

Simultaneous Flow Cytometric Detection of Basophil Activation Marker CD63 and Intracellular Phosphorylated p38 Mitogen-Activated Protein Kinase in Birch Pollen Allergy

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Background: Phosphorylation of p38 MAPK is a crucial step in IgE-receptor signaling in basophils. The relation of p38 MAPK to the well-validated diagnostic cell surface marker CD63 has not been evaluated in a clinical allergy model.

Methods: Expression of CD63 and phosphorylation of p38 MAPK were analyzed flow cytometrically in anti-IgE-gated basophils from 18 birch pollen allergic patients, five grass pollen allergic patients, and five healthy individuals, after 3 and 20 min of stimulation with recombinant major birch pollen allergen (rBet v 1). Additional time points and the influence of p38 MAPK inhibitor SB203580 were studied in birch pollen allergic patients.

Results: Phospho-p38 MAPK and CD63 were expressed dose-dependently in birch pollen allergic patient basophils within 1 minute of rBet v 1 stimulation. P38 MAPK phosphorylation was fastest and subsided gradually while CD63 expression remained elevated for at least 20 min. Inhibition of p38 MAPK significantly inhibited CD63 upregulation. With optimal stimulation of the cells (1 µg/mL), sensitivity and specificity for the discrimination between patients and a group of control individuals (grass pollen allergic patients and healthy controls) were 94% and 100% for CD63 at 3 and 20 min and for phospho-p38 MAPK at 3 min.

Conclusion: Antigen-induced p38 MAPK phosphorylation in human basophils essentially contributes to CD63 upregulation. It is a sensitive and specific intracellular marker for allergy diagnosis and offers new insight into the mechanisms of basophil activation. © 2008 Clinical Cytometry Society

Key terms: basophils; CD63; p38 mitogen-activated protein kinase; signal transduction; Bet v 1 protein; allergy; pollen allergy; flow cytometry

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In most patients, allergy diagnosis can reliably be established by history taking, skin testing, and measurement of specific IgE, but in more complex cases, functional in vitro tests are needed. Flow cytometric assessment of in vitro antigen-induced basophil activation (basophil activation test: BAT) is increasingly recognized as a valuable additional tool for allergy diagnosis. Overviews of the general principles, potential applications, and perspectives of the BAT can be found elsewhere (1–6). At present, the most frequently applied and clinically best validated BAT technique relies upon identification of basophils by anti-IgE and assessment of their activation status by detection of CD63. In most settings, surface membrane upregulation of CD63, a lysosomal membrane-associated protein, correlates well with histamine release from activated basophils (7–9), and it has shown excellent sensitivity and specificity in several clinical applications [for review: (1–6)]. The apparent lack of sensitivity of the BAT in drug allergy (10–12) and the fact that there is no immediate effect of (semi-)rush allergen-specific immunotherapy on antigen-induced CD63 expression (13,14), argue that CD63 expression might not fully represent the complexity of basophil degranulation. Therefore, much is expected from studies addressing the intracellular signaling pathways that are leading to the main basophil effector functions, including mediator release, leukotriene generation, and cytokine production. Some of these pathways have been identified in purified basophils from healthy individuals: as a consequence of high affinity IgE receptor (FcεR1) cross-linking, proximal tyrosine kinases Lyn and Syk are rapidly activated, kick-starting a signaling cascade that involves calcium currents and several protein kinases, among which is p38 mitogen-activated protein kinase (MAPK). In vitro phosphorylation of p38 MAPK is crucial to all three basophil effector functions. In contrast, activation of the Ras/Raf/extracellular signal-regulated kinase (ERK) pathway in basophils merely contributes to leukotriene generation (15–21). It is not known, however, whether this applies to allergic reactions in vivo. Therefore, we employed a flow cytometric technique which was recently described by our group (22), to study antigen-induced p38 MAPK phosphorylation in combination with the well-known diagnostic marker CD63, in the clinical model of birch pollen allergy. We aimed to further investigate the kinetic relationship of both molecules and whether this accounts for potential differences in diagnostic performance.

MATERIALS AND METHODS

Patient and Control Samples

Endotoxin-free heparinized whole blood samples (Vacuette, Greiner Labortechnik GmbH, Kremsmünster, Austria) were obtained from 18 patients with birch pollen allergy who were sensitized to Bet v 1 [Bet v 1 is the major allergen from birch (*Betula verrucosa*) pollen (2)]. We also included five patients with grass pollen allergy without birch pollen allergy and five healthy individuals

without classical inhalant allergies. Diagnosis of birch or grass pollinosis was based on a history of rhinoconjunctivitis and/or asthma during relevant seasons, confirmed by a positive skin prick test for birch or grass pollen (HAL, Haarlem, The Netherlands) and the presence of specific serum IgE (sIgE) for birch pollen and recombinant Bet v 1 or grass pollen, respectively (ImmunoCAP FEIA, Phadia, Uppsala, Sweden). Absence of birch pollen allergy in the group of grass pollen allergic patients, or of other classical inhalant allergies in the healthy individuals, required a negative history and negative skin and sIgE tests (sIgE < 0.35 kU_A/L) for birch pollen or a panel of common inhalant allergens, respectively. The study was conducted during the tree pollen season and most patients were treated with antihistamines and/or local glucocorticosteroids. Patients receiving systemic glucocorticoids or allergen-specific immunotherapy were excluded from the study.

