

P38 Mitogen-Activated Protein Kinase Signal Transduction in the Diagnosis and Follow Up of Immunotherapy of Wasp Venom Allergy

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Background: P38 mitogen-activated protein kinase (MAPK) is known to govern IgE-mediated basophil activation. Intracellular phosphorylated p38 MAPK (Pp38 MAPK) in IgE-activated basophils can be quantified flow cytometrically.

Objectives: To study whether Pp38 MAPK constitutes a potential novel read-out for flow-assisted diagnosis of hymenoptera venom allergy and to investigate whether this marker allows follow-up of successful venom immunotherapy (VIT).

Methods: Fifty-two patients with documented wasp venom allergy and seven wasp-stung asymptomatic control individuals were enrolled. Wasp venom-induced basophil activation was analyzed flow cytometrically with anti-IgE, anti-CD63, and anti-Pp38 MAPK to assess their activation status before starting immunotherapy. To assess whether p38 MAPK constitutes a candidate marker for monitoring VIT, we repeated the basophil activation test (BAT) in 25 patients on the fifth day of a build-up immunotherapy. In addition, we investigated whether the Pp38 MAPK-based BAT could contribute in the decision of discontinuing VIT in a cross-sectional analysis in 13 patients receiving treatment for 3 years and 14 patients receiving treatment for 5 years.

Results: Patients exhibited a dose-dependent basophil activation with phosphorylation of p38 MAPK and upregulation of downstream CD63. In contrast, stung controls demonstrated a dose-dependent but “abrogated” signal transduction in basophils with less and shorter duration of the phosphorylation of p38 MAPK and without subsequent upregulation of CD63. When repeated after 5 days of VIT and when investigated cross-sectionally after 3 years or 5 years of maintenance therapy, no effect of VIT on the phosphorylation of p38 MAPK was demonstrable.

Conclusions: This study discloses that not only basophils from patients, but also from the stung control individuals, respond to wasp venom stimulation with phosphorylation of p38 MAPK, although to a lesser extent. No clear effect of VIT on the phosphorylation of p38 MAPK was shown. Thus, although p38 MAPK provides an additional tool in the diagnosis of wasp venom allergy, it does not contribute to the decision whether to stop successful VIT. © 2010 International Clinical Cytometry Society

Key terms: basophils; CD63; immunotherapy; p38 mitogen-activated protein kinase; signal transduction; hymenoptera venom

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In most individuals, hymenoptera stings give rise to a transient and bothersome local reaction characterized by pain, redness, and swelling. However, for those who have an IgE-mediated allergy to components of the venom, a sting may elicit life-threatening, even fatal reactions. In such patients, correct diagnosis is a prerequisite

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site for effective management, such as specific venom immunotherapy (VIT) (1). In VIT, increasing doses of venom allergen are administered to the patient until the maintenance dose is reached. Various treatment regimens are available. In the semirush protocol adopted at our department, the required time to reach the maintenance dose of 100 µg is 5 days. In more conventional protocols, the maintenance dose is reached in a few months. In the majority of patients, therapy is stopped after 5 years, irrespective of IgE and/or skin test results (1).

The current diagnostic procedure of hymenoptera venom allergy includes skin tests and quantification of venom-specific IgE (sIgE) (2). Sometimes, however, the culprit insect remains unidentified. In these cases, measuring the upregulation of CD63 or CD203c in the basophil activation test (BAT), which is based on flow cytometric immunophenotyping of venom-challenged basophils, can constitute a valuable additional tool for the correct identification of the offender and to start VIT accordingly (3–9). However, as addressed elsewhere (10), there might be room for further improvement. Sainte-Laudy et al. (11) demonstrated that the sensitivity of the BAT could probably benefit from application of upstream activation markers. Recently, we provided evidence that flow cytometry constitutes a unique tool for simultaneous quantification of the degranulation marker CD63 and the upstream and intracellular located signaling molecule p38 mitogen-activated protein kinase (p38 MAPK) (12).

The primary objective of this study was to investigate p38 MAPK phosphorylation as a novel read out for wasp venom-challenged basophils in the discrimination between patients with severe wasp venom allergy and stung control individuals.

