

ORIGINAL PAPER

Combined analysis of intracellular signalling and immunophenotype of human peripheral blood basophils by flow cytometry: a proof of concept

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Clinical and Experimental Allergy

Summary

Background The signal transduction pathways and control mechanisms involved in IgE-mediated basophil activation remain incompletely understood.

Objectives To investigate whether basophilic intracellular signal transduction and immunophenotype can be analysed simultaneously by flow cytometry.

Methods Basophils in whole blood were stimulated with anti-IgE and latex antigen at various concentrations and during different time courses. Phosphorylation of p38 mitogen-activated protein kinase (MAPK) as a representative of the intracellular signal transduction pathway and surface expression of CD63 was assessed simultaneously flow cytometrically. The effect of pre-incubation with IL-3 was assessed.

Results Stimulation of the basophils with anti-IgE and allergen induces a rapid phosphorylation of p38 MAPK that peaks between 1 and 5 min and returns to baseline levels after 60 min. In contrast, CD63 up-regulation demonstrates a maximal but more continuous expression that peaks approximately 5 min later than phosphorylation of p38 MAPK. Specific inhibition of p38 MAPK reduced or almost completely abrogated up-regulation of CD63. Pre-incubation of the basophils with IL-3 produces a rapid p38 MAPK phosphorylation over basal levels, but this was weaker and shorter than for anti-IgE stimulation. Pre-incubation of the basophils with IL-3 did not potentiate anti-IgE-induced phosphorylation of p38 MAPK and did affect spontaneous or IgE-mediated CD63 up-regulation.

Conclusions This study provides the proof that the flow cytometer allows an integrated analysis of basophilic intracellular signalling and immunophenotyping. Owing to its technical simplicity, the low number of cells required and rapid analysis, the technique seems promising for use in the clinic as a diagnostic tool or to monitor therapy.

Capsule summary This study is the first to provide evidence for a combined analysis of basophilic intracellular signalling and immunophenotyping by flow cytometry. Owing to its technical simplicity, the low number of cells required and rapid analysis, the technique seems promising for use in the clinic as a diagnostic tool or to monitor therapy.

Keywords allergy, basophil activation, CD63, flow cytometry, IgE, IL-3, intracellular signalling, p38 MAPK

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Introduction

Upon encounter of a specific allergen that cross-links high-affinity IgE receptors [Fcε receptor I (FcεRI)], basophils not only synthesize and secrete quantifiable mediators but also up-regulate the expression of different activation markers such as CD63 and CD203c that can be quantified flow cytometrically (for review: Ebo et al. [1]). The mechanisms that govern basophil activation and

culminate in mediator release are complex and remain incompletely elucidated. Nevertheless, the different pathways involved in basophil activation and degranulation are known to be rapidly propagated by consecutive protein phosphorylation cascades [2–7].

With the advent of monoclonal antibodies directed against phosphorylated epitopes and intracellular staining techniques, we reasoned that flow cytometry might constitute an additional instrument to investigate intracellular

signalling in basophils. Moreover, as the technique allows integrating immunophenotyping of individual cells, it might capture data that are inaccessible for techniques that are performed on homogenous cell populations and from which results represent an average of isolated cells analysed.

On several occasions, p38 mitogen-activated protein kinase (MAPK) has been demonstrated to be a pivotal regulator of basophil activation following stimulation with different secretagogues [4, 6, 8]. Therefore, we developed a flow cytometric technique allowing combined exploration of basophilic p38 MAPK phosphorylation and surface staining of CD63. For this purpose, we evaluated different permeabilization strategies as well as stimulation conditions. Various concentrations and time course treatments with IL-3, anti-IgE and specific allergen-induced were applied.

Materials and methods

Basophil activation

Endotoxin-free heparinized whole-blood samples were obtained from healthy donors. Aliquots of 100 µL whole blood were stimulated with 100 µL anti-IgE (0.1, 1 and 10 µg/mL) (clone G7-8; Pharmingen, BD Bioscience, Erembodegem, Belgium) or 100 µL activation buffer (Hank's balanced salt solution with 1 mol/L HEPES and 7.5% NaHCO₃, pH = 7.4) as a negative control during different time-points. Reactions were stopped by placing the tubes on ice.

Effect of incubation temperature

To determine the effect of incubation temperature, aliquots of blood or anti-IgE (0.1 and 10 µg/mL) were pre-warmed for 15 min on 37 °C or brought to room temperature before the start of the stimulation experiments.

