was used as a control (dotted line). The light grey-shaded curve (STV) shows the negative control (IgE and OmAb/IgG untreated cells). (b) The bar charts represent the percentage of IgE^B-positive cells and (c) the percentage of mean fluorescence intensity (MFI) for IgE^B with respect to OmAb/hIgG untreated cells (without OmAb or hIgG). The first bar indicates the negative control (IgE and OmAb/IgG untreated cells). Results are expressed as the mean $\pm$ SD values of three independent experiments. Statistical significance (** P < 0.01, *** P < 0.001) is relative to OmAb/IgG untreated cells (second bar). (d) Time-course of IgE^B dissociation mediated by OmAb in LAD2 cells. Results are expressed as the mean $\pm$ SD values of three independent experiments. Statistical significance (** P < 0.01; **** P < 0.0001) is relative to OmAb/IgG untreated cells.

physiological blood levels of OmAb (25–60 μ g/mL) [19], required a longer incubation time (24 h) in order to be effective (Fig. 1d).

Simultaneously, we studied fresh blood basophils that already had pre-bound IgE. The cells were incubated for different times and with varying doses of OmAb or

hIgG. After 24 h of incubation, bound IgE levels were significantly decreased in the presence of OmAb (Fig. 2a). Interestingly, we detected a significant IgE dissociation as early as after 3 h of OmAb incubation (Fig. 2b–c). Likewise, we observed a similar effect when using low OmAb doses; however, a longer incubation time was needed to achieve a significant dissociation of IgE from the basophils' surface (Fig. 2c).

OmAb removes IgE from FcεRI in mast cells and basophils without involving receptor endocytosis

To determine whether OmAb was able to reduce FcεRI expression apart from dissociating IgE from its receptor, we evaluated the receptor expression levels in mast cells and basophils (Fig. 3). Both short- (4 h) and long-term (24 h) incubations with OmAb did not affect FcεRI expression in mast cells (Fig. 3a) in spite of causing a drastic reduction of pre-bound IgE levels. However, longer incubations (12–24 h) induced a decrease in the levels of receptor expression in basophils (Fig. 3b). It has been previously reported that monomeric IgE is capable of stabilizing FcεRI in the cell membrane and of increasing its expression as a mechanism to amplify

its signal [20]. For this reason, it is important to note that this effect on the reduction of FcεRI expression is likely a consequence of the absence of IgE bound to the receptor, and not a direct effect of OmAb on the receptor's expression.

Effects of OmAb in mast cell and basophil activation

Next, we studied the physiological relevance of IgE dissociation on mast cell activation, compared to the case of basophil activation. To this end, IgE^B-sensitized mast cells were incubated with OmAb (or hIgG as a control) for 4 h and were subsequently challenged with streptavidin (STV) for 30 min. As shown in Fig. 4a, OmAb reduced mast cell degranulation, as measured by the CD63 activation marker. OmAb *per se* does not affect CD63 expression (data not shown). To rule out the possibility that OmAb also reduces cell activation as a result of an increase in the levels of cellular apoptosis, we determined whether mast cell viability was compromised in the presence of OmAb. Annexin V-7AAD staining was performed in cells that were incubated with human IgG or OmAb. No differences in cell viability were observed (Fig. 4b).

