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Sequential allergen desensitization of basophils is non-specific and may involve p38 MAPK

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Abstract

Background: Sequential allergen desensitization provides temporary tolerance for allergic patients. We adapted a clinical protocol to desensitize human blood basophils *ex vivo* and investigated the mechanism and allergen specificity.

Methods: We included 28 adult, grass allergic subjects. The optimal, activating allergen concentration was determined by measuring activated CD63⁺ CD193⁺ SS^{Low} basophils in a basophil activation test with 8 log-dilutions of grass allergen. Basophils in whole blood were desensitized by incubation with twofold to 2.5-fold increasing allergen doses in 10 steps starting at 1 : 1000 of the optimal dose. Involvement of p38 mitogen-activated protein kinase (MAPK) was assessed after 3 min of allergen stimulation ($n = 7$). Allergen specificity was investigated by desensitizing cells from multi-allergic subjects with grass allergen and challenging with optimal doses of grass, birch, recombinant house dust mite (rDer p2) allergen or anti-IgE ($n = 10$).

Results: Desensitization reduced the fraction of blood basophils responding to challenge with an optimal allergen dose from a median (IQR) 81.0% (66.3–88.8) to 35.4% (19.8–47.1, $P < 0.0001$). CD63 MFI expression was reduced from 68 248 (29 336–92 001) to 30 496 (14 046–46 179, $P < 0.0001$). Basophils from multi-allergic subjects were desensitized with grass allergen. Challenge with grass allergen resulted in 39.6% activation (15.8–58.3). An unrelated challenge (birch, rDer p2 or anti-IgE) resulted in 53.4% activation (30.8–66.8, $P = 0.16$ compared with grass). Desensitization reduced p38 MAPK phosphorylation from a median 48.1% (15.6–92.8) to 26.1% (7.4–71.2, $P = 0.047$) and correlated with decrease in CD63 upregulation ($n = 7$, $r > 0.79$, $P < 0.05$).

Conclusion: Desensitization attenuated basophil response rapidly and non-specifically at a stage before p38 MAPK phosphorylation.

Currently, the only treatment modifying the underlying disease mechanism causing allergy is allergen immunotherapy (AIT). The disease-modifying effect is induced soon after initiating treatment and persists for years after discontinuation (1, 2). The mechanism behind AIT is not fully understood, but involves blocking IgG antibodies, a skewing from a Th2 towards a Th1 response and increased number and activity of regulatory T cells (1–3). This happens after weeks of treatment, yet an effect can already be seen after a few days of ‘rush up-dosing’ (4, 5). Desensitization is proposed to contribute to this early tolerance (2, 3). Desensitization has been used to treat patients with allergy to antibiotics since the

1950s (6). A standardized drug desensitization protocol has been established and treatment expanded to include allergy to chemotherapeutics and to monoclonal antibodies (7).

The desensitization mechanism has been investigated in many studies, but is still not fully characterized. Mast cells and basophils definitely play key roles in desensitization, as they are the effector cells of the immediate, IgE-driven hypersensitivity reaction. During this reaction, the body is re-exposed to the allergen, which cross-links two specific IgE molecules presented on high-affinity IgE receptors (FcεRI) on the cell membrane of mast cells or basophils. This initiates an activation cascade resulting in calcium mobilization and

finally degranulation of histamine and other inflammatory mediators and synthesis of interleukins and leukotrienes (8). When desensitization is performed, either as rapid drug desensitization or as allergen immunotherapy up-dosing, mediator release is blunted and fewer or no symptoms occur. Little is known about the exact mechanism, but changes in the FcεRI-signalling cascade involving Syk, SHIP and the mitogen-activated protein kinases (MAPKs) (9, 10) have been proposed along with slow mediator depletion and activation of the tolerogenic histamine receptor 2 (11). There are conflicting reports in the literature concerning allergen specificity of desensitization (12–16).

