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## The Inflammatory Response Induced by Aspartic Proteases of *Candida albicans* Is Independent of Proteolytic Activity<sup>∇</sup>

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The secretion of aspartic proteases (Saps) has long been recognized as a virulence-associated trait of the pathogenic yeast *Candida albicans*. In this study, we report that different recombinant Saps, including Sap1, Sap2, Sap3, and Sap6, have differing abilities to induce secretion of proinflammatory cytokines by human monocytes. In particular Sap1, Sap2, and Sap6 significantly induced interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), and IL-6 production. Sap3 was able to stimulate the secretion of IL-1 $\beta$  and TNF- $\alpha$ . All Saps tested were able to induce Ca<sup>2+</sup> influx in monocytes. Treatment of these Saps with pepstatin A did not have any effect on cytokine secretion, indicating that their stimulatory potential was independent from their proteolytic activity. The capacity of Saps to induce inflammatory cytokine production was also independent from protease-activated receptor (PAR) activation and from the optimal pH for individual Sap activity. The interaction of Saps with monocytes induced Akt activation and phosphorylation of I $\kappa$ B $\alpha$ , which mediates translocation of NF- $\kappa$ B into the nucleus. Overall, these results suggest that individual Sap proteins can induce an inflammatory response and that this phenomenon is independent from the pH of a specific host niche and from Sap enzymatic activity. The inflammatory response is partially dependent on Sap denaturation and is triggered by the Akt/NF- $\kappa$ B activation pathway. Our data suggest a novel, activity-independent aspect of Saps during interactions of *C. albicans* with the host.

*Candida albicans* is a generally harmless commensal yeast that colonizes mucosal surfaces of the gastrointestinal or urogenital tract in most healthy people. In severe cases of infection, *C. albicans* may reach the bloodstream due to damage of barriers, for example, after polytrauma or surgery, or via penetration of the gastrointestinal mucosa in susceptible hosts, thereby causing hematogenously disseminated candidiasis (31). *C. albicans* has developed a battery of virulence factors that enables the fungus to colonize host tissues, to escape the immune response, and to cause disease (5, 22, 62). A variety of different virulence factors contribute to the infective process of the fungus, and these factors can be expressed in either a qualitative or quantitative manner, depending on the site of infection, the stage of infection, and the nature of the host immune response (12, 19).

The secretion of aspartic proteases (Saps), encoded by a gene family with 10 members, has long been recognized as a virulence-associated trait of this pathogenic yeast (34, 35). Individual Sap proteases have been purified from *C. albicans* culture supernatants (18) or by recombinant expression in *Pichia pastoris* (3) or *Escherichia coli* (24, 25), and their biochemical properties have been characterized (2, 3, 55). For example, the optimum pHs for individual Saps have been established as between pH 2.0 and 5.5 for Saps 1 to 3 and

between pH 3.0 and 7.0 for Saps 4 to 6 (3). Sap9 and Sap10 have been identified as glycosylphosphatidylinositol (GPI)-anchored proteases which target proteins necessary for both cellular processes and host-pathogen interactions (1).

The expression of all *SAP* genes has been monitored *in vivo* during interaction with host cells in several studies (10, 37, 46, 49, 56, 57, 60). Furthermore, earlier studies have shown that Saps are recognized by the host and that anti-Sap antibodies are produced by patients (47).

The role of *C. albicans* Saps during human infection has not been clarified completely. Saps have been described as critical virulence factors involved in promoting adhesion to, invasion of, and damage to epithelial cells and tissue (9, 21, 33, 35, 64). Consistent with this view, it has been reported that the aspartic protease inhibitor pepstatin A is able to inhibit the invasion of *C. albicans* during interaction with oral or intestinal epithelial cells (9, 36). In addition, inhibiting Sap activity by using pepstatin A has been shown to reduce human epithelial damage (27, 36, 52). Furthermore, it has been shown that mutants lacking Saps 1 to 3 cause a reduced proinflammatory response of epithelial cells (49, 51). Another report showed that Saps 4 to 6, but not Saps 1 to 3, play an important role in survival and escape of *C. albicans* during interaction with macrophages (3). A more recent study on the cell surface-associated protease Sap9 showed that this protease can efficiently and rapidly degrade the antimicrobial peptide histatin 5, produced by oral epithelial cells, resulting in the loss of the anti-*Candida albicans* potency of histatin 5 (32). Another recent report indicates that deletion of *SAP9* leads to a mitigated release of reactive oxygen intermediates (ROI) by human polymorphonuclear

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leukocytes (PMNs) and decreases *C. albicans*-induced apoptosis triggered by ROI formation (17). Furthermore, Saps 1 to 3 have been shown to mediate evasion of human complement attack via degradation of complement components (13).

Pathogens initially interact with the innate immune system via binding between microorganism-specific chemical signatures (pathogen-associated molecular patterns [PAMPs]) and pattern recognition receptors (PRRs) on antigen-presenting cells. PAMPs bind specifically to two classes of PRRs, namely, Toll-like receptors (TLRs) and C-lectin-like receptors. PAMP-PRR interaction triggers signaling responses that culminate in release of proinflammatory cytokines. Moreover, it has been demonstrated that several microbial virulence factors are able to link PRRs and to activate the proinflammatory response. *Porphyromonas gingivalis* fimbriae, a major virulence factor of this pathogen, are able to link CD14 and CD11b/18, following which TLRs are recruited as signal receptors (14). The M1 protein of *Streptococcus pyogenes* binds to TLR2 and TLR4 (54). Both the native mannose-bound MP65 protein (a  $\beta$ -glucanase adhesin) and the recombinant protein (rMP65) of *Candida albicans* were efficiently bound and taken up by macrophages and dendritic cells in a process involving both clathrin-dependent and clathrin-independent mechanisms. Moreover, the RGD sequence is involved in rMP65 uptake, to some extent. After internalization, rMP65 was partially localized in lysosomes and resulted in efficient protein degradation and presentation to CD4<sup>+</sup> T cells (42).