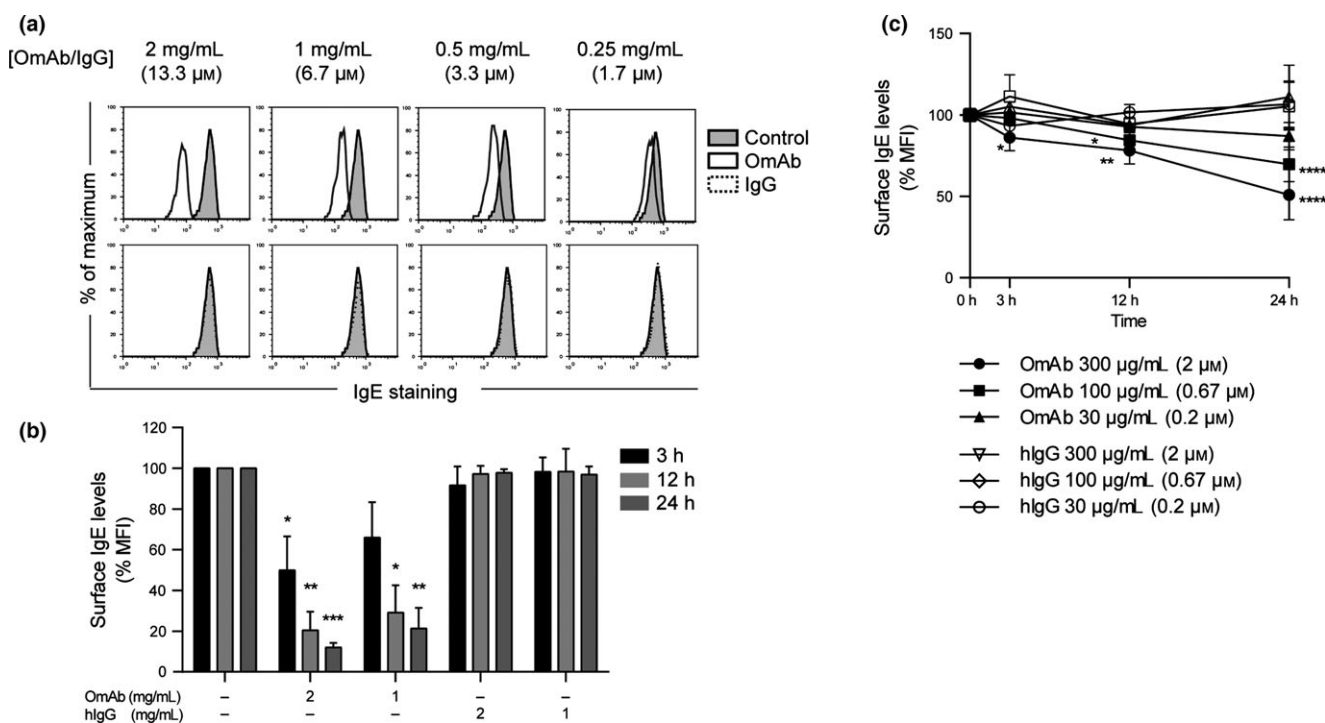


Fig. 2. OmAb removes surface-bound IgE in primary basophils. Primary basophils from 3 non-atopic donors were incubated with different concentrations of OmAb/hIgG for 3, 12, and 24 h, respectively, and subsequently stained for IgE. (a) The histograms show surface-bound IgE levels after 24 h of incubation with OmAb (black line), hIgG (dotted line) and untreated cells (light grey shaded). (b) The bar chart represents the percentage of MFI for IgE with respect to OmAb/hIgG untreated cells. Results are expressed as the mean $\pm$ SD values of three independent experiments. Statistical significance (* P < 0.05, ** P < 0.01, *** P < 0.001) is relative to OmAb/hIgG untreated cells (first three bars). (c) Time-course of IgE dissociation mediated by OmAb in basophils. Results are expressed as the mean $\pm$ SD values of three independent experiments. Statistical significance (* P < 0.05, ** P < 0.01, **** P < 0.0001) is relative to OmAb/hIgG untreated cells.