Basophil Activation, Fixation, and Staining

Blood samples and reagents were prewarmed for 15 min at 37°C. Thereafter, 100 µL of blood was incubated at 37°C with 100 µL of anti-IgE (10 µg/mL, clone G7-18, Pharmingen, BD Bioscience, Erembodegem, Belgium) as a positive control, recombinant birch pollen allergen Bet v 1 (0.0001–0.01–1 µg/mL, kindly provided by Staller-gènes, Antony, France), or activation buffer (Hanks' balanced salt solution with 1 mol/L HEPES and 7.5% NaHCO₃, pH = 7.4) as a negative (unstimulated) control. The rBet v 1 concentrations were chosen from preparative dose finding experiments over the range of 0.000001–10 µg/mL. After the indicated time periods, reactions were stopped by placing the tubes on ice. Basal phosphorylation was determined by immediately putting prewarmed, unstimulated tubes on ice. Basophils were stained on ice with 20 µL 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 µL of monoclonal phycoerythrin (PE)-conjugated anti-human CD63 (clone H5C6, BD Biosciences) for 20 min. Subsequently, red blood cells were lysed and leukocytes were fixed with 2 mL BD Phosflow Lyse/Fix Buffer (BD Biosciences) for 20 min at room temperature. Cells were washed in D-PBS (Dulbecco's phosphate buffered saline, pH 7.4, Invitrogen) and permeabilized with 0.3% saponin (Acros, Geel, Belgium) in PBS for 30 min at 4°C. Cells were resuspended in 100 µL 0.3% saponin in PBS and stained with 10 µL anti-phospho-p38 MAPK (T180/Y182) conjugated with Alexa Fluor 647 (BD Biosciences) for 30 min at room temperature in the dark. Afterwards cells were washed and resuspended in PBS with 0.1% NaN₃.

Inhibition of p38 MAPK

Specific p38 MAPK inhibitor SB203580 stock solution (1 mg/mL in DMSO, Sigma-Aldrich) was diluted in activation buffer (final concentration 1 and 10 µmol/L) and added to the cells at the start of prewarming. Concentra-