Serine protease enzymes are able to activate protease-activated receptors (PARs), a newly identified family of G-protein-coupled receptors. As a consequence, proteases have the potential to modulate the inflammatory response (16).

The first cell interaction of foreign antigens with cell surface receptors leads to the stimulation of signal transduction cascades, starting with phosphatidylinositol 3-kinase (PI3K) activation, which is one of the most prevalent signal transduction events, if not the most prevalent event, associated with mammalian cell surface receptor activation (15). PI3K recruits pleckstrin homology domain-containing proteins, including Akt and phosphoinositide-dependent protein kinase, to the plasma membrane. The PI3K/Akt signaling cascade is crucial to widely divergent physiological processes, which include cell cycle progression, differentiation, transcription, translation, apoptosis, endocytosis, motility, and metabolism (30).

Given the many roles of *C. albicans* secreted aspartic proteases during interaction with the host through different mechanisms, we questioned whether Saps (i) are able to interact with PARs and (ii) retain immunomodulatory potential during interaction with host immune cells. Furthermore, we analyzed the response of primary human phagocytic cells (monocytes) to different purified Saps, as well as the biological and molecular events that accompany this interaction.

## MATERIALS AND METHODS

**Aspartic protease production.** Recombinant *C. albicans* aspartic proteases rSap1, rSap2, rSap3, and rSap6 and the *Malassezia furfur* lipase MfLip1 were expressed as recombinant proteins by use of *Pichia pastoris* clones, produced in our laboratory (MfLip1) or kindly provided by Michel Monod, Lausanne, Switzerland (Sap1, -2, -3, and -6), as previously described (3, 4).

Recombinant proteins were harvested from *P. pastoris* culture supernatants. Aspartic proteases were purified via anion-exchange chromatography, followed by desalting by passage through a Sephadex G25 column, and lipase was con-

centrated using Amicon Ultra-15 centrifugal filter units (Millipore). The activity of proteases and inhibition by pepstatin A under standard reaction conditions were verified by using a fluorescence-based casein assay (Molecular Probes). The culture supernatant was first desalted, and low-molecular-weight solutes were removed by passage through a Sephadex G25 column (PD10 column; Pharmacia), using 10 mM sodium citrate buffer, pH 6.5. The recombinant proteases Sap1, Sap2, and Sap3 recovered from the first purification step were loaded on a small polypropylene column filled with 0.5 ml of DEAE-Sepharose (Pharmacia), washed with the same buffer, and eluted with 100 mM sodium citrate buffer at pH 5.0. Sap6 protein recovered from the Sephadex G25 column was loaded on a hydroxyapatite column (0.1 g in a small polypropylene column). The column was first washed with 50 mM sodium phosphate buffer (pH 7.0), and the Sap isoenzymes were eluted with 150 mM sodium phosphate buffer (pH 7.0). The eluted proteases were further purified through an affinity chromatography system (EndoTrap; Profos AG) to remove possible endotoxin contamination. After purification, rSAPs tested negative for endotoxin contamination in a *Limulus* assay (E-toxate; Sigma) with a sensitivity of 10 pg/ml of *Escherichia coli* lipopolysaccharide (LPS). Nevertheless, selected experiments were carried out at least once in the presence of 10  $\mu$ g/ml of polymyxin B (Sigma), a polycationic antibiotic, to neutralize any undetected contamination with bacterial LPS.

In selected experiments, we used native Sap2 purified as previously described (29, 58). The Sap2 produced by *C. albicans* strain 10261, grown in YBD medium (0.2% yeast extract, 2% glucose, 0.2% bovine serum albumin [BSA]), was purified by DEAE-Sephadex A-25-120 chromatography and concentrated by ultrafiltration. The purified enzyme gave one band with a molecular weight of 43,000 in an analysis by SDS-PAGE.

In addition, we also used culture supernatants from *C. albicans* strains grown in YCB-BSA and containing high concentrations of native Sap2 as previously described (20). Briefly, *Candida albicans* CAF2-1 and CEC987 were pregrown overnight in SD medium (6.7 g yeast nitrogen base without amino acids and 20 g glucose per liter) at 30°C and then diluted 1:100 in YCB-BSA medium (23.4 g yeast carbon base and 4 g BSA per liter). The pH of YCB-BSA medium was adjusted to 4.0, and the culture was incubated at 30°C for 48 h. The secretomes from these cultures were used in experiments to stimulate proinflammatory cytokine secretion by monocytes.

Proteases and LPS were heat inactivated by autoclaving (exposure to 120°C for 20 min).

In selected experiments, the aspartyl protease pepsin from porcine gastric mucosa (Sigma) was used as a negative control.

**Monocyte isolation.** Heparinized venous blood was obtained from healthy donors and diluted with RPMI 1640 (Gibco-BRL). Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation over Ficoll-Hypaque Plus (Pharmacia Biotech, Uppsala, Sweden), recovered, washed twice, suspended in RPMI 1640 supplemented with 10% fetal calf serum, 100 U penicillin/ml, and 100  $\mu$ g streptomycin/ml, plated in cell culture flasks (Corning Incorporated), and incubated for 1 h at a density of  $2 \times 10^6$  to  $3 \times 10^6$ /ml. The adherent cells recovered were >95% CD14<sup>+</sup>, as evaluated by flow cytometry.

**Cytotoxicity assay.** Monocytes ( $2.5 \times 10^5$ ) were treated with different doses of recombinant Saps (rSaps) ranging from 1 to 50  $\mu$ g/ml for 4 h and 18 h at 37°C, and cell viability was evaluated by the use of an ATP bioluminescence kit (Via Light kit; Cambrex). Briefly, 100  $\mu$ l of each sample was added to a 96-well culture plate, 50  $\mu$ l of lysis reagent was added to each well, and after 10 min of incubation, 100  $\mu$ l of ATP monitoring reagent (AMR Plus) was added to each sample. After 2 min of incubation at room temperature, luminescence was measured by a luminometer (Infinite 200; Tecan).