In spite of the high efficiency of VIT, an effective instrument for monitoring this therapy is still lacking. Also, the question regarding the optimal duration of VIT remains unanswered. Both, venom-specific IgE and venom skin test (VST) fail to reflect the induction of clinical tolerance and seem of little predictive value for future (deliberate) sting reactions (13–17). Recently, we demonstrated that flow-assisted analysis of in vitro activated basophils based on the CD63 expression might constitute a tool for monitoring VIT. However, an effect of the treatment was only demonstrated after a maintenance course of 6 months and with submaximal stimulation of the cells (18). As p38 MAPK plays a pivotal role in IgE-mediated basophil activation (12,19), we anticipated that this signaling molecule could represent a read out for follow-up of early and maintenance VIT. Therefore, in a subgroup of patients that were responsive in the BAT before starting therapy, the test was repeated after 5 days of semirush VIT, and a separate cross-sectional analysis was performed after 3 and 5 years of VIT to evaluate the contribution of p38 MAPK BAT in the decision whether to stop VIT.

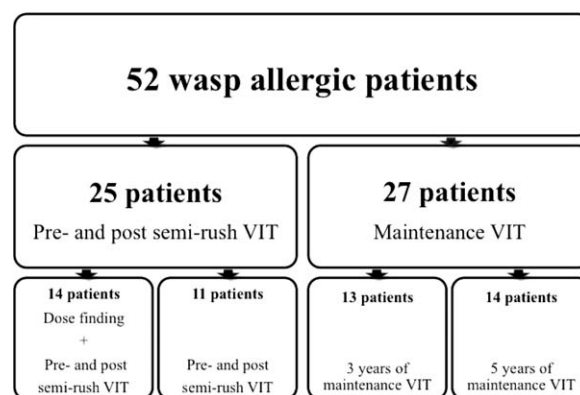


Fig. 1. Number of patients in the different groups. VIT, venom immunotherapy.

MATERIALS AND METHODS

Patients and Stung Controls

Fifty-two [35 men and 17 women; age 46 (6–74) years] patients with a systemic reaction to a wasp (*Vespa vulgaris*) sting were enrolled (Fig. 1). In all patients, clinical suspicion of wasp venom allergy was confirmed by a positive intradermal VST using an intradermal endpoint titration method (10^{-4} – $1\mu\text{g/mL}$ solutions of wasp venom; Pharmalgen, ALK-Abelló, A/S, Denmark), wasp venom sIgE (*Vespa vulgaris*) (Immuno-CAP FEIA, Phadia AB, Brussels, Belgium), and/or CD63 based BAT (2).

Dose-finding experiments with wasp venom for the BAT comprised a comparative analysis between 14 patients [10 men and 4 women; aged 49 (6–69)] years and seven healthy volunteers [3 men and 4 women; aged 49 (25–63)] years with a previous wasp sting without symptoms and negative venom sIgE.

To study whether p38 MAPK constitutes a candidate marker for monitoring VIT, the BAT was repeated in 25 patients [14 men and 11 women; age 45 (6–70) years], receiving semirush VIT. Experiments were performed before the start of VIT and on the fifth day of a build-up therapy before the final injection but after administration of a cumulative dose of venom equivalent to several wasp stings. Analyses were restricted to patients responsive to an IgE stimulation (positive control) in the p38 MAPK-based BAT before the start of therapy. Whether the BAT could contribute in the decision of discontinuing, VIT was studied in a separate cross-sectional analysis in 13 patients [10 men and 3 women; age 46 (30–71) years] receiving treatment for 3 years and 14 patients [11 men and 3 women; age 57 (31–74) years] receiving treatment for 5 years. Table 1 summarizes the relevant characteristics of the patients in longitudinal follow-up and those analyzed cross-sectionally. This study was approved by the local ethics committee.

Table 1
General Characteristics of the Patients

	Group pre- and post semirush VIT	Cross-sectional group after 3 years of maintenance therapy	Cross-sectional group after 5 years of maintenance therapy
N	25	13	14
M/F	14/11	10/3	11/3
Age	45 (6–70)	46 (30–71)	57 (31–74)
Total IgE (kU/L)	128 (7–1397)	96 (5–863)	75 (13–2653)
Wasp IgE (kUa/L)	2.71 (<0.35–>100.00)	3.34 (0.38–12.50)	2.77 (<0.35–96.80)
Percentage positive skin test for wasp	73	62	57
Severity of reaction	All patients were selected if they presented with a compelling history of anaphylaxis (such as urticaria, angioedema, bronchospasm, hypotension, shock), which is $\geq$ grade 2 according to the classification of Mueller (20)		

No significant differences between groups ($P < 0.05$).