In the literature, several *in vitro* desensitization experiments performed on cultured mast cells (12, 13), purified basophils (17, 18) and peripheral mononuclear blood cells (15, 16) are described. Furthermore, desensitization in these experiments was often performed with subthreshold allergen doses in the presence or absence of extracellular calcium and did not address allergen concentrations optimal for response (15, 17–20). Although subthreshold desensitization has been the standard method for investigating desensitization mechanisms, it does not properly reflect the situation of *in vivo* rapid drug desensitization. The aim of this study was to investigate the effects of allergen desensitization of blood basophils by an adaptation of the standardized protocol for drug desensitization focusing on making the conditions as close to *in vivo* circumstances as possible. We explored allergen specificity as well as the role of p38 MAPK during desensitization.

Materials and methods

Reagents

Human serum albumin (HSA, 10%) was from Sigma-Aldrich (St. Louis, MI, USA). Phosphate-buffered saline (PBS) was from Thermo Fisher Scientific, Oxoid (Hampshire, UK). FACS lysing solution, Phosflow Lyse/Fix buffer, p38 MAPK (pT180/pY182) PerCP-Cy 5.5 (catalogue no. 560406) and anti-IgE (catalogue no. 555894) were acquired from BD Biosciences (San Jose, CA, USA). Anti-CD193 Alexa 647 (catalogue no. 310710) and anti-CD63 FITC (catalogue no. 312004) were acquired from Biolegend (San Diego, CA, USA). Grass and birch pollen Aquagen and rDer p2 (recombinant house dust mite allergen) were a gift from ALK-Abelló (Hørsholm, Denmark).

Subjects

This study included 28 patients with a history of symptomatic rhino-conjunctivitis [by ARIA definitions (21)] and/or allergic asthma [by GINA definitions (22)] on exposure to grass pollen. Exclusion criteria were age <18, serious comorbidity, pregnancy, fever or cold on the day of blood sampling, statins, cyclosporine (up to 3 months previously), beta-blocker, ACE inhibitor, tri-cyclic antidepressants, oral corticosteroids and allergen immunotherapy. For subject characteristics, see Table 1.

Table 1 Clinical characteristics

Clinical characterization	
Male	12/28
Age (range)	37.6 (20–63)
Years with allergy (range)	18.0 (0–56)
Total IgE (kU/l)	903.0
Grass-specific IgE (kU/l)	12.7
Birch-specific IgE (kU/l)	8.7
House dust mite-specific IgE (kU/l)	12.0
Grass allergy	28/28
Birch allergy	16/28
House dust mite allergy	17/28
Other allergies	24/28
Skin prick test pos/neg	27/28
Rhinitis	23/28
Conjunctivitis	16/28
Asthma	12/28

All patients had a history of symptomatic rhino-conjunctivitis and/or allergic asthma on exposure to grass pollen and a positive skin prick test (except for one whose dermatitis did not allow skin prick testing). Data presented as mean (range) or positive/total. IgE data for three patients are missing.

Multi-allergic subjects ($n = 5$) were selected for allergen specificity tests based on comparable levels of specific IgE against grass pollen (mean total serum IgE 3483.4 kU/l; mean specific serum IgE 29.5 kU/l) and either birch pollen ($n = 2$; mean specific serum IgE 9.9 kU/l) or Der p2 ($n = 3$; mean specific serum IgE 40.5 kU/l), and comparable reactivity to the allergens in a basophil activation test (BAT). The subjects ($n = 5$) used for specificity tests with anti-IgE fulfilled inclusion criteria.

The regional ethics committee approved the protocol. Participants gave written, informed consent. Experiments comparing two different protocols or parameters were performed in parallel.

Basophil activation test

Heparinized whole blood (80 µl) was incubated with $1.6 \cdot 10^{-9}$ – 10^{-2} g/l grass pollen allergen at log intervals and titrated volumes of anti-CD193 Alexa 647 and anti-CD63 FITC at 37°C for 30 min. PBS was used as negative control. Samples were lysed and fixed with FACS lysing solution and analysed on a BD FACS Canto II flow cytometer. Basophils were identified as SSC^{low} CD193^{bright} by BD FACS Diva software. The PBS sample was used to set the cut-off of maximum 2% activated cells. Activated basophils were identified as CD63^{bright} cells in the basophil population. The concentration giving maximal activation was noted.