**Cytosolic calcium determination.** The cytosolic calcium level was determined by using a fluorescent dye as previously described (41, 59). Fura-2 (4  $\mu$ l of a 1 mM solution in dimethyl sulfoxide [DMSO]) was added to monocyte suspensions (about  $30 \times 10^6$  cells) in serum-free medium and incubated for 60 min at 37°C in the dark. Cells were harvested by centrifugation at  $600 \times g$  for 10 min and finally suspended ( $10^7$  cells/ml) in HBSS (140 mM NaCl, 5.3 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 5 mM glucose, 25 mM HEPES; pH 7.4). A total of  $10^6$  cells per sample were stimulated with different rSaps (20  $\mu$ g/ml), and the Ca<sup>2+</sup> level was determined for the following 90 min. As a positive control, 5  $\mu$ M ionomycin was added for 10 min to obtain maximal fluorescence. EDTA (10 mM) was added to chelate the Ca<sup>2+</sup> from Fura-2 as a negative control. Fluorescence was measured with a spectrophotofluorimeter (excitation at 380 nm and emission at 510 nm) (Infinite 200; Tecan). Cytosolic calcium concentrations were calculated as follows:  $[Ca^{2+}] = K_d \times (F - F_{min}) / (F_{max} - F)$ , where  $F$  is the experimentally measured fluorescence intensity,  $F_{min}$  is the measured fluorescence intensity in the absence of Ca<sup>2+</sup> (in the presence of EDTA),  $F_{max}$  is the measured fluorescence intensity of Ca<sup>2+</sup>-saturated dye (ionomycin-treated cells), and  $K_d$  is the dissociation constant of Fura-2 (224 nM).



**Determination of proinflammatory cytokine production.** Monocytes ( $2 \times 10^6$ /ml) were incubated with different doses of rSaps, ranging from 1 to 50  $\mu\text{g/ml}$ , or with LPS, ranging from 0.001 to 10  $\mu\text{g/ml}$ , for 18 h. As a negative control, a recombinant protein, lipase (15  $\mu\text{g/ml}$ ), obtained with the same *Pichia pastoris* system, and pepsin (1 to 50  $\mu\text{g/ml}$ ) were used in selected experiments. In parallel experiments, monocytic cells were treated with pepstatin A (15  $\mu\text{M}$ ), polymyxin B (10  $\mu\text{g/ml}$ ), or 10 to 50  $\mu\text{g/ml}$  of mouse anti-human PAR1 (ATAP2; raised against amino acids 42 to 55, located inside the tethered ligand domain of PAR1); mouse anti-human PAR2 (SAM11); a mouse monoclonal IgG2a antibody raised against amino acids 37 to 50 (SLIGKVDGTSHVTG), located inside the human PAR2 cleavage site; or mouse anti-human PAR3 (8E8), raised against amino acids 31 to 47, which include the cleavage site to inhibit the enzymatic activity of Saps. To study the role of Akt in the intracellular signaling activated by proteases, cells were pretreated with 5  $\mu\text{M}$  wortmannin for 40 min before adding Saps. The presence of human tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and IL-6 in culture supernatant fluids was measured with an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences Pharmingen).

**Protease FITC labeling.** Proteases and CTLA-4 F(ab) $_2$  (Ancell) were labeled with fluorescein isothiocyanate (FITC), using a Fluoro Tag FITC conjugation kit (Sigma). Briefly, fresh FITC solution in carbonate-bicarbonate buffer was added to the rSap solutions, and samples were incubated for 2 h at room temperature with gentle stirring. The labeled proteins were purified from unconjugated fluorescein by use of a Sephadex G25 M column.

**Protease association with human monocytes.** The association of rSaps with human monocytes was analyzed by flow cytometry. Monocytes were incubated with different doses of FITC-conjugated rSaps and CTLA-4 F(ab) $_2$  for different periods at 37°C in the presence of 5% CO $_2$ . After incubation, cells were collected by centrifugation, fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS), washed twice, and resuspended in PBS containing 0.5% bovine serum albumin and 0.1% sodium azide. Cells attached to or containing Saps were monitored using a FACScan flow cytometer (Becton Dickinson). To analyze the specific association of FITC-labeled proteins in selected experiments, monocytes were first incubated with unlabeled rSaps (20  $\mu\text{g/ml}$ ) and then treated with FITC-conjugated rSaps at 20  $\mu\text{g/ml}$  for 30 min.

**Determination of phosphorylated Akt and I $\kappa$ B $\alpha$ .** The levels of Akt and I $\kappa$ B $\alpha$  phosphorylation in monocytes were measured by flow cytometry after 4 h of stimulation with rSap1, rSap2, rSap3, rSap6, or pepsin (20  $\mu\text{g/ml}$ ). Phosphorylated proteins were detected with rabbit anti-human phospho-I $\kappa$ B $\alpha$  or with rabbit anti-human phospho-Akt (Cell Signaling Technology). Cells were first fixed in 3% paraformaldehyde for 10 min and then permeabilized in 0.1% saponin at 4°C for 10 min. Primary antibody incubation was followed by incubation with tetramethyl rhodamine isocyanate (TRITC)-conjugated goat anti-rabbit IgG (Sigma). Phosphorylation of phospho-I $\kappa$ B $\alpha$  or phospho-Akt was analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA).

**Statistical analysis.** Statistical significance was determined using analysis of variance (ANOVA). Results are presented as means  $\pm$  standard deviations (SD).