Basophil Activation

The combined technique of immunophenotyping and analysis of intracellular signalling is described elsewhere (12). In short, endotoxin-free heparinized whole blood samples (Vacuette, Greiner Labortechnik GmbH, Kremsmünster, Austria) were obtained from all patients and stung control individuals. Aliquots of 100- μ L whole blood were challenged at 37°C with 100 μ L of anti-IgE (10 μ g/mL, clone G7-18, Pharmingen, BD Bioscience, Erembodegem, Belgium) as positive control, wasp venom (lyophilized extract that is also applied for skin tests and VIT, used at concentrations 0.01, 0.1, 1, and 10 μ g/mL, ALK-Pharmalgen), or activation buffer (Hank's balanced salt solution with 20 μ M HEPES and 7.5% NaHCO₃, pH = 7.4, without IL-3, negative control) for 3 or 20 min, two points of time at which an optimal stimulation of Pp38 MAPK, respectively, CD63 is seen (12,21). The tubes were then placed on ice to stop the reaction.

Immunophenotyping

Basophils were stained with 20 μ L of monoclonal anti-human IgE (clone GE-1, Sigma-Aldrich Chemic GmbH, Steinheim, Germany) that was labeled with Alexa Fluor 488 (Molecular Probes, Invitrogen, Paisley, UK) and 10 μ L of monoclonal phycoerythrin (PE)-conjugated anti-human CD63 (clone H5C6, BD Biosciences) for 20 min on ice. Subsequently, cells were lysed and fixed with 2-mL BD Phosflow (BD Biosciences) for 20 min at room temperature.

Basophil Permeabilization and Intracellular Staining of p38 MAPK

To stain intracellular Pp38 MAPK, fixed cells were washed in phosphate-buffered saline (PBS, pH 7.4, Invitrogen) and permeabilized with 300 μ L of 0.3% saponin (Acros, Geel, Belgium) in PBS for 30 min at 4°C. Cells were resuspended in 100 μ L of 0.3% saponin in PBS and stained with 10 μ L of anti-Pp38 MAPK (T180/Y182) conjugated with Alexa Fluor 647 (BD Biosciences) for 30 min at room temperature in the dark. Afterward, cells were washed with saponin buffer 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). IgE staining and side scatter were applied to gate out at least 500 basophils expressing a high density of surface-bound IgE. According to Roederer et al. (22), fluorescence minus one was used to set the marker for positive cells. Results were expressed as a percentage of positive basophils for intracellular Pp38 MAPK and membrane-expressed CD63. A control setting of ice-chilled blood was used to determine basal phosphorylation.

Statistical Analysis

All results were expressed as median (range). The Mann-Whitney *U* test, Friedman, Wilcoxon, and Spearman's rank correlation test were applied where appropriate. Two graph-receiver operating characteristics curve analysis was performed to calculate the optimal threshold value that corresponds to the best sensitivity and specificity. All statistic calculations were performed using SPSS version 16.0.

RESULTS

Dose-Finding Experiments

At 3 and 20 min, stung controls and patients demonstrated comparable net anti-IgE induced phosphorylation of p38 MAPK and CD63 expression (data not shown).

A representative sample of wasp venom-induced phosphorylation of p38 MAPK is shown in Figure 2. The results of venom-induced CD63 upregulation and phosphorylation of p38 MAPK for different stimulation concentrations are displayed in Figures 3a and 3b. When challenged with wasp venom, patients showed a significant dose-dependent net phosphorylation of p38 MAPK and net upregulation of CD63 that was already demonstrable after 3 min (Fig. 3a) ($P < 0.001$). Stimulation with the lowest concentration of 0.01 μ g/mL used in our experiments resulted in a small but significant phosphorylation of p38 MAPK ($P = 0.03$). Phosphorylated p38 MAPK (Pp38 MAPK) further increased for the concentration of 0.1 μ g/mL and reached a plateau for 1 and