Sequential allergen desensitization

Heparinized whole blood (2 ml) was incubated in a water bath at 37°C through 10 steps with twofold to 2.5-fold increasing doses of allergen added every 10 min (Fig. 1). The starting dose (mean starting allergen concentration

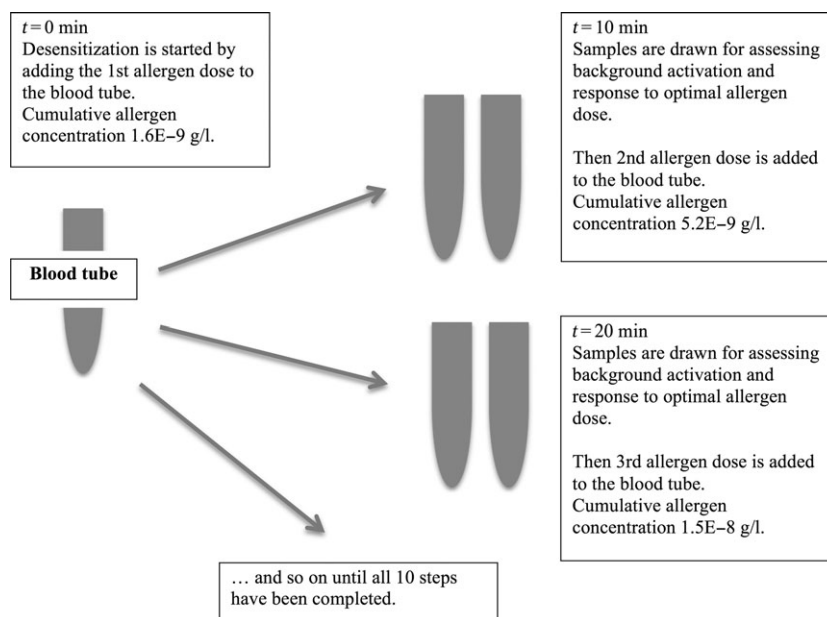


Figure 1 Sequential allergen desensitization. The allergen doses are added to the blood tube containing 2 ml heparinized whole blood. At each time point, two samples are drawn for assessing background activation and to challenge with the allergen dose

3.4×10^{-8} g/l, range 1.6×10^{-13} – 1.6×10^{-7} g/l) was approximately 1 : 1000 of the final concentration, which was equal to the concentration giving maximal activation found during the BAT (mean optimal allergen concentration 2.3×10^{-5} g/l, range 1.1×10^{-9} – 1.1×10^{-3} g/l). This great variation of concentration ranges between subjects is due to their basophils reacting maximally at different allergen concentrations. Before adding the next allergen dose, two samples of 80 μ l were drawn; one to measure background activation in the desensitized blood and one to challenge with the optimal allergen dose as established in the BAT. Allergen concentration was corrected for the change in sample volume. Samples were labelled, treated and analysed as described above for BAT.

Allergen specificity

BATs were performed with grass pollen and either birch pollen or rDer p2 to ensure similar activation and establish the optimal allergen concentrations. Grass pollen was used to desensitize basophils in 2.5 ml blood according to the original procedure, but before each allergen dose, three samples were drawn, the additional sample to challenge with an optimal dose of the unrelated allergen (birch pollen, rDer p2 or anti-IgE).

p38 MAPK

Basophils in whole blood were desensitized according to the protocol. Non-desensitized control samples were kept alongside in the water bath. Cells were labelled with anti-CD193

giving maximal activation in the BAT. Desensitization is completed in 10 steps equalling 100 min. The final, cumulated allergen concentration corresponds to the allergen concentration giving maximal activation in the BAT.

and anti-CD63 as described for BAT. The challenge samples were incubated with grass pollen for 3 min and then placed in ice-cooled water to stop the reaction. Samples were lysed and fixed using the Phosflow Lyse/Fix buffer. Cells were permeabilized with a 0.3% saponin solution for 30 min at 4°C and stained with antiphospho-p38 antibody for 30 min at room temperature in the dark. The basophil population was identified as described for BAT. The non-desensitized, non-challenged control samples were used to set the gate between phosphorylated and nonphosphorylated p38 MAPK.