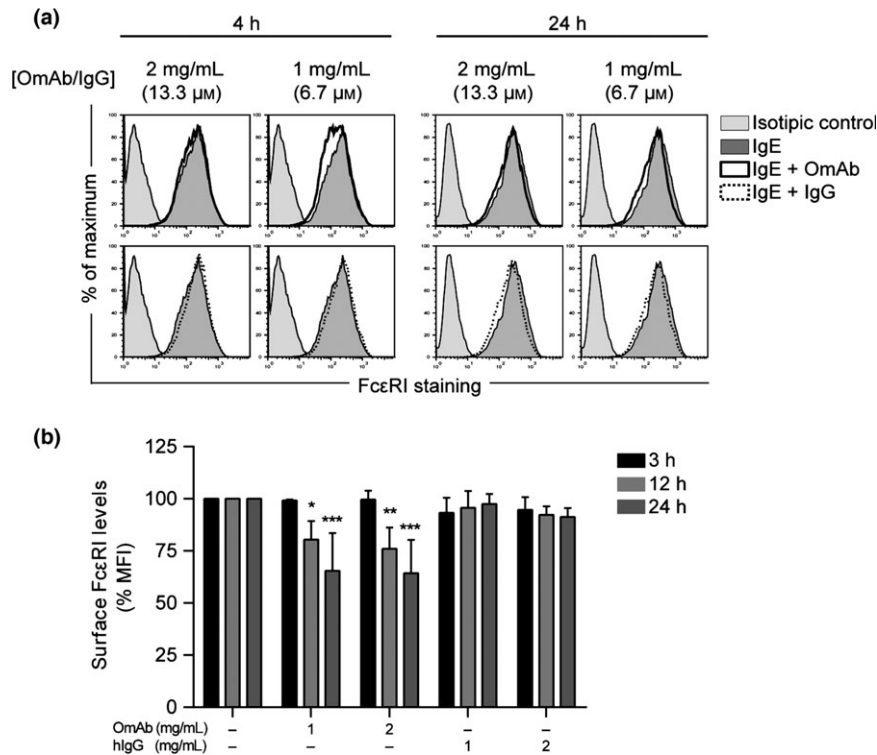


Fig. 3. The reduction of pre-bound IgE from the cell surface is not due to a decrease in FcεRI levels. (a) Histograms of surface FcεRI staining of previously sensitized LAD2 cells after incubation with OmAb for 4 and 24 h at different doses (black line). hIgG was used as a control (dotted line). Data are representative of three independent experiments. (b) The bar chart represents the percentage of MFI for FcεRI, with respect to OmAb/hIgG untreated cells for human basophils incubated with OmAb at different times and doses. Results are expressed as the mean $\pm$ SD values of three independent experiments. Statistical significance (* P < 0.05, ** P < 0.01, *** P < 0.001) is relative to OmAb/IgG untreated cells (first bar).

Under similar conditions, OmAb significantly reduced basophil degranulation at different time points, as measured by means of CD63 staining, achieving significant differences in CD63 expression after 24 h of OmAb incubation (Fig. 4c). Finally, similarly to the case of mast cells, OmAb did not alter basophil viability (Fig. 4d).

OmAb attenuates IgE-mediated activation of human mast cells

In order to further examine this mechanism of action, we analysed the phosphorylation status of IgE^B-sensitized mast cells which were treated with OmAb. LAD2 cells were sensitized with IgE^B, washed to remove unbound IgE^B, and incubated with OmAb for 4 h. Thereafter, the cells were challenged with STV for 1 or 5 min. The cells were then lysed as described in section Material and Methods, and their phosphorylation pattern was evaluated by means of a western blot analysis. As shown in Fig. 5a, global tyrosine phosphorylation was reduced in the presence of OmAb (lanes 5–9) compared to the case of cells that had been incubated without OmAb (lanes 2–4). Moreover, this reduction in the pTyr signal

proved to be dose-dependent. Human IgG, which was used as a control, had the same effect on cells as that of the incubation with media alone (lanes 10–14).

We subsequently evaluated the phosphorylation status of Syk, PLCγ, LAT, and ERK, effectors of FcεRI-dependent events. The phosphorylation status of these molecules was reduced, indicating that incubation with OmAb decreased both proximal events regulated by Syk, PLCγ, and LAT, and downstream events governed by ERK (Fig. 5a,b).