## RESULTS

Secreted aspartic proteases (Saps) are important virulence factors of *Candida albicans* and may also contribute to the induction of an inflammatory host immune response (35, 51). To analyze a possible correlation between Sap secretion and the host immune response, we used the following recombinant secreted Saps: rSap1, rSap2, and rSap3, representing the Sap family subgroup Sap1-3, and rSap6, representing the Sap family subgroup Sap4-6. We tested the ability of secreted aspartic proteases to induce proinflammatory cytokines by coincubating human monocytes isolated from blood from healthy donors with the different rSaps. The results shown in Fig. 1A indicate that human monocytes recognized all Saps, but the capacity of human monocytes to secrete IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 varied in response to different Saps. In particular, Sap1, Sap2, and Sap6 induced significant amounts of IL-1 $\beta$ , TNF- $\alpha$ , and IL-6. rSap3 was a strong inducer of TNF- $\alpha$ , while it was a poor inducer of IL-1 $\beta$  and IL-6. In contrast, the lipase of *M. furfur* and pepsin

from porcine gastric mucosa, used as a control, did not cause significant induction of cytokines (Fig. 1B). A dose-dependent analysis performed with all rSaps showed that the optimal dose to stimulate TNF- $\alpha$  secretion was around 20  $\mu\text{g/ml}$  (Fig. 1B). Similar results were obtained when additional cytokines, for example, IL-6 or IL-1 $\beta$ , were tested (data not shown). To exclude the possibility of the production of proinflammatory cytokines being influenced by LPS contamination, we performed experiments in the presence of polymyxin B (10  $\mu\text{g/ml}$ ). The results showed no differences for all proteases tested, while, as expected, TNF- $\alpha$  production induced by LPS was strongly reduced (Fig. 1C).

To compare a native Sap to the cognate recombinant protease, we also used purified native Sap2 and a culture supernatant containing high concentrations of native Sap2. The secretome was collected from the cultures of two strains of *C. albicans* (CAF2-1 and CEC987) grown in YCB-BSA medium for 48 h. Similar production of TNF- $\alpha$  was induced by native and recombinant Sap2 (Fig. 1D). The secretomes of both strains, diluted 1:10 and 1:100, were tested. The secretome diluted 1:10 in both cases stimulated the secretion of TNF- $\alpha$  (Fig. 1D).

Preliminary experiments were performed to test for possible cytotoxicity of rSaps by treating monocytes with the recombinant proteases at doses ranging from 1 to 50  $\mu\text{g/ml}$ . We observed no cytotoxicity after 4 h and 18 h of incubation for all rSaps, except rSap6, which was able to induce cytotoxicity in 40% of cells at a high dose (50  $\mu\text{g/ml}$ ). Based on the previous results, a concentration of 20  $\mu\text{g/ml}$  was used in the subsequent experiments. The capacity of rSap1, rSap2, rSap3, and rSap6 to induce cellular activation was also confirmed by their ability to induce a Ca $^{2+}$  influx, and rSap1 was the best stimulator (data not shown).

Given the fact that most, if not all, of the biological effects of Saps have been attributed to their enzymatic activity (35, 50), we tested whether induction of inflammatory cytokines in monocytes was affected by treatment of Saps with pepstatin A, an aspartic protease inhibitor. Figure 2A shows that the addition of pepstatin A did not affect the production of TNF- $\alpha$  and IL-6, suggesting that proteolytic activity is not necessary to cause induction of these cytokines. Since it has been reported that Saps 1 to 3 have different pH optima for their enzymatic activity in the pH range of 2.0 to 5.5 (3), we tested the potential of rSap1, rSap2, and rSap3 to induce cytokine production at their optimal pHs (pHs 4 and 5). No production of cytokines was observed at these selected pH values (data not shown). Therefore, no cytokines were produced when Saps 1 to 3 were most active. Under these experimental conditions, even LPS was unable to induce cytokine production. This could be due to the difficulty of macrophages to accomplish vital biological functions at these acidic pH values.

Next, we analyzed whether the denaturation and inactivation of rSaps influenced the stimulation of cytokine production by monocytes. To this end, Saps were denatured by autoclaving and then used to stimulate the cells. Denaturation of rSaps strongly reduced the proinflammatory cytokine production induced by rSap2, rSap3, and rSap6 (Fig. 2B). In particular, the denaturation of these Saps strongly reduced the production of TNF- $\alpha$  and IL-1 $\beta$ , while proinflammatory cytokine induction by rSap1 was not modified. The induction of cytokine secretion