Effect of pre-activation with interleukin-3

IL-3 is a well-characterized potentiatory factor of IgE-dependent basophil stimulation [9]. To evaluate the effect of IL-3, blood samples were incubated with different concentrations (2, 10 and 50 ng/mL) of IL-3 (PeproTech Inc., Rocky Hill, NJ, USA) for 15 min at 37 °C preceding activation. Subsequently, cells were stimulated with anti-IgE for 3 and 20 min.

Antigen stimulation

Blood from three patients with a well-documented IgE-mediated allergy for latex was used. Different concentrations of latex (10^{-8} , 10^{-6} , 10^{-4} , 10^{-2} and 1 µg/mL,

Stallergenes, Antony, France) were tested at different stimulation periods (0, 3, 10, 20, 40 and 60 min).

Immunophenotyping

To estimate activated basophils, cells were stained with 20 µL of monoclonal anti-human IgE (clone GE-1, Sigma-Aldrich Chemic GmbH, Steinheim, Germany) that was labelled with Alexa Fluor 488 (Molecular Probes, Invitrogen, Paisley, UK) as described by the manufacturer and 10 µL of monoclonal phycoerythrin (PE)-conjugated anti-human CD63 (clone H5C6, BD Biosciences) for 20 min on ice. Subsequently, cells were lysed/fixed with 2 mL BD Phosflow (BD Biosciences) for 20 min at room temperature.

Cell permeabilization

To stain intracellular proteins, different permeabilization strategies based on methanol 90% in phosphate-buffered saline (PBS), BD Phosflow Perm III (BD Biosciences) or saponin 0.3% (Acros, Geel, Belgium) in PBS (pH 7.4, Invitrogen) were tested. Cells were suspended in one of these solutions for 30 min at 4 °C. Except for the saponin protocol, cells were washed and re-suspended with staining buffer (PBS with 0.5% fetal calf serum). Cells in the saponin protocol were washed and resuspended in 100 µL 0.3% saponin in PBS.

Staining of phospho-p38 mitogen-activated protein kinase

Ten microlitres of anti-phospho-p38 MAPK (T180/Y182) conjugated with Alexa Fluor 647 (BD Biosciences) was added and incubated for 30 min at room temperature in the dark. Afterwards, the cells were washed and resuspended in PBS with 0.1% NaN₃.

Flow cytometric analysis

Flow cytometric analysis of the basophil activation was performed on a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA). IgE-staining and side scatter were applied to gate out at least 500 basophils that expressed a high density of surface-bound IgE. According to Herzenberg *et al.* [10], fluorescence minus one (FMO) was used to set the marker for positive cells. Results are expressed as percentage positive basophils for phosphorylated p38 MAPK and CD63.

Because phosphorylation of the intracellular signalling molecules is a rapid-onset process, samples were studied at different time-points (0, 1, 3, 5, 10, 20, 40 and 60 min). A control setting of ice-chilled blood was used to determine basal phosphorylation.

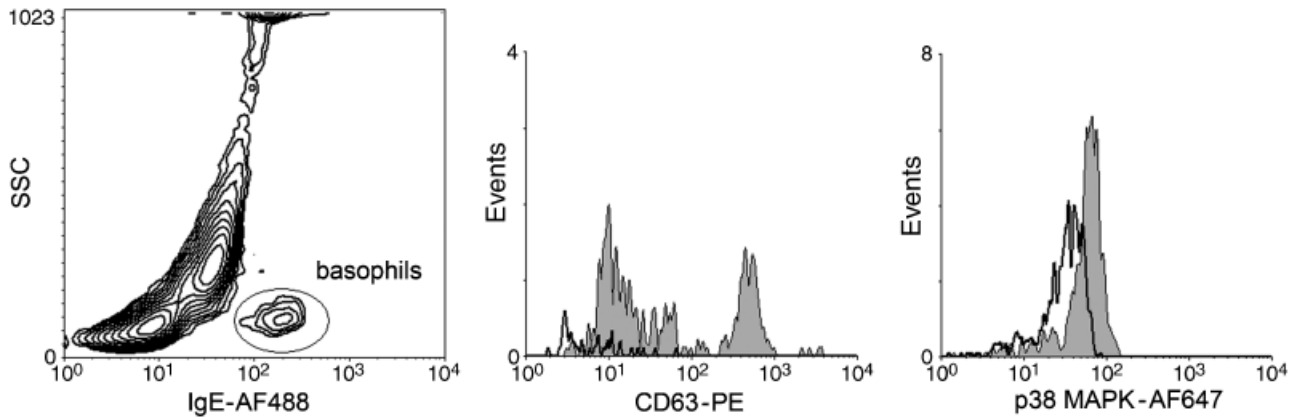


Fig. 1. Effect of permeabilization strategy. Open histograms represent permeabilization with methanol, and closed histograms represent staining with saponin. Only the saponin-based permeabilization procedure allowed a combined surface staining with CD63 (mid panel). Phosphorylation of p38 mitogen-activated protein kinase (MAPK) that was demonstrable with all permeabilization protocols (right panel). PE, phycoerythrin.