OmAb inhibits leukotriene release in mast cells and basophils

ERK1/2 phosphorylates cytoplasmic phospholipase A2, which is involved in the production of the eicosanoid precursor arachidonic acid [21]. Leukotrienes are a product of the 5' lipoxygenase pathway of arachidonic acid metabolism and are generated in leucocytes and other immunocompetent cells, such as mast cells, eosinophils, neutrophils, monocytes, and basophils. We therefore assessed the ability of OmAb to inhibit leukotriene synthesis in mast cells and basophils. Our results demonstrated that OmAb inhibits cysteinyl leukotriene (CysLT)

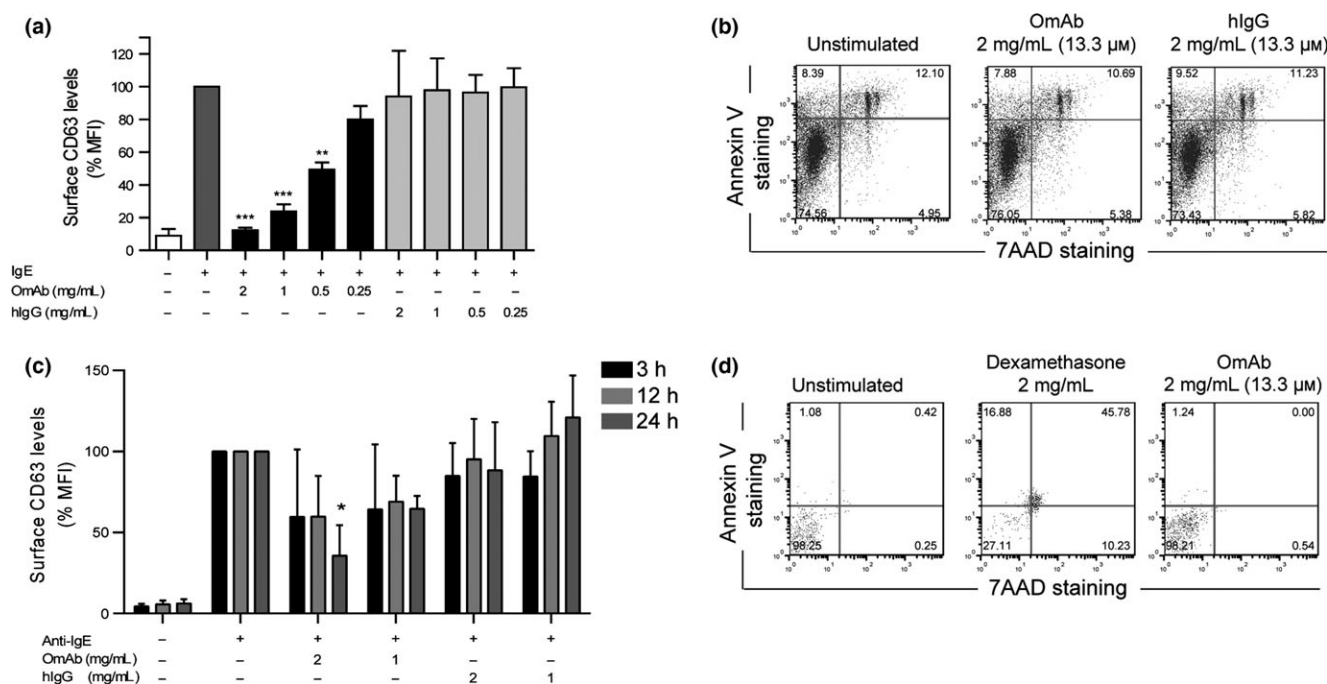


Fig. 4. OmAb inhibits IgE-mediated degranulation in LAD2 cells and primary basophils. (a) LAD2 cells were sensitized with IgE^B overnight and washed to remove unbound IgE^B. The cells were stained for CD63 after an IgE^B challenge. The bar charts represent the percentage of MFI for CD63, with respect to OmAb/IgG untreated cells (second bar). hlgG was used as a control. The first bar indicates the negative control (IgE and OmAb/IgG untreated cells). The data represent the mean $\pm$ SD values of three independent experiments. Statistical significance (** $P < 0.01$, *** $P < 0.001$) is relative to OmAb/IgG untreated cells. (b) LAD2 cells were incubated with OmAb (or hlgG as a control) for 4 h, and cell apoptosis (by Annexin V/7AAD staining) was evaluated. (c) Human primary basophils from three non-atopic donors were incubated with different concentrations of OmAb for 3, 12 and 24 h, respectively. Basophils were stimulated with 1 μ g/mL anti-IgE for 40 min, and CD63 expression was analysed. The bar charts represent the percentage of MFI for CD63 with respect to the OmAb/IgG untreated cells. The first bars indicate the negative control (IgE and OmAb/IgG untreated cells). The data represent the mean $\pm$ SD values of 3 independent experiments. Statistical significance (* $P < 0.05$) is relative to OmAb/IgG untreated cells. (d) Basophil apoptosis was measured after the incubation of the donor cells for 24 h at 37°C with 2 mg/mL OmAb, 2 mg/mL dexamethasone as a positive apoptosis control, or stimulation buffer as a negative control. Cell apoptosis was evaluated by Annexin V/7AAD staining.