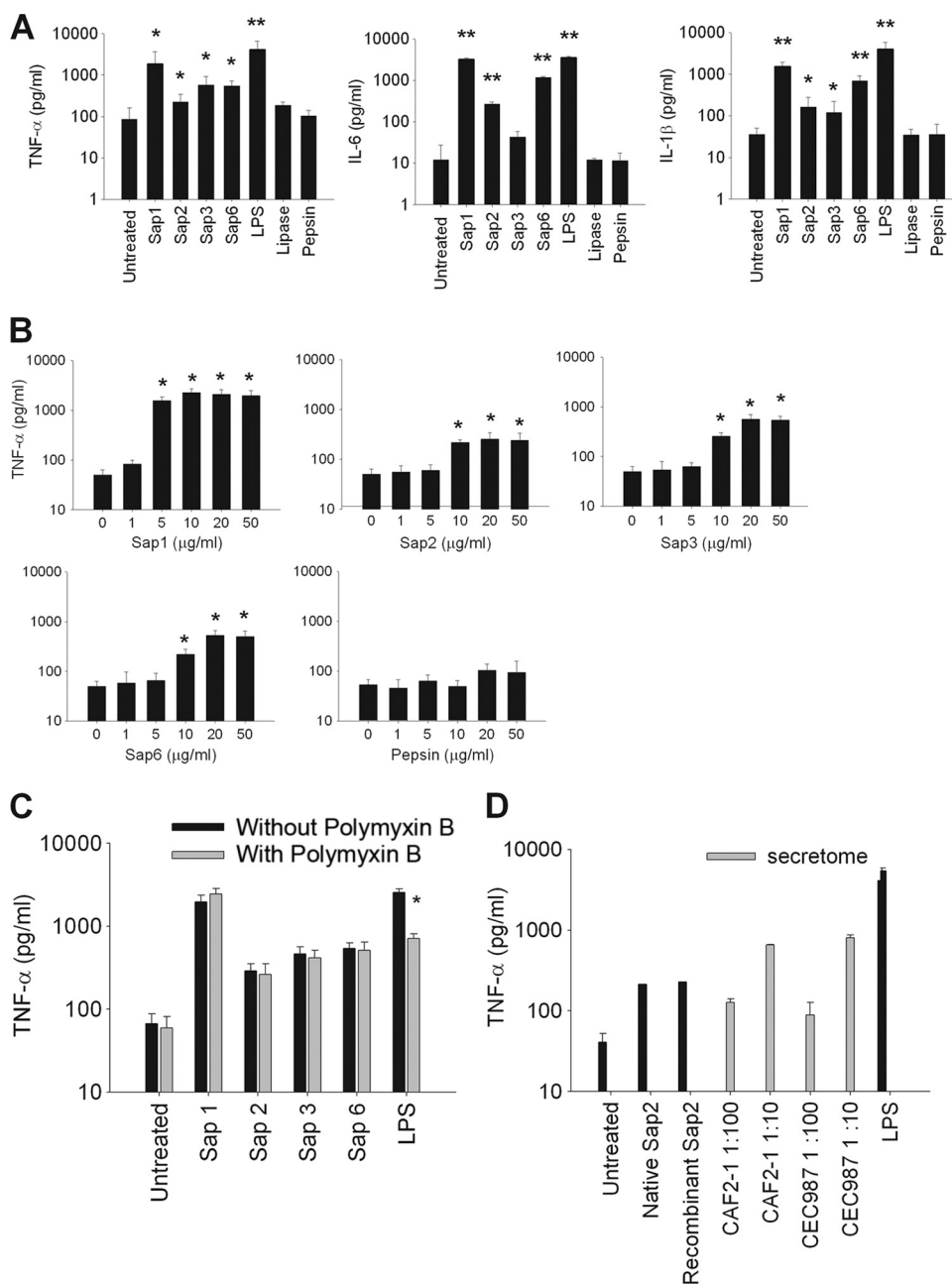


FIG. 1. TNF- $\alpha$ , IL-6, and IL-1 $\beta$  production from monocytes after stimulation with *Candida albicans* rSaps. (A) Monocytes were treated with rSap1, rSap2, rSap3, and rSap6 (20  $\mu$ g/ml), with lipase of *M. furfur* (20  $\mu$ g/ml), with pepsin from porcine gastric mucosa, or with LPS (1  $\mu$ g/ml) or were left untreated and then were incubated for 18 h at 37°C. After incubation, supernatants were recovered and tested for the presence of TNF- $\alpha$ , IL-6, and IL-1 $\beta$ . (B) Monocytes were treated with different concentrations of rSaps or pepsin, ranging from 1 to 50  $\mu$ g/ml, or were left untreated and then were incubated for 18 h at 37°C. (C) Monocytes were treated with different rSaps (20  $\mu$ g/ml) and LPS (1  $\mu$ g/ml) in the presence or absence of 10  $\mu$ g/ml of polymyxin B. (D) Monocytes were treated with native Sap2, rSap2, and culture supernatant. Data are expressed as means  $\pm$  SD for three independent experiments. Statistical analysis was performed with an ANOVA test for multiple comparisons. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (rSap-treated cells versus untreated cells).

was not modified when LPS, whether autoclaved or not, was used at a dose of 1  $\mu$ g/ml (Fig. 2B). The dose of LPS was selected by testing TNF release after stimulation with various doses of LPS (0.001, 0.01, 0.1, 1, and 10  $\mu$ g/ml). The results showed that the best stimulation occurred at 1  $\mu$ g/ml and 10  $\mu$ g/ml (data not shown).

Receptors of the PAR family are expressed in phagocytic

cells, serve as sensors of serine proteases, e.g., thrombin, mast cell tryptase, trypsin, and cathepsin G, in these cells (40), and are considered key initiators of the inflammatory response. Since sensing via PARs initiates with a proteolytic digest, we tested the possible involvement of these receptors in the Sap-induced proinflammatory cytokine production. To this end, we blocked PAR1, PAR2, and PAR3, which are expressed mainly