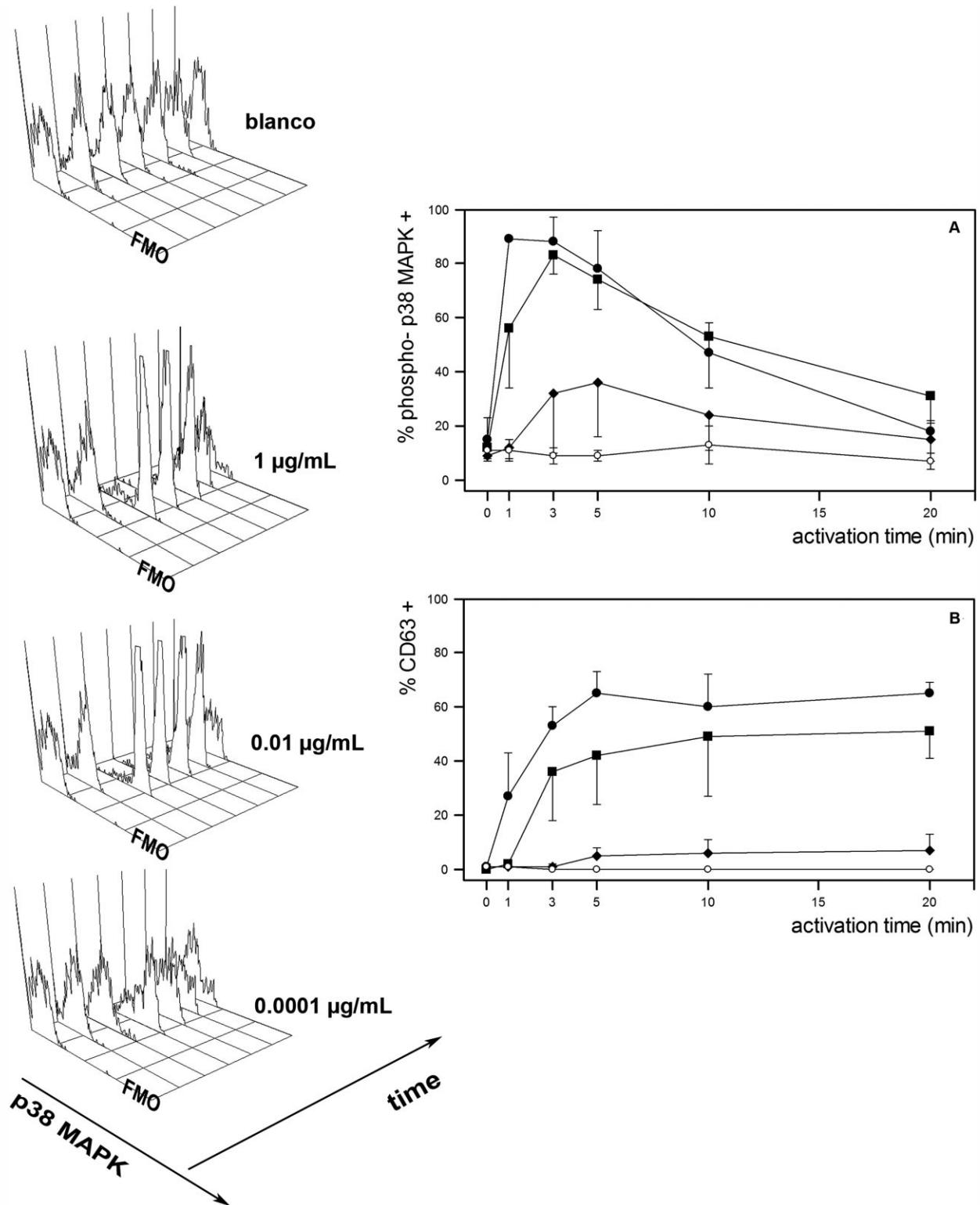


FIG. 1. Kinetics of p38 MAPK phosphorylation and CD63 upregulation in rBet v 1-stimulated basophils from birch pollen allergic patients ($N = 3$). **A:** Flow cytometric detection of intracellular phosphorylation of p38 MAPK in basophils, incubated with buffer (open circles), or stimulated with 0.0001 $\mu\text{g/mL}$ (diamonds), 0.01 $\mu\text{g/mL}$ (squares) or 1 $\mu\text{g/mL}$ (closed circles) rBet v 1, and immediately put on ice (0 min) or activated for 1, 3, 5, 10, or 20 min. Results are expressed as mean $\pm$ SEM of the percentage phospho-p38 MAPK positive basophils, displaying fluorescence intensity above the level of the 99th percentile of a fluorescence-minus-one (FMO)-sample. **B:** Cell surface expression of CD63 in rBet v 1-stimulated basophils. Histograms of phospho-p38 MAPK in basophils from one representative patient are shown to illustrate the effect of buffer incubation (blanco) or rBet v 1 stimulation (1, 0.01, 0.0001 $\mu\text{g/mL}$) for the same time periods as in A.