release in IgE^B-sensitized mast cells (Fig. 6a) and basophils (Fig. 6b). Overall, our data prove that OmAb can inhibit leukotriene synthesis and secretion, thus explaining the ability of OmAb to reduce leukotrienes during sustained inflammation in asthma and allergic reactions [22–24].

Discussion

IgE plays a central role in the pathophysiology of allergic diseases. It was also recently proposed that IgE plays a role in the amplification of inflammation favouring the infiltration of immune cells into secondary lymphoid organs [25, 26]. OmAb is effective in treating allergic diseases other than asthma, providing new insights into unexpected roles for IgE [27–31]. In the case of chronic urticaria, the exact mechanism of OmAb is still unknown. Moreover, OmAb has been reported to control physical urticarias [32–34]. Thus, OmAb could have additional properties that act directly on mast cells and basophils.

In this study, we described an OmAb activity in mast cells that was already proposed to act in basophils [15]. This mode of action may be particularly relevant to chronic urticaria, in which case mast cells play a key role. We showed that OmAb inhibits mast cell activation by mediating the reduction of IgE from its receptor. The relevant experiments were performed side by side with basophils. Inhibition occurred after only a few hours and was consistent with the rapid response observed in a minority subset of patients suffering from chronic urticaria that could not be explained by FcεRI internalization resulting from the disappearance of free IgE. A possible explanation for this result is that OmAb is able to disturb the IgE-FcεRI balance by rapidly sequestering any free-IgE molecule. This hypothesis is supported by the dissociation constant (K_d) reported for IgE:OmAb (0.02–7 nM; which is dependent on the nature of the IgE [35]) and IgE:FcεRI (0.1 nM) complexes [36]. Thus, OmAb would not only act by preventing new IgE from binding, as stated for some time now, but also by sequestering IgE from FcεRI after it has already bound.