Statistical analyses

Log-transformed dose–response curves were fitted with 4-parameter nonlinear regression. Data are presented as median values in curves and median with interquartile range (IQR) in scatter plots. Results were compared with a two-tailed, Wilcoxon matched-pairs signed-rank test. Correlation was tested with Spearman's rank correlation. The level of statistical significance was $P < 0.05$. All analyses were performed with GRAPHPAD PRISM 6.0.

Results

Sequential allergen desensitization effectively attenuates basophil response

The allergen concentration giving maximal activation was determined (Fig 2A). Background activation and response to the allergen dose giving maximal activation in the BAT were plotted as a function of allergen concentration. Results are

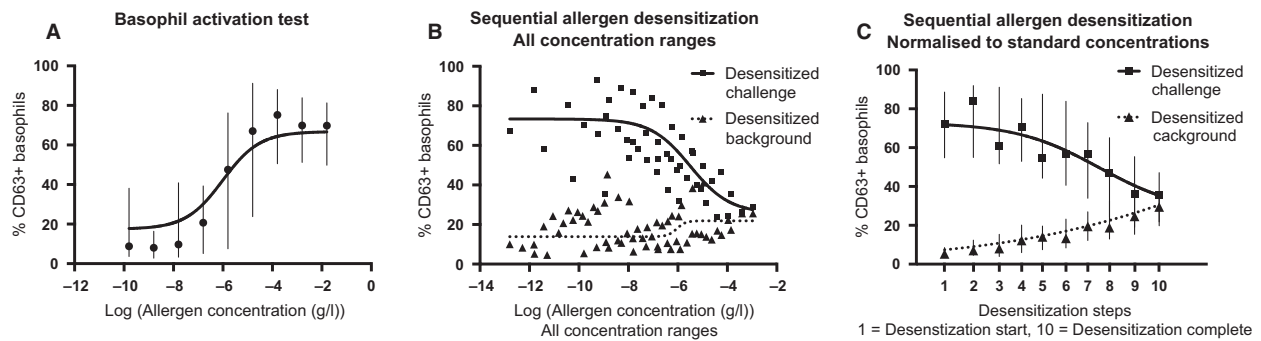


Figure 2 Desensitization of all subjects ($n = 28$), not including repeated analyses. Dose–response curves were fitted to a 4-parameter nonlinear regression model. Maximal activation was determined with a basophil activation test (A). Desensitization

presented as concentration ranges (Fig. 2B) and desensitization steps for a better overview (Fig. 2C).

Desensitization in 10 doubling steps of allergen, starting at 1 : 1000 of the optimal allergen concentration, reduced the fraction of basophils responding to challenge with an optimal allergen concentration from a median 81.0% (IQR 66.3–88.8) at baseline to 35.4% (IQR 19.8–47.1; $P < 0.0001$) after desensitization (Fig. 3A). Individual desensitized basophils responded to a lesser degree as the CD63 mean fluorescence intensity (MFI) of challenged samples decreased from 68 248 (IQR 29 336–92 001) to 30 496 (IQR 14 046–46 179; $P < 0.0001$; Fig. 3B). The fraction of CD63⁺ cells (35.4% compared with 29.5%, $P = 0.009$; Fig. 3A), but not MFI (30 496 compared with 25 676, $P = 0.26$; Fig. 3B), increased significantly by maximal allergen challenge after completed desensitization. Sensitivity calculated during sequential desensitization (mean logEC50 of -5.8 [95% CI -6.7 – (-4.9)] was comparable to the sensitivity to allergen of basophils in untreated whole blood [-6.0 (95% CI -6.9 – (-5.1)), $P = 0.19$, Fig. 2A–B]. Sequential allergen desensitization was superior to keeping control samples in the water bath and desensitizing with a single, suboptimal allergen concentration (data not shown).

curves are plotted with the actual allergen concentrations (B, further explained in Methods) and the desensitization steps from 1 to 10 (C). Symbols and error bars represent median and interquartile range (A, C).