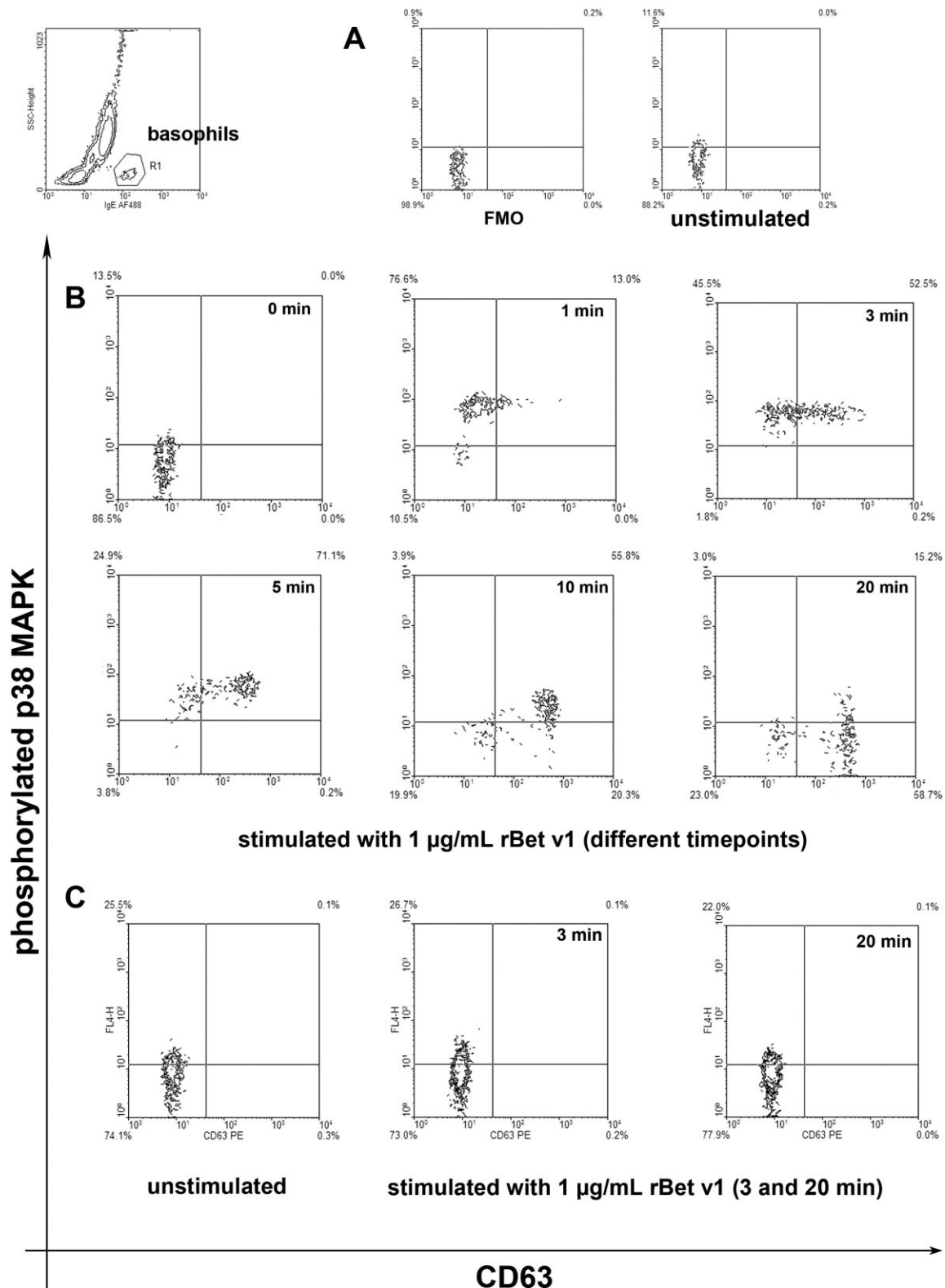


Fig. 2. Combined flow cytometric analysis of CD63 and phospho-p38 MAPK in anti-IgE-gated basophils from a birch pollen (BP) allergic patient (**A** and **B**) and a grass pollen allergic patient (**C**). Contour plots from representative samples demonstrate gating strategy, unstimulated values (**A** and **C**) and time kinetics (0, 1, 3, 5, 10, and 20 min) (**B** and **C**) of phospho-p38 MAPK and CD63 in response to rBet v 1 stimulation (1 μ g/mL). Quadrants are based on the 99th percentile of an FMO-sample. In the BP allergic patient (**B**), different stages of activation can be observed, with p38 MAPK phosphorylation preceding CD63 upregulation and being uniformly present, whereas CD63 is only expressed on part of the basophils. In CD63-negative basophils, p38 MAPK is more quickly dephosphorylated than in CD63-positive cells. In contrast, rBet v 1 has no effect on p38 MAPK phosphorylation in the GP allergic patient (**C**).

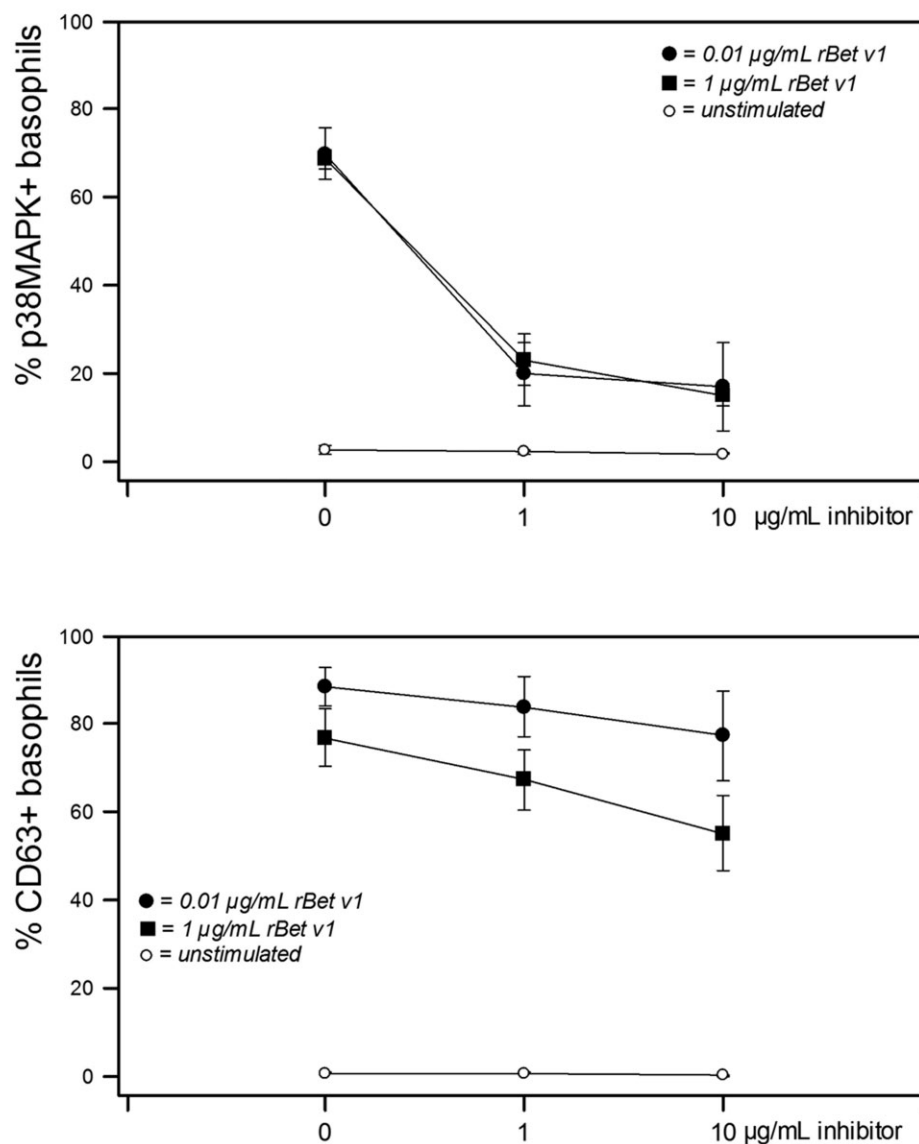
A

FIG. 3. Effect of p38 MAPK-specific inhibitor SB203580 on rBet v 1-induced basophil activation. SB203580 (1–10 µmol/L) was added to the cells prior to incubation with buffer (unstimulated) or rBet v 1 (0.01–1 µg/mL) and results are expressed as mean $\pm$ SEM of the p38 MAPK phosphorylation at 3 min and CD63 expression at 20 min ($N = 3$) (A). Results from the lowest concentration of rBet v 1 (0.0001 µg/mL) are not shown because of a lack of activation in the absence of SB203580 in two patients. Density plots from a representative sample are shown for the 3 min (B) and 20 min (C) time point.

tions of SB203580 higher than 10 µmol/L are toxic to basophils, due to the effect of DMSO.