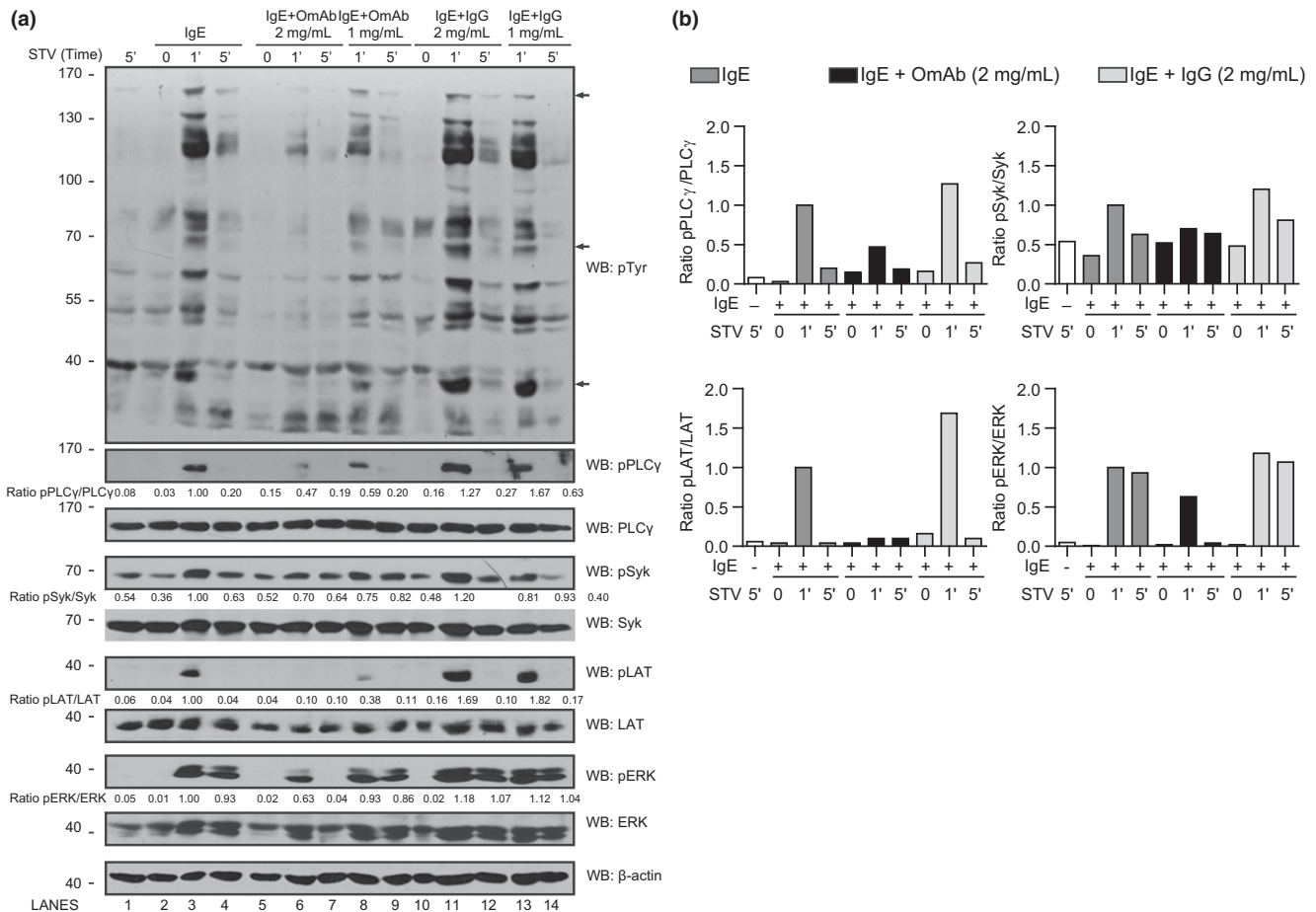


Fig. 5. OmAb incubation attenuates IgE-dependent signalling activation. LAD2 cells were sensitized with IgE^B and subsequently incubated for 4 h with OmAb or hIgG as a control. IgE^B was subsequently cross-linked with 200 ng/mL STV for the indicated times. (a) Specific mAb that recognizes phosphorylated-Tyr was used to analyse the general activation status. Specific mAbs against p-PLC γ _{Y783}, p-Syk γ ₃₅₂, p-LAT γ ₁₉₁, p-ERK γ _{T202/Y204} were used to analyse the phosphorylation status of these proteins in LAD2 whole-cell lysates. The total levels of these proteins were also measured, and β -actin was used as a loading control. (b) The bars graphics correspond to western blot quantitation by densitometry.