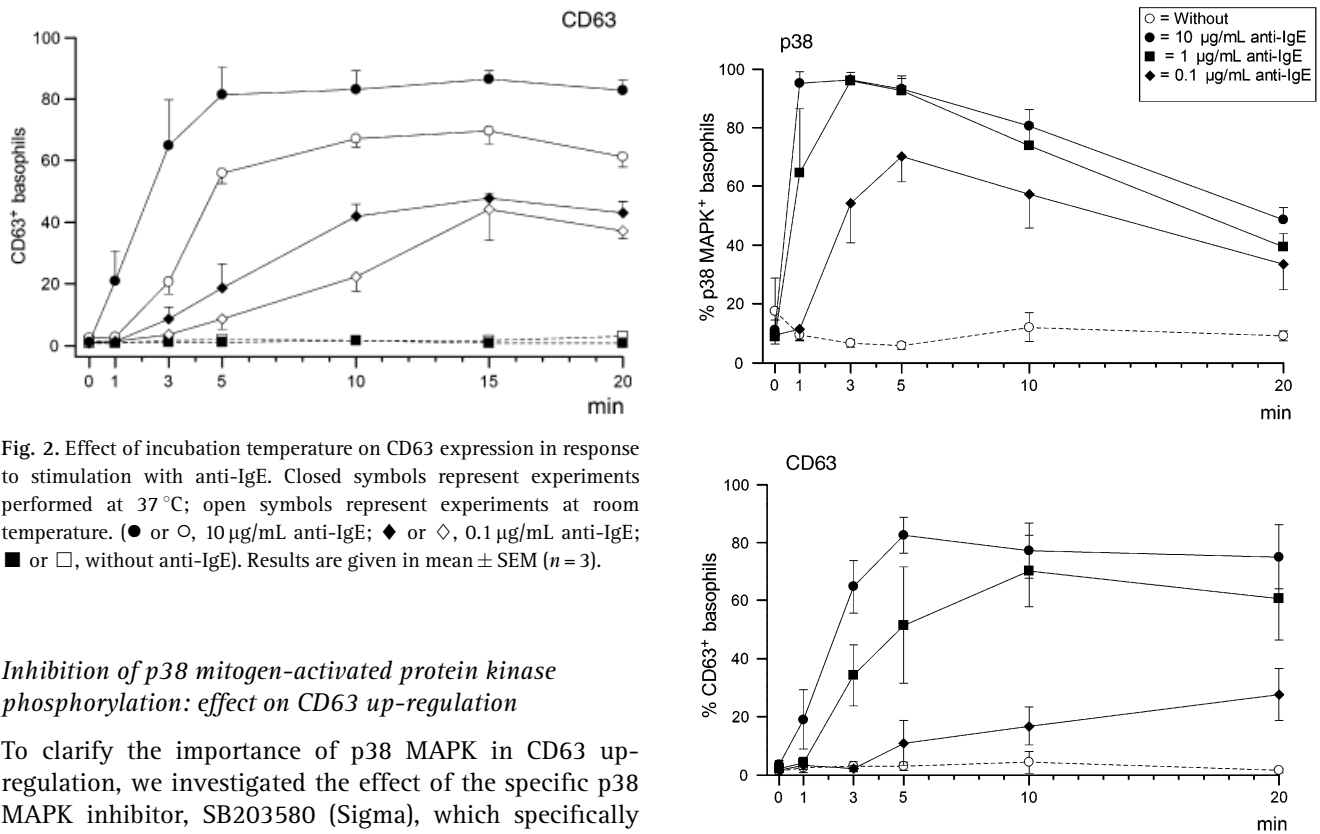


Fig. 2. Effect of incubation temperature on CD63 expression in response to stimulation with anti-IgE. Closed symbols represent experiments performed at 37 °C; open symbols represent experiments at room temperature. (● or ◐, 10 µg/mL anti-IgE; ◆ or ◑, 0.1 µg/mL anti-IgE; ■ or □, without anti-IgE). Results are given in mean $\pm$ SEM ($n = 3$).