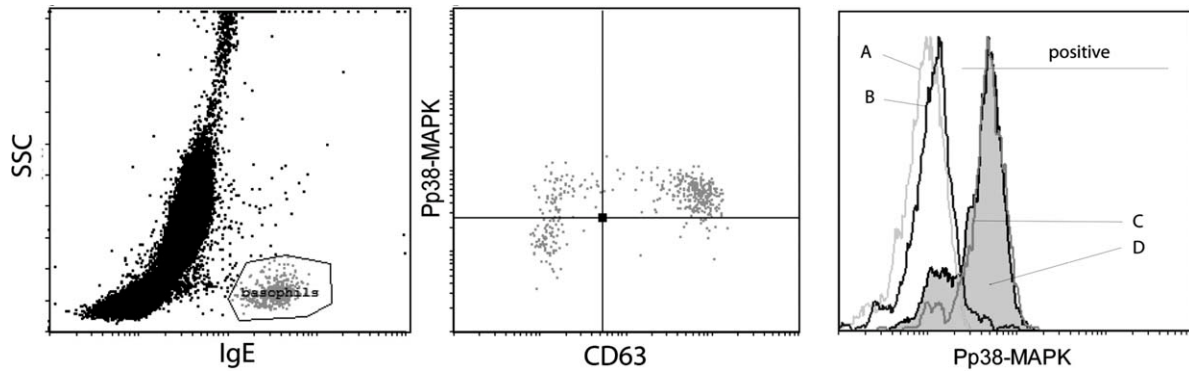


FIG. 2. A representative sample of wasp induced phosphorylation of p38 MAPK (Pp38 MAPK). The left panel shows the basophil selection according to the side-scatter characteristics and intense IgE labeling. The middle panel shows wasp induced upregulation of Pp38 MAPK and CD63 in the gated basophils. In the right panel histograms for Pp38 MAPK upregulation in basophils are shown, in four different settings, namely fluorescence minus one (light gray line), unstimulated (black line), allgE stimulated (dark gray line), and wasp stimulated (gray, filled). Fluorescence minus one was set on resting cells.

10 $\mu\text{g/mL}$. Phosphorylation of p38 MAPK was rapid and already demonstrable after 3 min (Fig. 3a) but significantly declined at 20 min (Fig. 3b). Upregulation of CD63 demonstrated a largely comparable dose-response but increased slightly at 20 min.

In contrast, stung controls demonstrated a dose-dependent phosphorylation of p38 MAPK (Fig. 3a) ($P < 0.001$), whereas CD63 expression remained essentially unaltered and was comparable to spontaneous CD63 expression (Fig. 3a). However, phosphorylation of p38 MAPK in stung controls and patients displayed distinct characteristics. In stung controls, phosphorylation of p38 MAPK was restricted to the two highest stimulation concentrations, was less pronounced (Fig. 3a), and short-lived with a return to basal values within 20 min (Fig. 3b).

According to the above-described kinetics of phosphorylation of p38 MAPK, which are in line with our prior data (12,21), further evaluation of the diagnostic performance of the BAT was performed at a 3-min stimulation time.

Sensitivity and Specificity

Table 2 summarizes the sensitivity, specificity, and cut-off values according to receiver operating curves for Pp38 MAPK and CD63 at 3-min stimulation time. Although the concentration of 0.1 $\mu\text{g/mL}$ did not stimulate the basophils maximally, this concentration resulted in a 100% sensitivity and specificity for the Pp38 MAPK BAT. Actually, as a result of phosphorylation of p38 MAPK in the stung controls, the specificity of this read-out decreased to 86% for the concentrations of 1 and 10 $\mu\text{g/mL}$. The CD63 upregulation appeared to be too insensitive for diagnostic purposes for the lowest concentration of 0.01 $\mu\text{g/mL}$ assessed by the current Pp38 MAPK/CD63 protocol. In contrast, for the higher stimulation concentrations, the CD63 read-out demonstrated a 100% sensitivity and specificity.