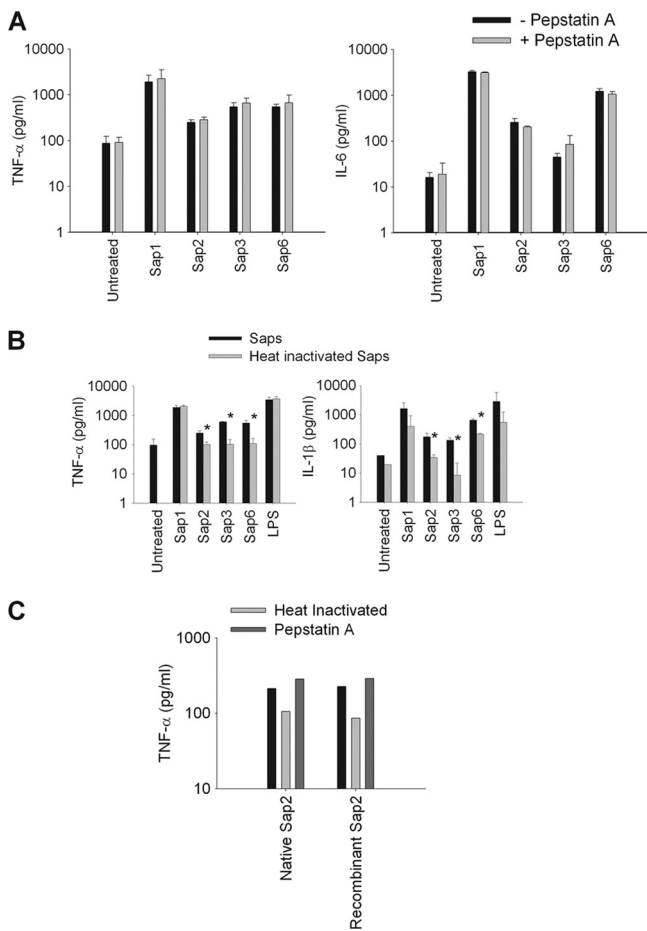


FIG. 2. Effects of heat inactivation of Saps, pepstatin A treatment, and anti-human PAR antibodies on cytokine production induced by Saps. Monocytes were treated with rSap1, rSap2, rSap3, and rSap6 (20  $\mu$ g/ml) in combination with pepstatin A (15  $\mu$ M) (A) or with heat-inactivated rSap1, rSap2, rSap3, rSap6, or LPS (1  $\mu$ g/ml) (B). (C) Effect of pepstatin A (15  $\mu$ M) or heat inactivation of native or recombinant Sap2 (20  $\mu$ g/ml) on TNF- $\alpha$  production. After incubation, supernatants were recovered and tested for the presence of TNF- $\alpha$ , IL-1 $\beta$ , or IL-6. Data are expressed as means  $\pm$  SD for three independent experiments. Statistical analysis was performed with an ANOVA test. \*,  $P < 0.05$  (heat-inactivated rSap-treated cells versus rSap-treated cells).

in monocytes (53), by using a mouse anti-human PAR1 antibody directed against the tethered ligand domain and mouse anti-human antibodies to PAR2 or PAR3, specific for the cleavage sites. The data obtained showed that the blockage of PAR1, PAR2, and PAR3 did not affect the production of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  induced by rSap1 (data not shown). The induction of cytokines by rSaps in the presence of the aspartic protease inhibitor pepstatin A implied a direct interaction of rSaps with monocytic cells. To verify a direct interaction of rSaps with human monocytes, the interaction of FITC-labeled rSaps with monocytes was assayed using flow cytometry. Sap1 and Sap2, which showed high-level stimulation of proinflammatory cytokines, were chosen for this experiment. The results shown in Fig. 3 indicate a dose-dependent association of rSap1 and rSap2 with monocytes. Clearly, the interaction of rSap2 with cells was greater than that of rSap1.

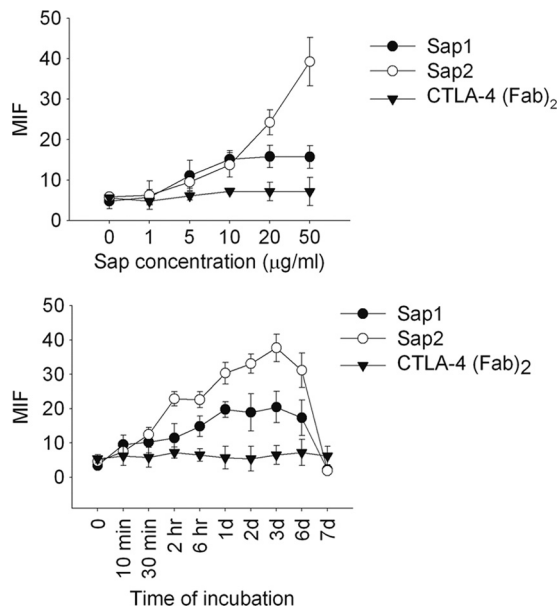


FIG. 3. Association of FITC-Saps with monocytes. Monocytes treated with different concentrations of FITC-labeled rSap1 or rSap2 or with FITC-conjugated CTLA-4 F(ab)<sub>2</sub> for different times were analyzed by flow cytometry to assess rSap binding. FITC-rSap or FITC-CTLA-4 F(ab)<sub>2</sub> binding by monocytes is expressed as the mean fluorescence intensity (MIF). Data are expressed as means  $\pm$  SD for three independent experiments.

Both Saps showed similar kinetics of association, reaching a plateau within 1 day and maintaining it for 4 days, after which the level declined, to become undetectable after 7 days of incubation. This could be due to degradation or extrusion of rSaps. Because the CTLA-4 receptor is not expressed on monocytes, FITC-conjugated CTLA-4 F(ab)<sub>2</sub> was used as a negative control. This protein was not found to be associated with the cells at any tested concentration (Fig. 3).

The PI3K-Akt network mediates intracellular signals to regulate a variety of cellular responses, including cellular activation, protein synthesis, cell cycling, and survival (6, 39). Given that PI3K is involved in the signal transduction pathway associated with cell activation (15), we considered the possibility that Sap-induced activation may be related to phosphatidylinositol phosphate (PIP3) recruitment. Since recent evidence indicates that PI3K-dependent activation of Akt is linked to an increase of PI3K activity (15), we evaluated Akt and I $\kappa$ B phosphorylation in cells stimulated with different rSaps. Monocytes treated with rSap1, rSap2, rSap3, or rSap6 for 1 h showed increased activation of pAkt compared to unstimulated cells (Fig. 4A).