Inhibition of p38 mitogen-activated protein kinase phosphorylation: effect on CD63 up-regulation

To clarify the importance of p38 MAPK in CD63 up-regulation, we investigated the effect of the specific p38 MAPK inhibitor, SB203580 (Sigma), which specifically blocks enzyme activity by coupling to the ATP pocket of the kinase without a direct action on other kinases of the MAP family [11–13]. SB203580 stock solution (1 µg/mL in DMSO, Sigma) was diluted in an activation buffer (1 and 10 µmol/L) and added to the cells before activation. Basophils of three healthy control individuals were stimulated with two concentrations of anti-IgE (0.1 and 10 µg/mL), whereas cells of three patients were stimulated with two concentrations of latex (10^{-2} and 1 µg/mL) for 3 and 20 min. Basophil immunophenotyping and intracellular staining were performed as described above.

Fig. 3. Time kinetics of anti-IgE-induced phosphorylation and up-regulation of CD63 ($n = 3$). Results expressed as mean $\pm$ SEM.

Results

Combined staining of phosphorylated p38 mitogen-activated protein kinase and CD63: influence of the permeabilization procedure

Stimulation of basophils with anti-IgE resulted in a significant phosphorylation of p38 MAPK that was

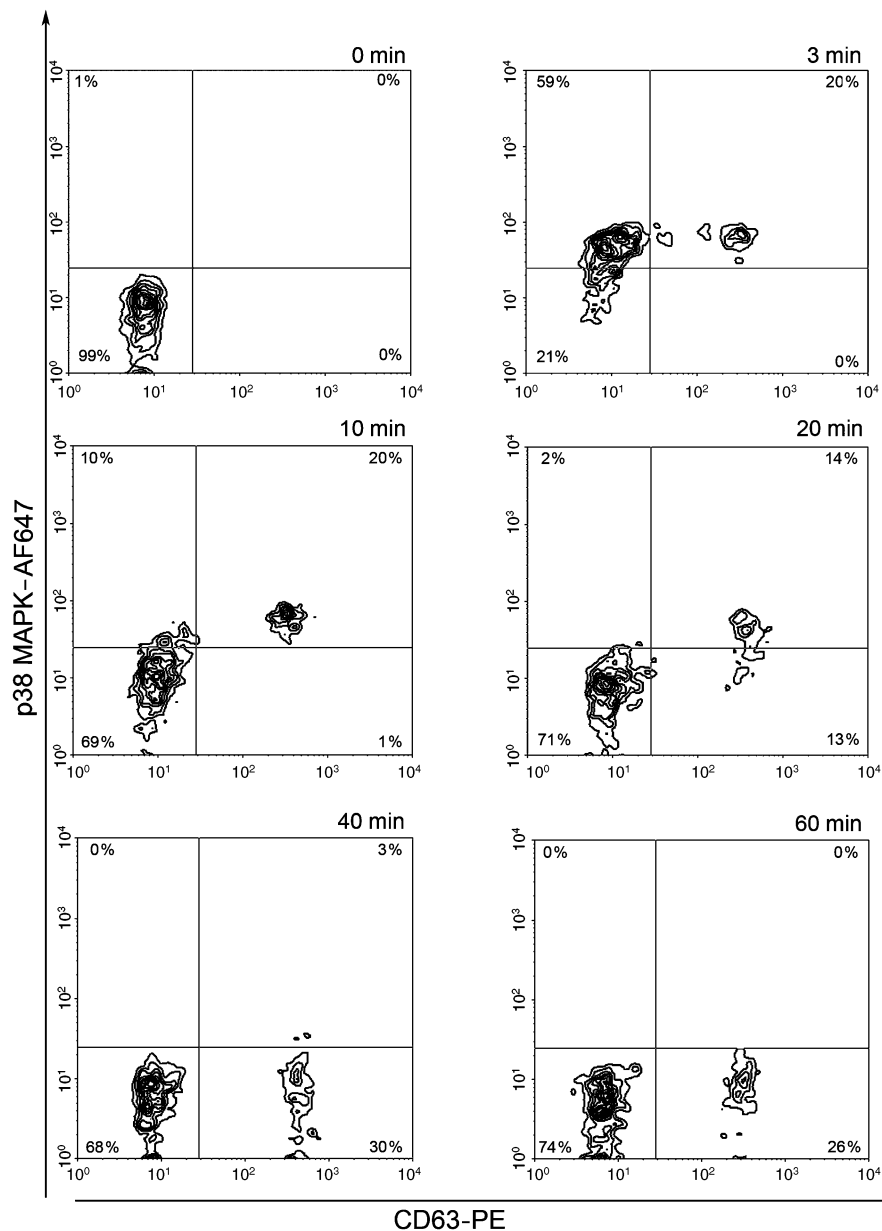