Grass pollen desensitization is not allergen specific

Allergen-specific desensitization has been reported for murine bone marrow-derived mast cells (BMMCs) (12), but not for blood basophils during ultra-rush AIT (23). We desensitized basophils with grass pollen allergen and challenged with optimal concentrations of both grass and the unrelated allergen in parallel tubes (Fig. 4). Basophil reactivity to grass was reduced from a median 78.4% (IQR 50.8–90.2) to 39.6% (IQR 15.8–58.3; $n = 10$; $P = 0.002$), while reactivity to the unrelated allergen (birch ($n = 2$), rDer p2 ($n = 3$) or anti-IgE ($n = 5$)) was reduced from 84.3% (IQR 77.8–89.1) to 53.4% (IQR 30.8–66.8; $n = 10$, $P = 0.002$). There was no significant difference in the challenge values between grass and the unrelated allergens at baseline ($P = 0.38$) or after desensitization ($P = 0.16$).

Desensitization occurs upstream of p38 MAPK

p38 MAPK, one of the early signal transduction molecules activated by IgE-FcεRI-induced signalling (10), was upregulated after 3 min of stimulation with allergen at optimal concentration (Fig. 5A). Sequential grass desensitization

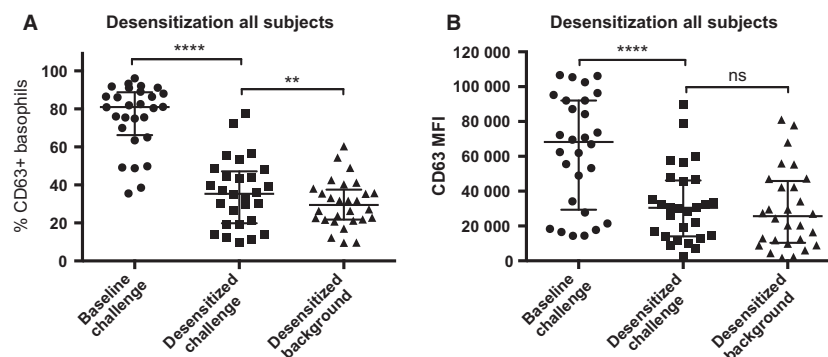


Figure 3 Activation of basophils challenged at baseline and after desensitization was compared along with the background activation of the desensitized cells (A). CD63 mean fluorescence intensity

(MFI) was also assessed in these settings (B). Error bars represent median $\pm$ IQR. ns = $P > 0.05$; ** = $P < 0.01$; **** = $P < 0.0001$.

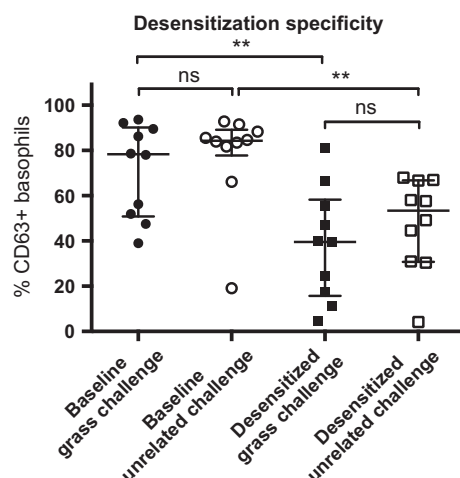


Figure 4 Allergen specificity tested by desensitizing basophils with grass allergen and challenging with an unrelated allergen ($n = 10$). Challenge at baseline and after desensitization was compared for both grass and an unrelated allergen (birch, rDer p2 or anti-IgE). Error bars represent median with IQR. ns = $P > 0.05$. ** = $P < 0.01$.