Since Akt has been implicated in regulating the activation of proinflammatory cytokine genes (8, 11), we investigated whether rSap-mediated activation of proinflammatory cytokines in monocytes occurs via phosphorylation of I $\kappa$ B $\alpha$ , which would allow the translocation of NF- $\kappa$ B into the nucleus (23). After 4 h of coinubation of monocytes with rSap1, rSap2, rSap3, and rSap6, activation of I $\kappa$ B $\alpha$  was observed (Fig. 4B), and the latter was related to gene transcription of proinflammatory cytokines (26). As a negative control, pepsin was used

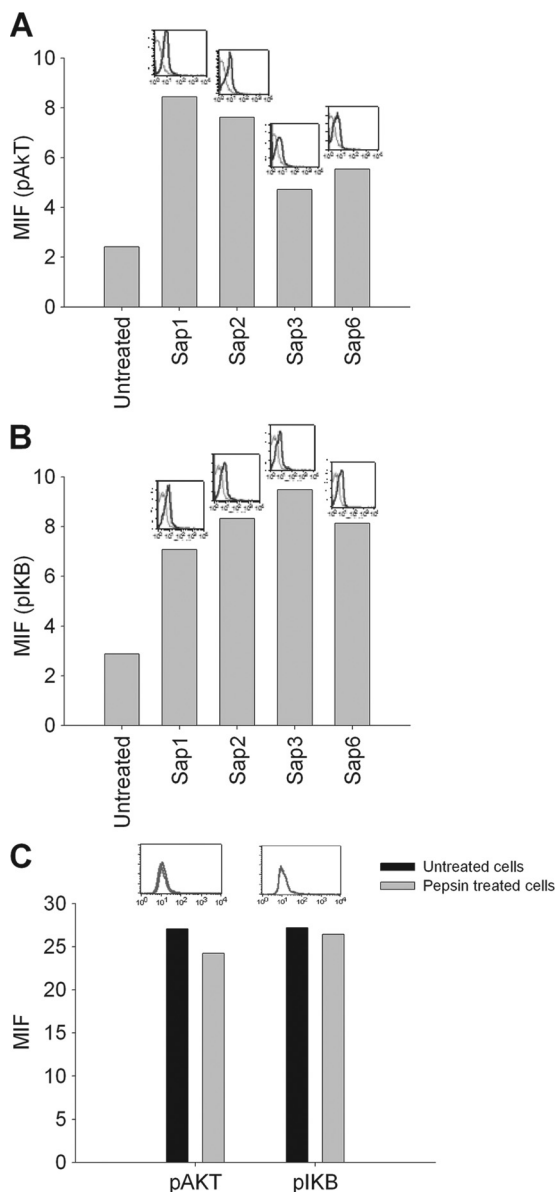


FIG. 4. pAkt and pIκB expression in monocytes treated with Saps. Monocytes were left untreated or were stimulated with rSap1, rSap2, rSap3, or Sap6 (20 μg/ml) (A and B) or with pepsin (20 μg/ml) (C) for 4 h. After incubation, cells were fixed in 3% paraformaldehyde, permeabilized in 0.1% saponin, and incubated with rabbit anti-human phospho-Akt or phospho-IκBα and with TRITC-conjugated goat anti-rabbit IgG. Fluorescent cells were analyzed by cytofluorimetric analysis and are expressed as the mean fluorescence intensity (MIF). Data are representative of three independent experiments with similar results.

in parallel experiments. This aspartic protease was found to be unable to affect Akt and IκB pathways (Fig. 4C).

Wortmannin is known to be a potent inhibitor of the PIP3-Akt pathway (38, 43), so we evaluated whether the treatment of monocytes coincubated with wortmannin was able to inhibit the secretion of proinflammatory cytokines. Indeed, 5 μM wortmannin drastically reduced IL-1, TNF-α, and IL-6 secretion in rSap-treated monocytes (Fig. 5).

Taken together, our results indicate that Saps stimulate cy-

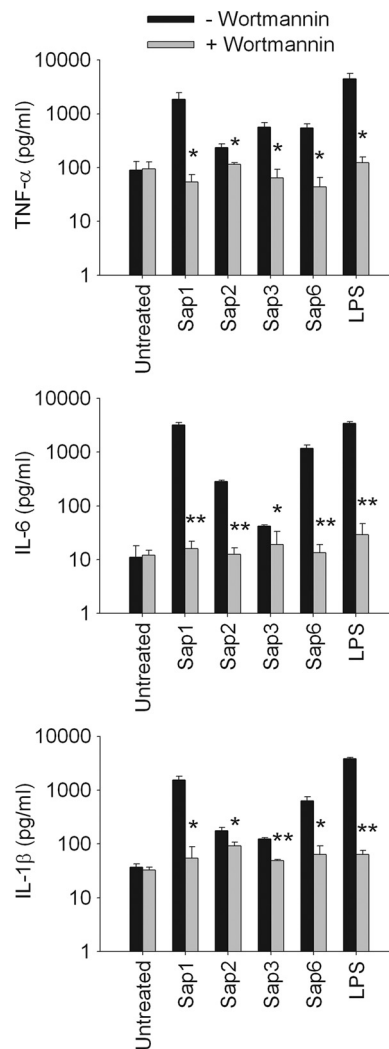


FIG. 5. Effect of Akt signaling inhibition on cytokine production induced by rSaps. Monocytes were left untreated or were pretreated with 5 μM wortmannin for 40 min and then treated with rSap1, rSap2, rSap3, or rSap6 (20 μg/ml) or with LPS (1 μg/ml) for 18 h at 37°C. After incubation, supernatants were recovered and tested for the presence of TNF-α, IL-6, and IL-1β. Data are expressed as means ± SD for three independent experiments. Statistical analysis was performed with an ANOVA test for multiple comparisons. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (rSap-plus-wortmannin-treated cells versus rSap-treated cells).

tokine production independently of their enzymatic activity and that signal transduction is mediated via Akt.

### DISCUSSION

*Candida* infections are a clinical problem of growing importance. The incidence of infections has grown dramatically over the past 2 to 3 decades, and it is very likely that this trend will continue in the years to come. *C. albicans* is the most common fungal pathogen and is the fourth leading cause of nosocomial infections (28, 45), with a high mortality rate for systemic infection (61). A variety of fungal virulence attributes are involved in the infection process, and some of these attributes are thought to have distinct roles during infection; for example,



Sap proteases have the potential to degrade tissue barriers during invasion, to inactivate host defense molecules, or to digest host proteins for nutrient supply (35). Many aspects of their activities have been studied, and it has been proposed that some particular Saps may be required and optimized for certain host niches and different stages of infection (36). Indeed, different members of the Sap family are known to be active at a broad range of pH values, ranging from pH 2.0 to 6.5; therefore, *C. albicans* provides proteases for a broad range of host niches (50).