Fig. 4. Representative density plot of time kinetics of phosphorylation of p38 mitogen-activated protein kinase (MAPK) and CD63 up-regulation by IgE⁺ basophils in response to stimulation with latex. After 3 min, $\pm 80\%$ of the cells demonstrate phosphorylated p38 MAPK; in only 20% of the cells is up-regulation of CD63 expression demonstrable. Phosphorylation of p38 MAPK declines rapidly and returns to baseline values after 20–40 min, whereas CD63 up-regulation remains on a plateau for over 60 min. Note the bimodal distribution of CD63 up-regulation. PE, phycoerythrin.

demonstrable with all permeabilization protocols. However, only the saponin-based permeabilization procedure allowed a combined surface staining with CD63 (Fig. 1). This protocol was adopted for further experiments.

Effect of incubation temperature

As shown in Fig. 2, pre-warming of the cells and reagents for 15 min at 37 °C markedly enhanced the onset and

magnitude of anti-IgE-mediated CD63 up-regulation without affecting spontaneous expression. When cells were stimulated with 10 µg/mL of anti-IgE, up-regulation of CD63 already peaked after 5 min. In contrast, for the lower stimulation concentration of 0.1 µg/mL, CD63 up-regulation was maximal after 10–15 min.

Pre-warming of the cells did not alter the kinetics of phosphorylation of p38 MAPK (data not shown). Therefore, in further experiments blood and reagents were pre-warmed at 37 °C before stimulation.