Follow-Up of VIT

Recently, it has been demonstrated that the CD63-based BAT might constitute a method for monitoring venom immunotherapy (VIT) (18). However, as recently confirmed by others (23), a treatment effect was only demonstrable, provided that the cells were stimulated

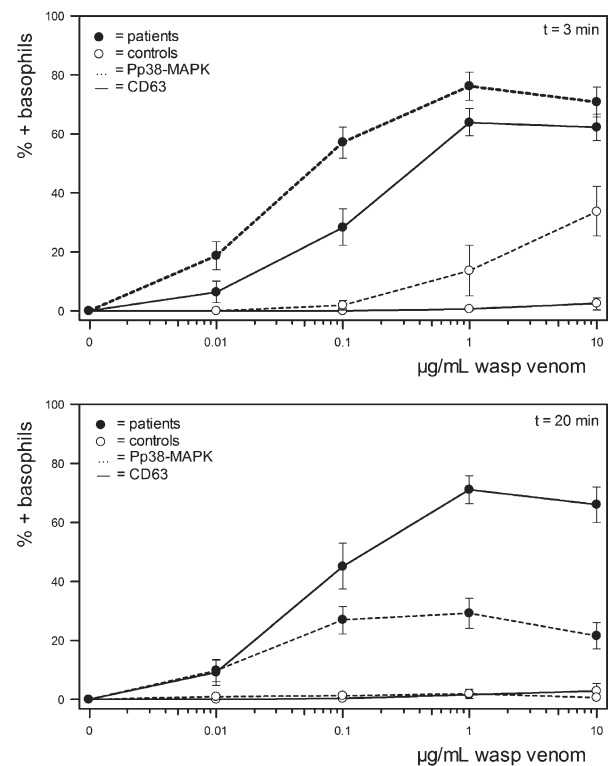


FIG. 3. Dose-finding experiments of the phosphorylated p38 MAPK (Pp38 MAPK)/CD63 assay for a stimulation period of, respectively, 3 (Fig. 2a) and 20 min (Fig. 2b). Closed symbols represent patients ($n = 14$), open symbols represents controls ($n = 7$). Pp38 MAPK is represented by the dashed line, upregulation of CD63 by the full line.

Table 2
Sensitivity and specificity

CD63				P 38 MAPK		
Wasp concentration	Sensitivity	Specificity	Cut off value (% positive basophils)	Sensitivity	Specificity	Cut off value (% positive basophils)
10µg/mL	100%	100%	20%	100%	86%	45%
1µg/mL	100%	100%	14%	100%	86%	42%
0.1µg/mL	100%	100%	1%	100%	100%	17%
0.01µg/mL	50%	86%	1%	93%	100%	1%

Sensitivity and specificity of wasp induced upregulation of CD63 and Pp38 MAPK for a stimulation time of 3 min in the discrimination of wasp allergic patients and healthy controls.

submaximally and not when the cells were challenged maximally. Consequently, follow-up studies in this study were restricted to the stimulation with 0.1 µg/mL wasp venom, as this concentration results in an optimal sensitivity and submaximal basophil response in the current protocol, which combines phosphorylation of p38 MAPK and CD63 expression.

Figure 4 shows the individual time course of the Pp38 MAPK BAT during VIT after stimulation of the cells in 25 patients with 0.1 µg/mL. This figure displays that a high-dose 5-day semirush hyposensitization therapy did not result in a decrease of phosphorylation of p38 MAPK. Before treatment, incubation of the basophils with 0.1 µg/mL of wasp venom resulted in a median (range) percentage of Pp38 MAPK positive basophils of 61% (20–86%). After the semirush hyposensitization therapy, corresponding median (range) percentage of Pp38 MAPK-positive basophils was 62% (12–88%).

Figure 4 also summarizes the cross-sectional results for the patients treated for 3 or 5 years. There was no significant difference for Pp38 MAPK between these groups when compared with the patients before VIT. The wasp venom-specific IgE antibodies became negative (<0.35 kUa/L) in 4 (31%) patients treated

for 3 years and in 5 (36%) patients treated for 5 years.

DISCUSSION

Recently, we provided the proof of concept that flow cytometry constitutes a promising method to simultaneously analyze immunophenotype and intracellular signaling of IgE-dependent activation of basophils. Moreover, we also showed that antigen-induced upregulation of CD63 is dependent on the phosphorylation of p38 MAPK (12,21). Therefore, the primary objective of this study was to investigate whether phosphorylated p38 MAPK constitutes a novel read-out for flow-assisted diagnosis of hymenoptera venom allergy.