reduced p38 phosphorylation after a 3-minute allergen challenge from a median 48.1% (IQR 15.6–92.8) to 26.1% (IQR 7.4–71.2, $P = 0.047$). However, p38 phosphorylation was not completely inhibited, causing an increase from a median 8.9% (IQR 3.2–18.5) to 26.1% (IQR 7.4–71.2, $P = 0.03$) following allergen challenge of desensitized cells (Fig. 5A).

We explored the relationship between change in p38 MAPK and CD63 as both were upregulated by activation and attenuated by desensitization. Decrease in phosphorylation of p38 MAPK after desensitization correlated with decrease in upregulation of CD63, both as fraction of positive cells ($R_s = 0.79$, $P = 0.048$; Fig. 5B) and when comparing MFI ($R_s = 0.96$, $P = 0.0028$; Fig. 5C).

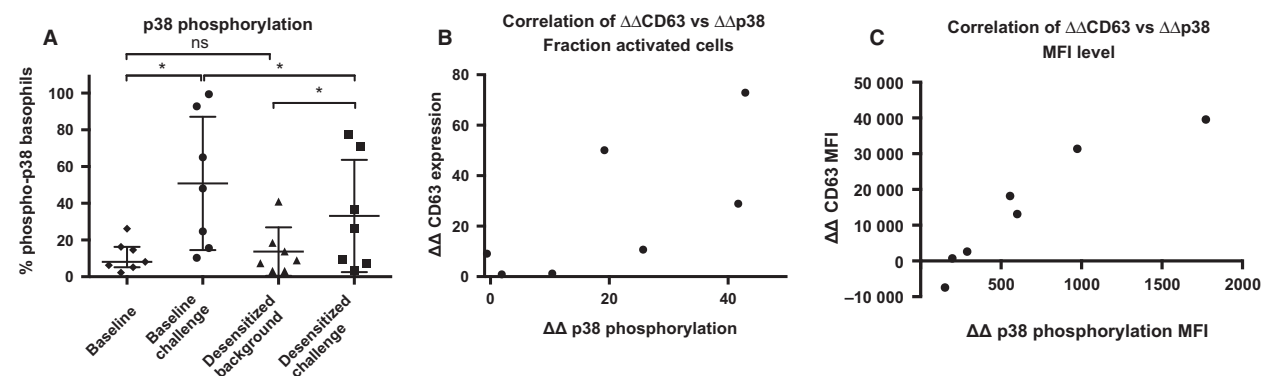


Figure 5 Phosphorylation of p38 MAPK was compared in untreated (baseline) and desensitized blood both with and without a 3-minute challenge with grass pollen allergen ($n = 7$; A; data are

Discussion

We found that the allergen activation of peripheral blood basophils of allergic patients could be significantly inhibited by sequential addition of doubling doses of allergen, starting at a functionally defined dose of 1 : 1000 of the allergen dose giving maximal activation. The allergen dose accumulated during desensitization equalled the optimal allergen dose. This is the first time (sequential) desensitization of whole blood basophils is done at allergen doses optimal for activation, but the price was a small number of basophils activated at each step. However, addition of yet another maximal dose only leads to a 5.9% further increase in activated basophils. Desensitization reduced the number of responsive basophils. It also reduced the amount of CD63 upregulated to the surface of each cell to less than half of the values measured before desensitization. The marked decrease in MFI could translate to a reduction in degranulation events in the individual basophil granulocyte possibly caused by fewer fusion events with the cell surface or lack of fusion between granules during compound exocytosis (24).

This process mimics a recent protocol for clinical drug desensitization (25), reaching allergen concentrations optimal for activation rather than subthreshold levels as previously described (9, 17). During desensitization, we found significant activation of the basophils. This was also found in clinical desensitization (25) and rush AIT (26) where 33% and 2–36% of patients, respectively, had to be treated for allergic symptoms. These observations may be explained by the sequential background activation we observe *ex vivo*.