Furthermore, we found that OmAb not only inhibits CD63 expression upregulation (a degranulation marker) but also IgE-dependent proximal and distal signalling events. In our study, Syk phosphorylation was reduced. This kinase plays an essential role in early and late mast cell responses by binding to the phosphorylated Fc ϵ RI γ chain ITAMs after receptor aggregation, resulting in a conformational change that increases the enzymatic activity of Syk [37] and leads to downstream signal propagation. Activated Syk phosphorylates LAT, which acts as a scaffold for a multi-molecular signalling complex that includes PLC γ , among other molecules [38]. Therefore, we determined that the IgE-dependent/Syk-LAT-PLC γ signalling axis was affected by the OmAb treatment.

Our data clearly demonstrate that OmAb also reduces ERK phosphorylation and suppresses subsequent events, such as leukotriene synthesis, in mast cells and basophils, which explains the effects of OmAb on sustained inflammation in asthmatic patients [39].

Thus, we proved that OmAb can reduce IgE bound to the cell surface of mast cells, causing a reduction of all IgE-dependent events in a time- and dose-dependent manner.

Our data show some experiments performed both at supraphysiological doses (2 mg/mL (13 μ M) and physiological concentrations (30 μ g/mL (0.2 μ M)). With regard to the doses of OmAb, pharmacodynamics data [35] have demonstrated that after the administration of a single subcutaneous dose of OmAb (300 mg), its mean serum concentration is of 33.1 ± 10.4 μ g/mL, ($n = 23$). In a study performed in a Japanese population, Hayasi [19] obtained a serum concentration of 25–60 μ g/mL after the administration of a single dose of OmAb 300 mg, whereas Kornmann [40] obtained a maximum serum concentration of 121.9 μ g/mL after the administration of two doses of OmAb 450 mg on day 1 and day 15 of the study. The dose of OmAb is not adjusted to body weight and IgE concentration for the treatment of chronic

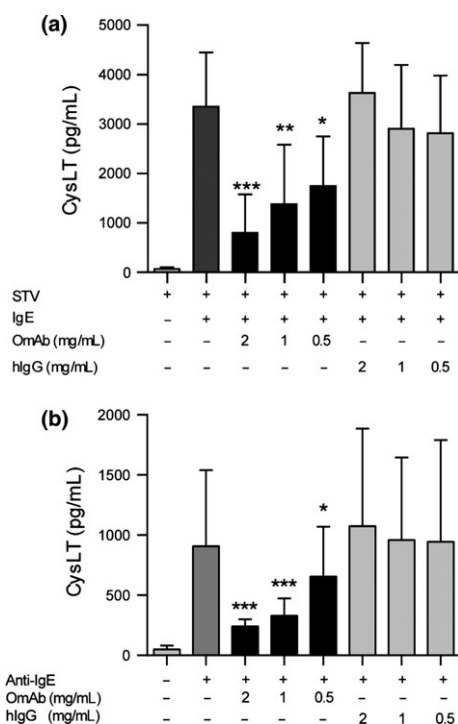


Fig. 6. OmAb inhibits IgE-mediated leukotriene secretion. (a) LAD2 cells were sensitized overnight with IgE^B, washed several times and then incubated for 4 h with OmAb or hIgG as a control. Thereafter, cells were challenged with 200 ng/mL STV for 30 min. Supernatants were then used for cysteinyl leukotriene (CysLT) secretion quantification. The first bar indicates the negative control (IgE and OmAb/IgG untreated cells). The data represent the mean $\pm$ SD values of three independent experiments. Statistical significance (* P < 0.05, ** P < 0.01, *** P < 0.001) is relative to OmAb/IgG untreated cells. (b) Primary basophils from healthy donors were pre-incubated with OmAb or hIgG as a control for 24 h at different concentrations. The basophils were then activated with anti-IgE for 40 min. The supernatants were used to measure CysLT levels. The first bar indicates the negative control (OmAb/IgG untreated cells). The data represent the mean $\pm$ SD values of three independent experiments. Statistical significance (* P < 0.05, *** P < 0.001) is relative to OmAb/IgG untreated cells.

urticaria, thus, OmAb plasma and tissue levels may vary among individuals.