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 that expressed a high density of surface-bound IgE. Phospho-p38 MAPK positivity was expressed as the percentage of basophils with a fluorescence intensity above the level of the 99th percentile of a fluorescence-minus-one (FMO)-sample (23). For each time point of the incubation period (0, 1, 3, 5, 10, and 20 min), phosphorylation was recorded in both unstimulated and rBet v 1-stimulated samples. The net percentage of phospho-p38

MAPK- or CD63-positive basophils (rBet v 1-stimulated minus unstimulated) was used where appropriate.

Statistics

Data are expressed as percentages and did not follow a normal distribution. Receiver operating characteristics (ROC) analysis, the Friedman test, Mann-Whitney U test and Wilcoxon Signed Rank test were used where appropriate, for a significance level of $P < 0.05$. Statistical analysis was done with SPSS software version 14.0.

RESULTS

Kinetics of p38 MAPK Activation and Upregulation of CD63 in Response to Bet v 1 Stimulation in Birch Pollen Allergic Patients

To determine the optimal time point for diagnostic evaluation of p38 MAPK activation, the activation of p38

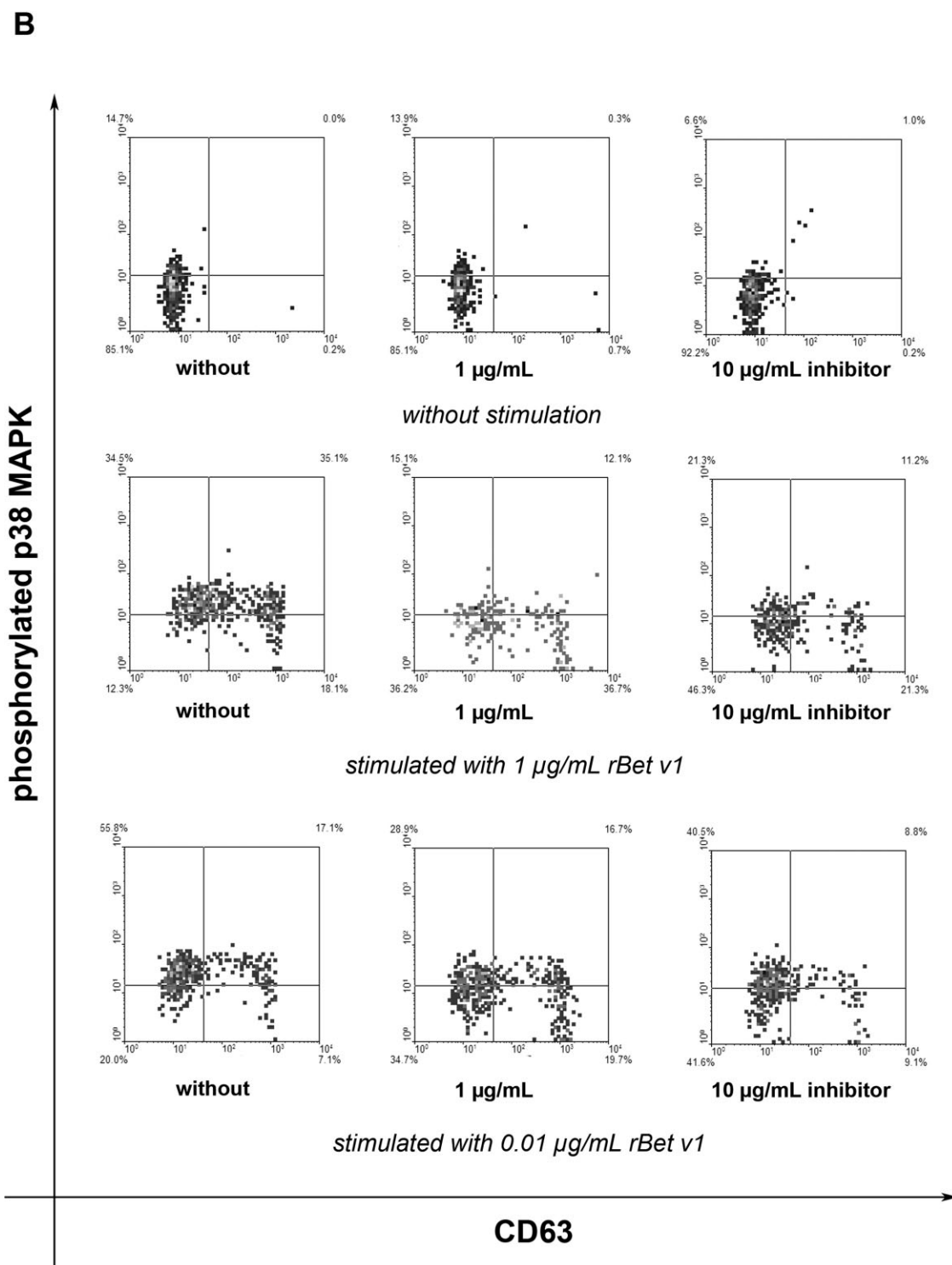


FIG. 3. (Continued)

MAPK and upregulation of CD63 were followed in birch pollen allergic patient basophils, in response to rBet v 1 stimulation (Fig. 1). Both the speed and level of activation of p38 MAPK were dose-dependent: a high dose of

rBet v 1 (1 $\mu\text{g/mL}$) activated the majority of basophils within 1 min, and the p38 MAPK phosphorylation gradually decreased over time to near-baseline values (Fig. 1A). This was mirrored by CD63 upregulation, which