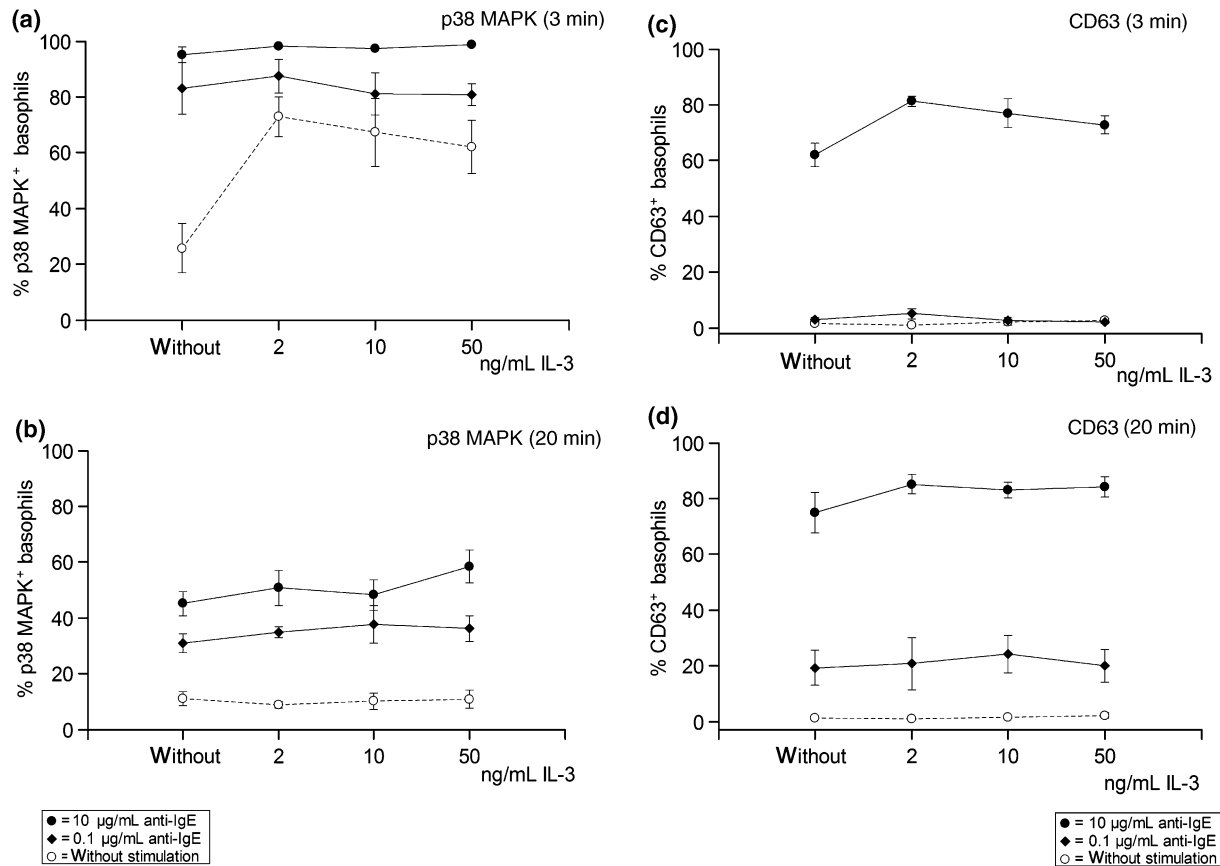


Fig. 5. Pre-incubation of the basophils with IL-3 results in a phosphorylation of p38 mitogen-activated protein kinase (MAPK) that is already demonstrable after 3 min (a) but returns to baseline values within 20 min (b). When IL-3 incubation was followed by anti-IgE stimulation, no synergism was apparent (a, b). Pre-incubation with IL-3 did not result in CD63 up-regulation and did not change the intensity or profile of anti-IgE-induced up-regulation of CD63 (c, d). Results are given in mean $\pm$ SEM ($n = 3$).

Kinetics of anti-immunoglobulin E stimulation

Phosphorylation of p38 MAPK (Fig. 3a) and up-regulation of CD63 (Fig. 3b) was found to be dose-dependent upon stimulation with anti-IgE. Phosphorylation of p38 MAPK peaked between 1 and 5 min and declined afterwards. In contrast, up-regulation of CD63 expression, reached its maximum between 5 and 10 min and remained on plateau for over 20 min. This difference in time kinetics is also evident from Fig. 4, which shows latex-specific stimulation of the cells.

Effect of pre-activation with interleukin-3

To assess the effect of IL-3 priming on phosphorylation of p38 MAPK and up-regulation of CD63, the basophils were incubated with a serial dilution of IL-3 for 15 min at 37 °C and subsequently stimulated with two concentrations of anti-IgE and buffer as a control for 3 and 20 min. Figure 5a shows that incubation with IL-3 induced a marked phosphorylation of p38 MAPK that was already demonstrable after 3 min and declined to basal values after

20 min (Fig. 5b). When IL-3 incubation was followed by anti-IgE stimulation, no synergism was apparent. IL-3 incubation for 15 min did not induce a statistically significant increase of anti-IgE-induced p38 MAPK phosphorylation (Figs 5a and b). Incubation with IL-3 did not result in CD63 up-regulation and did not change the intensity or profile of anti-IgE-induced up-regulation of CD63 (Figs 5c and d). Similar results were found for latex-specific stimulation (data not shown).

Kinetics of allergen-specific stimulation

The kinetics of phosphorylation of p38 MAPK and up-regulation of CD63 expression upon latex-specific activation paralleled the aforementioned findings for stimulation with anti-IgE. Allergen-specific phosphorylation of p38 MAPK and up-regulation of CD63 expression was found to be dose dependent. Phosphorylation of p38 MAPK peaked at 3 min and returned to basal values after 60 min (Fig. 6a). Up-regulation of CD63 expression peaked somewhat around 10 min and remained on a plateau for over 60 min (Fig. 6b).