In this study, phosphorylation kinetics of p38 MAPK and upregulation of CD63 expression upon venom-specific activation greatly parallel our prior findings regarding stimulation of basophils with latex (12) and with the recombinant major allergen from birch pollen (rBet v 1) (21).

In our prior studies with latex and rBet v 1, phosphorylation of p38 MAPK was restricted to basophils of patients and was completely absent in healthy control individuals. However, this study discloses that not only basophils from patients but also these from stung control individuals respond with phosphorylation of p38 MAPK although to a lesser extent and only after stimulation with higher concentrations of wasp allergen. In contrast to patients, stung control individuals demonstrate a signal transduction that is "abrogated" beyond the phosphorylation of p38 MAPK without upregulation of downstream CD63.

Despite the high efficiency of VIT, the mechanisms and biomarkers associated with clinical efficacy remain to be disentangled [for review, see Till et al. (24)]. Surveys monitoring negatiation of venom-specific IgE and skin test responses have repeatedly demonstrated that these tests are of limited predictive value and fail to reflect induction of clinical tolerance (13–17,25). In our cross-sectional analysis, only 29% of the patients demonstrated negative wasp venom IgE after 3 years of treatment and 36% after 5 years of treatment. This is in line with prior findings (18) and the data of van Halteren et al. (15) who observed negative sIgE in only 10% of their patients. On the other hand, anaphylaxis due to sting challenges has been reported in patients with negative venom-specific IgE or skin test (14,16,17,25).

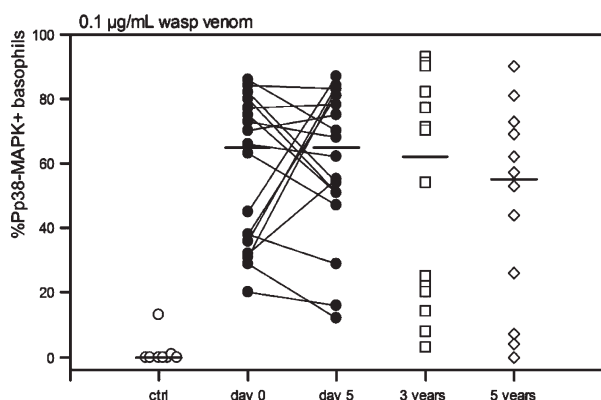


Fig. 4. Percentages of phosphorylated p38 MAPK (Pp38 MAPK)-positive basophils after stimulation of the cells with 0.1 µg/mL wasp venom in stung controls ($n = 7$, open circle) and wasp venom allergic patients ($n = 25$, closed circle) before treatment (day 0), and after semirush hyposensitization (day 5). Percentages of Pp38 MAPK positive basophils of a separate cross-sectional analysis in patients treated for 3 years ($n = 13$, open square) and 5 years ($n = 14$, open rhomb).

Therefore, current pragmatic practice is to offer VIT for a finite period of 3–5 years, irrespective of the persistence of a positive skin test or IgE result (26–28).

Although several studies showed that the BAT might constitute an instrument to monitor successful immunotherapy for inhalant (29–31) and venom allergy (5,12,23), none of these studies included intracellular signaling molecules. Therefore, in the second part of this study, we assessed whether Pp38 MAPK could serve as a new biological marker to monitor build-up and maintenance therapy. However, no effect of therapy on the Pp38 MAPK was demonstrable after the semirush period or after maintenance VIT for 3 or 5 years. Therefore, quantification of Pp38 MAPK using the BAT cannot contribute to the decision of discontinuing VIT safely.

In conclusion, this study re-emphasizes the potential of flow-assisted allergy diagnosis. Second, it confirms that signaling molecules such as Pp38 MAPK constitute a potential novel read-out for allergen-specific basophil activation. Finally, we demonstrated that phosphorylation of p38MAPK not always results in the upregulation of downstream CD63. It is anticipated that in particular conditions with IgE-independent activation of basophils (e.g. drug allergies), p38 MAPK might constitute a better (or sole) activation marker.

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