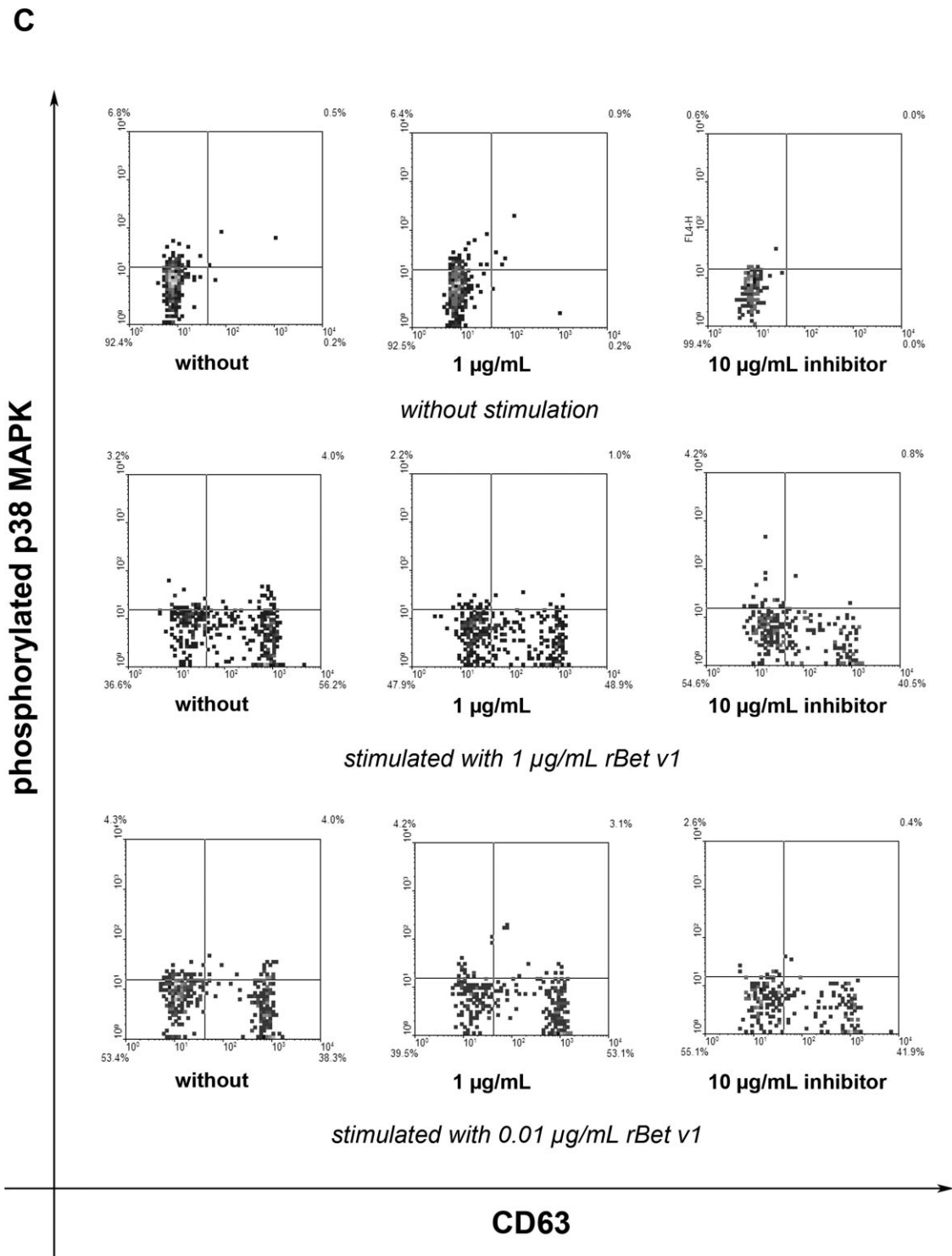


FIG. 3. (Continued)

quickly followed p38 MAPK phosphorylation. At high dose stimulation, it was maximal within 5 min, but remained so for more than 20 min (Fig. 1B). Notably, antigen stimulation on ice did not result in p38 MAPK phosphorylation or CD63 expression over baseline (Figs.

1A and 1B: 0 min). Figure 2 presents the kinetics of p38 MAPK activation and CD63 upregulation in representative samples of a birch pollen allergic patient (Figs. 2A and 2B) and a grass pollen allergic patient (Fig. 2C). Based on these findings, we further studied the 3 min

time point in addition to the 20 min previously adopted in the BAT, to explore the interdependence of rBet v 1-activated p38 MAPK and CD63 and their diagnostic value, in birch pollen (BP) allergic patients, grass pollen (GP) allergic patients, and healthy individuals.

P38 MAPK Inhibition Affects rBet v 1-Induced Upregulation of CD63

Having observed the close temporal relationship of p38 MAPK phosphorylation and upregulation of CD63, we studied the effect of adding the selective p38 MAPK inhibitor SB203580 to basophils from BP allergic patients, prior to rBet v 1 stimulation. There was little effect on the already negligible spontaneous p38 MAPK phosphorylation in resting basophils, but SB203580 profoundly inhibited rBet v 1-induced p38 MAPK auto-phosphorylation and also inhibited CD63 upregulation, particularly for the highest stimulation concentration (Fig. 3).