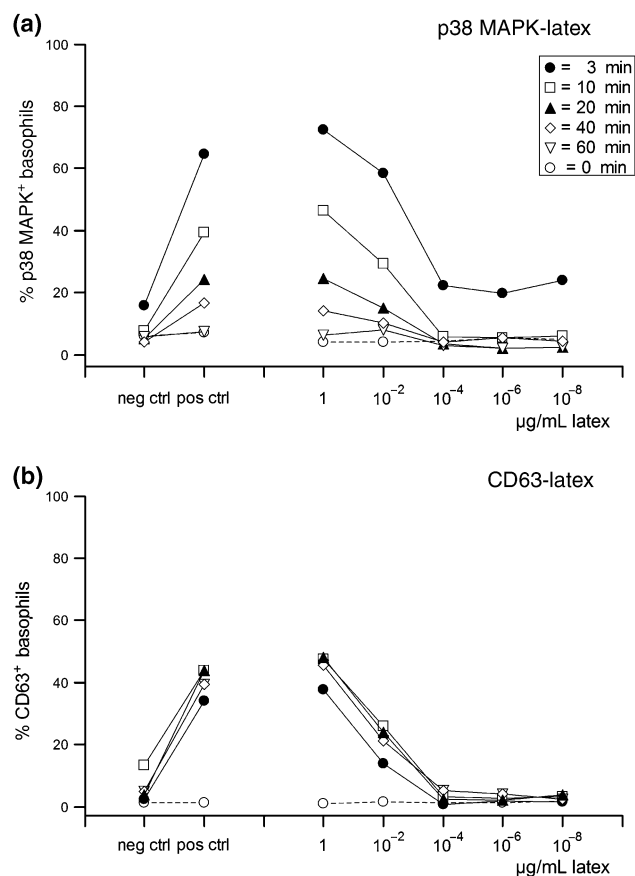


Fig. 6. Time kinetics of p38 mitogen-activated protein kinase (MAPK) phosphorylation (a) and CD63 expression (b) in response to stimulation with buffer (neg ctrl), anti-IgE (pos ctrl, 10 µg/mL) and various concentrations of latex antigen (10^{-8} up to 1 µg/mL). Phosphorylation of p38 MAPK peaks at 3 min and returns to basal values after 60 min (a). Up-regulation of CD63 expression peaked somewhat around 10 min and remained on a plateau for over 60 min (b). Comparative analysis shows that, in contrast to phosphorylation of p38 MAPK, the distribution of CD63 up-regulation was bimodal, i.e. positive and negative (or low borderline positive) subpopulations are detectable. Results are given in mean $\pm$ SEM ($n = 3$).

Comparative analysis shows that, in contrast to phosphorylation of p38 MAPK, the distribution of CD63 up-regulation was bimodal, i.e. positive and negative (or low-borderline positive) subpopulations are detectable. For example, 3 min of stimulation with latex (1 µg/mL) resulted in a phosphorylation of p38 MAPK in up to $72 \pm 20\%$ of the basophils ($n = 3$), whereas an up-regulated CD63 expression after 20 min was demonstrable in $48 \pm 22\%$ of the cells (Fig. 6).

Inhibition of p38 mitogen-activated protein kinase phosphorylation: effect on up-regulation of CD63

Addition of the selective p38 MAPK inhibitor SB203580 (1–10 µmol/L) significantly inhibited anti-IgE and latex-induced phosphorylation of p38 MAPK (Fig. 7).

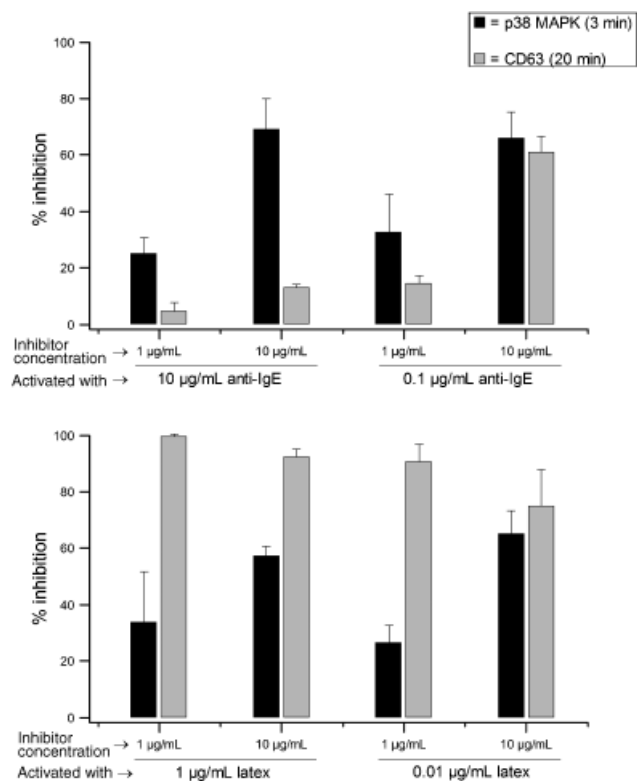


Fig. 7. Effect of pre-incubation with SB203580 on phosphorylation of p38 mitogen-activated protein kinase (MAPK) and expression of CD63, after stimulation of basophils with different concentrations of anti-IgE (top) and latex antigen (bottom). Results are given in mean $\pm$ SEM ($n = 3$).