Regarding the mechanism of action of OmAb on basophils, our results are consistent with those obtained by Eggel and colleagues [15]. Our research data reflect the physiological relevance of its action in mast cells and a comparison for both cells: mast and basophils with a physiological dose range of OmAb. While Eggel used a concentration of 50 μ M, we observed effects at a concentration as low as 0.6 μ M, which could be closer to that of the skin's physiological concentrations. In addition, using IgG as a control, we proved that the ability of OmAb to dissociate IgE was not a result of its immunoglobulin nature. The same experiments were performed with hIgG₁ instead of total hIgG to determine isotype specificity, without observing any

differences (data not shown). Both the histidine and the polysorbate contained in the OmAb solution as a diluent buffer were also analysed separately to rule out any undesirable effect, with no significant differences being found in this case either (data not shown).

It has recently been reported that OmAb does not inhibit mast cell or basophil activation *in vitro* [41]. Gericke's study was performed with low doses of OmAb (10 μ g/mL; 0.067 μ M), with the mast cells and basophils being incubated for 1 h or 24 h, respectively. Our results are consistent with that of Gericke's study, given that no effects were observed in the mast cells or basophils at such doses. We believe that any differences between both studies could possibly be explained by this fact.

The differences in the kinetics of IgE dissociation of mast cells and basophils that were observed in our study may be partly explained by the nature of the IgE. In human primary basophils, IgE is already bound to their surface receptor. In the case of mast cells, LAD2 were incubated with a single IgE clone from the Abbiotec company. In addition, this different IgE dissociation could also be explained by the differences between both types of cells in terms of the time elapsed until the decrease of Fc ϵ RI expression, as reported elsewhere [5, 6].

We did not observe the induction of any cellular apoptotic effects by OmAb *per se* in mast cells or basophils. However, monomeric IgE increases mast cell survival, mediator release, cytokine synthesis and Fc ϵ RI expression [42, 43]. Thus, the depletion of bound IgE may eventually decrease cell survival as well as Fc ϵ RI expression.

In conclusion, we proved that OmAb can dissociate pre-bound IgE from its receptor, Fc ϵ RI, in mast cells and basophils. We also demonstrated that OmAb can inhibit mast cell and basophil activation *in vitro* by directly inhibiting early and late IgE-dependent phosphorylation events, consequently leading to a decrease in pre-existing and newly synthesized mediators. This additional mechanism of action of OmAb explains its efficacy in controlling diseases such as chronic urticaria as rapidly as within 24 h. Thus, our study provides new information regarding the mechanism of action of OmAb in mast cells and completes Eggel's data on basophils. However, the role of this drug in an IgE-independent context remains unclear.

Acknowledgements

This study was funded by a grant from Novartis Spain and by grant Pi1200332 from the Health Research Fund of the Spanish Ministry of Economy and Competitiveness (Fondo de Investigaciones Sanitarias, Ministerio de Economía y Competitividad). RM was supported by

grant RD12/0013/0010 from the Carlos III Health Institute of Spain. RM, JB and MF belong to the Spanish Research Network on Adverse Reactions to Allergens and Drugs (RIRAAF: Red de Investigación de Reacciones Adversas Alérgenos y Fármacos) of the Carlos III Health Institute. We are indebted to the Citomics core facility of the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) for its technical help.

Conflict of interest

Dr. Ferrer is a member of the scientific advisory board of Novartis, a speaker for both said company and FAES,

has served on the scientific advisory board of Genentech, receives research grants from Novartis and has received the speaker honorarium title from MSD, Novartis, FAES and GSK. Antonio Valero reports having worked as a consultant for Faes Farma, Chiesi, Orion Pharma, MSD, Novartis, UCB, Uriach Pharma, GSK and Stallergenes; having been paid lecture fees by Novartis, GSK, Stallergenes, Chiesi, Leti and Thermofisher; and having received grant support from Novartis, Faes Farma and Uriach Pharma. Joan Bartra reports having served as a consultant for Novartis, Faes Farma and Hal Allergy; and having been paid lecture fees by Novartis, Stallergenes, UCB, Thermofisher, Menarini and Chiesi.

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