Intracellular phosphorylation of p38 MAPK and Expression of CD63 upon Basophil Stimulation with Recombinant Bet v 1 in Birch Pollen Allergic Patients, Grass Pollen Allergic Patients, and Healthy Individuals

Spontaneous phosphorylation of p38 MAPK in resting basophils was very limited and not significantly different in BP allergic patients, GP allergic patients, and healthy individuals (Fig. 4A). Three minutes of rBet v 1 stimulation elicited a clear phosphorylation of p38 MAPK in BP allergic patients, plateauing at the higher stimulation doses and affecting over 60% of the basophils. After 20 min, this phosphorylation had decreased significantly, but still remained significantly elevated over baseline values. In contrast, in GP allergic patients and healthy individuals, rBet v 1 did not induce significant p38 MAPK phosphorylation over baseline values (Fig. 4A), though one GP allergic patient showed a small dose-dependent increase in phospho-p38 MAPK after rBet v 1 stimulation, not accompanied by CD63 upregulation. Spontaneous expression of CD63 in resting basophils was negligible and comparable between BP allergic patients, GP allergic patients and healthy individuals. All 18 BP allergic patients were "responders," expressing CD63 in reaction to rBet v 1 and/or anti-IgE stimulation. After 3 and 20 min of stimulation with rBet v 1, a clear dose-dependent cell surface expression of CD63 was observed in BP allergic patient basophils (Fig. 4B). In contrast, there was neither CD63 expression in GP allergic patients nor healthy individuals (Fig. 4B).

Sensitivity and Specificity of Phospho-p38 MAPK and CD63 to Discriminate Birch Pollen Allergic Patients from Grass Pollen Allergic Patients and Healthy Individuals

Cut-off values for the discrimination between BP allergic patients and the group of GP allergic patients and healthy individuals were determined for each rBet v 1 dose and time point by receiver operating characteristics

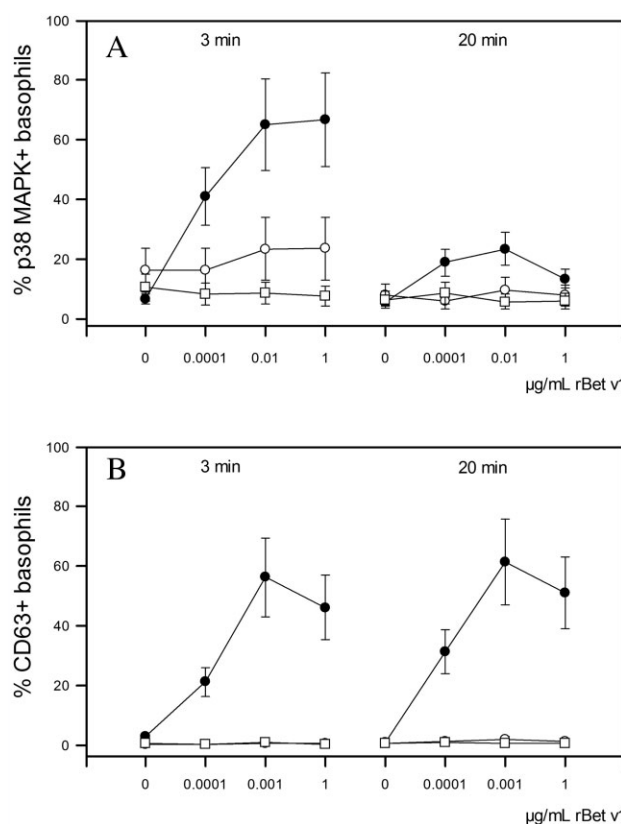


FIG. 4. Recombinant Bet v 1-induced activation of basophils from birch pollen allergic patients ($N = 18$, closed circles), grass pollen allergic patients ($N = 5$, open circles) and healthy individuals ($N = 5$, squares). **A:** Phospho-p38 MAPK in basophils incubated with buffer (0 µg/mL rBet v 1) and stimulated with rBet v 1 (0.0001, 0.01, and 1 µg/mL) for 3 and 20 min. Results are expressed as mean $\pm$ SEM of the percentage phospho-p38 MAPK positive basophils, based on the 99th percentile of a fluorescence-minus-one sample. P -values for the nonparametric comparison of stimulated and unstimulated phosphorylation in birch pollen allergic patients are <0.005 at 3 min and <0.02 at 20 min. In grass pollen allergic patients, p38 MAPK is not significantly phosphorylated upon rBet v 1 stimulation. **B:** Cell surface expression of CD63 in unstimulated blood samples and after rBet v 1 stimulation. For birch pollen allergic patients, P -values for nonparametric comparison of stimulated and unstimulated expression are all <0.01 .

(ROC) analysis. The corresponding sensitivity and specificity are displayed in Table 1. About 1 µg/mL of rBet v 1 resulted in optimal sensitivity and specificity of p38 MAPK phosphorylation, but only after 3 min of stimulation. Similarly, it showed optimal sensitivity and specificity of CD63 expression, with equal values after 3 and 20 min of stimulation (Table 1).