Additionally, we observed that inhibition of p38 MAPK prevented up-regulation of CD63 expression, a phenomenon particularly relevant for allergen-induced basophil activation (Fig. 7).

Discussion

Upon encounter of specific allergen that cross-links high-affinity IgE receptors (FcεRI), as a result of intracellular signal transduction, basophils synthesize and release different mediators that are responsible for the allergic inflammatory reaction. Although intensively studied, the molecular events and signal transduction pathways set into motion after receptor cross-linking for cell activation remain incompletely elucidated. Nevertheless, evidence has indicated that these pathways are rapidly propagated by consecutive protein phosphorylation cascades. One of the proteins to play a key role in IgE-mediated basophil activation and to control downstream degranulation is p38 MAPK [4, 6, 14]. Therefore, we developed a flow cytometric protocol for a combined exploration of p38 MAPK phosphorylation and surface staining of CD63 expression.

Preliminary investigations confirm that the permeabilization method and incubation temperature can

significantly influence the outcome of the experiments [15, 16]. The methanol-based permeabilization procedure had profound detrimental effects on the staining of CD63. Alternatively, pre-warming of the aliquots markedly enhanced the onset and magnitude of anti-IgE-mediated CD63 up-regulation without affecting its spontaneous expression.

Traditionally, analysis of signalling in basophils has been performed using Western blot techniques that have provided fundamental insights into the transduction pathways and regulatory mechanisms involved [3, 4, 6, 17, 18]. From our results, it emerges that flow cytometry provides qualitative results concerning dose responses and kinetics of p38 MAPK phosphorylation similar to the data obtained with the Western blot techniques. Stimulation of the basophils with anti-IgE and allergen induces a rapid phosphorylation of p38 MAPK that peaks between 1 and 5 min according to the stimulation strength. Subsequently, phosphorylation of p38 MAPK subsides and returns to baseline levels after 60 min. In contrast, IgE-mediated CD63 up-regulation demonstrates clear distinct time kinetics, with a maximal but more continuous expression that peaks approximately 5 min later than phosphorylation of p38 MAPK. Inhibition of p38 MAPK by SB203580 resulted in a reduced or almost completely abrogated up-regulation of CD63. Finally, short pre-incubation of the basophils with IL-3 produces a rapid p38 MAPK phosphorylation over basal levels, but this was less pronounced and shorter than for anti-IgE stimulation. Alternatively, short pre-incubation of the basophils with IL-3 did not potentiate anti-IgE-induced phosphorylation of p38 MAPK and was confirmed not to alter spontaneous or IgE-mediated CD63 up-regulation [19, 20].

However, the flow cytometric approach offers different opportunities. First, the technique allows to reduce the sampling volume considerably and to perform repetitive drawings in the same individual, thereby creating the opportunity to study the pharmacological effects of drugs or follow-up of treatment as for to the effect of venom-specific immunotherapy. Second, the flow cytometric technique does not require an *a priori* separation and purification of the basophils to ensure reliable measurements with negligible contribution from other cell contaminants. This enables to study the cells in their natural environment and to avoid potential interference from additional manipulations. Alternatively, the technique also significantly shortens the analysis time (3 h instead of 1–2 days) and thus renders it more accessible for clinical and research applications. Last but not the least, flow cytometry allows identification of cells with heterogeneity in responsiveness rather than providing data that represent a mean value for the total-isolated cell population. In this context, it is of note that we have detected differential distributions for p38 MAPK phosphorylation and CD63 up-regulation in response to stimulation with

anti-IgE and latex. In contrast to a global phosphorylation of p38 MAPK in almost all cells, the distribution of CD63 up-regulation upon IgE-mediated stimulation is bimodal, i.e. positive and negative subpopulations are detectable (see Fig. 4). The explanation of this dissociation between p38 MAPK phosphorylation and up-regulation of CD63 expression remains highly speculative. However, it is anticipated that multicolour flow cytometry, allowing simultaneous assessment of phenotype and analysis of several intracellular signalling pathways, might help to further reveal the underlying mechanism.

In summary, to our knowledge, this is the first study to demonstrate the flow cytometer as constituting a rapid alternative approach to study signalling pathways in human peripheral blood basophils. Moreover, the technique offers the unique opportunity for coupled phenotyping of the cells. It is anticipated that this integrated analysis will aid in revealing the mechanisms that govern basophil activation and degranulation.

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