Desensitization is currently considered a first, important step in AIT (2). Ultra-rush and semi-rush AIT is associated with a similar anergy of basophils (4, 11) and with a change in basophil sensitivity. We have not measured effect on basophil sensitivity after desensitization, but noted that the point of inflection of the baseline response in blood was comparable to the sensitivity of basophils before treatment, suggesting that sensitivity did not change during our desensitization protocol.

presented as median and IQR). Correlation of CD63 expression vs p38 phosphorylation as fractions of activated cells (B) and as MFI (C). * = $P < 0.05$.

We investigated allergen specificity by selecting multi-allergic donors with similar basophil reactivity to grass and either birch extract or rDer p2. Lack of allergen specificity was demonstrated by the significantly lower response observed when challenging grass-desensitized basophils with an optimal dose of an unrelated allergen. This non-specific desensitization may be explained by intracellular changes in basophils that would affect all signalling through FcεRI. It may be similar to the stunting of FcεRI-mediated activation during rush AIT (4), or it may be caused by stimulating the histamine receptor 2 (11). *Ex vivo* desensitization has been established as a non-specific procedure at subthreshold allergen concentration years ago (15, 16), but for the first time, we now present evidence that this is also true at concentrations optimal for activation. The mechanisms of desensitization may be different in mice and humans, *in vivo* and *ex vivo*, and further studies are needed to draw any definite conclusion.

p38 MAPK is an important early common mediator of FcεRI cross-linking in basophils (10, 27, 28) and has been implicated in the desensitization of BMMCs (12). It was phosphorylated after three minutes during activation of blood basophils. Desensitization significantly reduced the degree of phosphorylation, and reduction in phosphorylation of p38 MAPK correlated with reduction in basophil activation assessed as fraction of both activated cells and MFI. This suggests that p38 may participate in allergen-induced signal transduction of basophil granulocytes. Previous studies have shown that specific inhibition of p38 MAPK prevents activation and degranulation of human basophils (28, 29) emphasizing the importance of p38 MAPK in the common activation pathway, but this has never been shown in desensitization at a single-cell level. Phosphorylation of p38 MAPK is not affected by allergen immunotherapy after 3 years (30). Hence, changes in the activity of p38 seem to be an important factor in rapid desensitization but not in the long-term effects of allergen immunotherapy.

We describe an *ex vivo* model of allergen-induced desensitization of basophils that can be used to explore the mechanism underlying this important phenomenon. The relevance of this model to clinical desensitization or desensitization observed during AIT remains to be established. Our

model does not support conventional mechanisms regarding allergen specificity and mechanism, but this is consistent as intracellular changes should not be allergen dependent. As we continuously add allergen, we may induce a different mechanism than observed when desensitizing with only one subthreshold or suboptimal dose of allergen (17, 20). Further studies should examine changes in the intracellular signalling cascade during this protocol, as elucidating the mechanism would be helpful in monitoring desensitization as a separate process from immune deviation. This could lead to more personalized protocols for drug desensitization and allergen immunotherapy and thus help preventing adverse reactions.

In conclusion, the present model for allergen-mediated *ex vivo* desensitization of blood basophils with high allergen concentration attenuates intracellular signal transduction; it is independent of the activating allergen, reduces phosphorylation of p38 MAPK as well as the number of cells responding and the degree of activation of each basophil granulocyte.

Author contributions

Hans Jürgen Hoffmann designed the study, performed pilot studies, participated in data analysis and extensively reviewed the manuscript. Sara K. Witting Christensen performed the experiments, analysed the data and drafted the manuscript. Inge Kortekaas Krohn and Johannes Martin Schmid provided technical assistance and reviewed the manuscript. Jani Thuraiyah performed pilot studies and reviewed the manuscript. Tina Skjold provided clinical guidance and reviewed the manuscript. All authors reviewed the final manuscript.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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