DISCUSSION

Recently, we demonstrated that phosphorylated p38 MAPK can be quantified flow cytometrically as an early marker of anti-IgE- and antigen-induced basophil activation, with best results being obtained by using a saponin-based permeabilization technique (22). The tech-

Table 1
Sensitivity and Specificity of rBet v 1-Induced Basophil Activation Markers CD63 and Phospho-p38 MAPK in the Discrimination of Birch Pollen Allergic Patients from Grass Pollen Allergic and Healthy Controls

Activation marker	rBet v 1 stimulation		Cut-off value (% basophils)	Sensitivity (%)	Specificity (%)
	Duration (min)	Dose (μ g/mL)			
CD63	3	0.0001	0.1	80	80
		0.01	6.3	78	100
		1	4.4	94	100
	20	0.0001	2.2	73	100
		0.01	13.9	78	100
		1	7.9	94	100
Phospho-p38 MAPK	3	0.0001	7.1	80	100
		0.01	8.9	83	90
		1	31.1	94	100
	20	0.0001	1.2	73	70
		0.01	1.5	83	80
		1	0.9	83	80

Cut-off values are the net percentage (stimulated minus unstimulated) of CD63- or phospho-p38 MAPK-expressing basophils.

nique allowed us to study phospho-p38 MAPK in combination with CD63, which is a well-appreciated diagnostic marker in several clinical allergy models. Until then, studies on signaling molecules in clinical samples were hampered by the need for large sample volumes to perform Western blot, and most studies used passively sensitized, purified basophils from healthy individuals or non-specific stimuli such as anti-IgE or fMLP (*N*-formyl-methionyl-leucyl-phenylalanine) (15–21). In addition, the results provided by Western blot only represent the average of a cell isolate. In contrast, the present study evaluated rBet v 1-induced phospho-p38 MAPK and CD63 upregulation in basophils from birch pollen allergic patients, to analyze the role of p38 MAPK in clinically relevant basophil activation, and its potential application in allergy diagnosis. Out of several markers for basophil identification and detection of activation that have been described (4), we chose to evaluate CD63 in combination with IgE, because of its generally excellent flow cytometrical characteristics (unambiguous identification, bimodal upregulation) and clinical significance.

We studied the kinetics of p38 MAPK phosphorylation and CD63 upregulation for up to 20 min. Although p38 MAPK activation proceeded very fast, the speed was dose-dependent and we could detect major differences in the phosphorylation level within the first 3 min after the start of activation, depending on the rBet v 1 dose. The antigen-induced p38 MAPK phosphorylation wanes within 20 min, while CD63 upregulation is more sustained over time. Interestingly, activated basophils do not go on to express CD63 dephosphorylate p38 MAPK more quickly than their CD63-positive counterparts. This would indicate that p38 MAPK activation is situated upstream from CD63 upregulation, as could be expected since p38 MAPK has been shown to be crucially involved in basophil degranulation, leukotriene generation, and cytokine production (19). Indeed, inhibition of p38 MAPK with SB203580 also inhibited CD63 upregulation, although the level of inhibition was rather limited,

suggesting that alternative signaling pathways also connect IgE receptor cross-linking to CD63 upregulation. Remarkably, we previously found that natural rubber latex-induced CD63 upregulation was much more completely inhibited by SB203580 (22).

The finding that p38 MAPK activation is faster and more short-lived than CD63 upregulation suggests a classical signal transduction mechanism. Signal transduction through the MAPK family is based on amplification or diminution between different steps. It is tightly controlled by regulatory mechanisms, since most MAPK family members are involved in several essential cellular processes and prolonged activation might have detrimental (e.g. pro-apoptotic) effects (24). After its activation, p38 MAPK is rapidly dephosphorylated by cytoplasmic and nuclear phosphatases. In contrast, CD63 is associated with the end-stage of basophil activation and appears on the surface membrane after basophilic granule exocytosis (25).

Given their distinct contribution to the basophil activation cascade, it seemed interesting to evaluate the specificity and sensitivity of phospho-p38 MAPK and CD63 in detecting in vivo sensitization of basophils. For this purpose, we selected a group of 18 birch pollen allergic patients, with clear history, positive skin tests, and sIgE. To minimize heterogeneity in the patient group, only Bet v 1 allergic patients were included and therefore, we were able to use recombinant Bet v 1 for basophil activation. In addition to five healthy controls, we included five grass pollen allergic patients, to assess non-specific effects in patients suffering from another IgE-mediated allergy. Despite all tests having been conducted during the tree pollen season, baseline expression of CD63 and phosphorylation of p38 MAPK were not elevated in BP allergic patients. Upregulation of CD63 and phosphorylation of p38 MAPK were rBet v 1 dose-dependent and virtually confined to BP allergic patient samples. CD63 expression at 3 and at 20 min and p38 MAPK phosphorylation at 3 min of rBet v 1 stimulation

at optimal dose, showed excellent sensitivity and specificity in discriminating BP allergic patients from the group of GP allergic patients and healthy controls. In this study we had no clear indications of a diagnostic advantage of either molecule, be it in terms of sensitivity or specificity, but it would be very interesting to evaluate phospho-p38 MAPK in nonresponders (2,4), who do not upregulate CD63 in reaction to IgE receptor-mediated stimulation, and in patients with drug allergy, in whom the BAT has rather disappointing diagnostic sensitivity (10–12).

In conclusion, our data show that antigen-induced p38 MAPK phosphorylation precedes CD63 upregulation and CD63 expression depends at least partially on p38 MAPK. Although short-lived, phospho-p38 MAPK constitutes a sensitive and specific marker of antigen-specific basophil activation, which can be easily and rapidly assessed by flow cytometry. Entangling the role of p38 MAPK and CD63 in basophil activation might offer new insights into allergy diagnosis and treatment.

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