Here we report that different Saps show differing abilities to induce secretion of proinflammatory cytokines by human monocytes. In particular, Sap1, Sap2, and Sap6 strongly induced upregulation of IL-1 $\beta$ , TNF- $\alpha$ , and IL-6, while Sap3 was able to stimulate the secretion of IL-1 $\beta$  and TNF- $\alpha$ . The treatment of Saps with pepstatin A did not diminish their ability to induce cytokine secretion, and consequently, the stimulatory effect of Saps was independent from their proteolytic activity. Sap-induced inflammatory cytokine production was also independent from PAR activation and from the optimal pH for Sap activity, confirming that cytokine stimulation does not correlate with activity. Stimulation of cytokine production by monocytes seems to be regulated via NF- $\kappa$ B, since Saps induced Akt activation and phosphorylation of I $\kappa$ B $\alpha$ , which initiates translocation of NF- $\kappa$ B into the nucleus (63).

Previous studies showed that Saps are important virulence factors of *Candida albicans* during mucosal and disseminated infections and may also contribute to the induction of an inflammatory host immune response (50). In particular, it has been shown that mutants lacking either SAP1 or SAP2 caused reduced tissue damage in a model of vaginal infections using reconstituted human vaginal epithelium (RHVE) and had a significantly reduced potential to stimulate cytokine expression (49). In contrast, mutants lacking *SAP4* to *SAP6* induced similar levels of cytokines to those in the wild-type strain. Addition of the aspartic protease inhibitor pepstatin A strongly reduced the cytokine response evaluated in the RHVE model (49, 51). In our study, we documented a strong ability of Sap1, Sap2, and Sap6 to induce inflammatory cytokines, and these data seem to be consistent with the low capacity to induce inflammation of Sap null mutants lacking either *SAP1* or *SAP2* (51). However, in contrast with previous results (44), we were unable to mitigate the cytokine production by using pepstatin. There are at least two different explanations for these differences. First, the reduced induction of inflammation of Sap null mutants was associated with reduced tissue damage, which very likely also influenced the level of cytokine expression. We therefore propose that the previous induction of inflammation was induced primarily by fungal cells and their activity. Second, the previous study was based on epithelial cells only, while in this study human primary monocytes were analyzed.

Therefore, we report in this study, for the first time, that Saps are potent inducers of inflammatory cytokines, independent of their proteolytic activity. This view was supported by the observation that cytokine induction was independent from pH values necessary for optimal Sap activity. We therefore concluded that the antigenic structure of Saps caused the cytokine induction, a phenomenon previously observed for other virulence factors of *C. albicans*, such as the mannoprotein MP65, a well-known adhesin (42, 48). This conclusion is sup-

ported by the fact that heat denaturation of Sap proteins, and thus destruction of the antigenic structure, greatly mitigated proinflammatory cytokine release. However, the stimulatory activity of Sap1 appeared to be unaffected by heat treatment. This suggests that the stimulatory antigenic components of Sap2, Sap3, and Sap6 are heat labile, while these structures are heat stable in Sap1. Such immunogenic stability is consistent with earlier research indicating that the recognition of albumin, one of the most widely studied proteins, is only partially correlated with its native three-dimensional structure. Indeed, heat treatment and chemical denaturation (SDS treatment) of albumin are not able to significantly decrease its capability to bind to specific antibodies (44). Indeed, in our experimental system, Sap1 was the best stimulator of proinflammatory cytokine production. A possible explanation could be related to the different associations of rSaps with human cells, which may be crucial for inducing different levels of immunoregulation.

Our data indicate that Saps either bind to or are taken up by monocytes. This interaction of monocytes with rSaps was optimal within 1 to 4 days of incubation. After that time, the cells lost their capacity to bind to or take up Saps, suggesting either that Saps were degraded or extruded from cells after 7 days or that monocytes ceased their activity after day 4.

Given that the effects obtained with rSap2 were very similar to those obtained with native Sap2 and a culture supernatant from a *C. albicans* strain that contains a high level of Sap2, it seems feasible that these effects may also occur *in vivo*. Moreover, even though we do not know the exact concentrations of Saps secreted during infection, it is likely that high concentrations can be reached locally.

Serine proteases such as chymotrypsin and trypsin are released from host cells during inflammation and were originally thought to be primarily responsible for protein degradation. Different serine proteases differ in substrate specificity. For example, chymotrypsin prefers an aromatic side chain on the residue whose carbonyl carbon is part of the peptide bond to be cleaved. Trypsin prefers a positively charged Lys or Arg residue at this position. Recently, however, it was discovered that these proteases also function as signaling molecules through the activation of specialized G-protein-coupled receptors called PARs. So far, four PARs have been identified (PAR1 to -4) and have been detected in numerous cell types, including cells actively involved in the inflammatory response, such as neutrophils, and macrophages. PARs have a unique mechanism of receptor activation, since they all contain a serine protease cleavage site within the extracellular N terminus. Cleavage of this site results in the unmasking of a tethered ligand sequence which can then bind to and activate the receptor. The inflammatory activity induced by Saps was independent from PAR activation and, in particular, from PAR1, PAR2, and PAR3. These results support and extend the evidence given in a previous study in which PAR antagonists were unable to reverse the potentiation of cytokine production induced by soluble factors released by *C. albicans* (7).

The PI3K-Akt network mediates intracellular signals, which implies a variety of cellular responses, including cellular activation (6, 39). Given that PI3K is involved in the signal transduction pathway associated with cell activation (15), we considered the possibility that Sap-mediated activation might imply PI3K recruitment. Recent evidence indicates that PI3K-

dependent activation of Akt is synonymous with increased PI3K activity (15). Indeed, the activation of Sap-induced Akt implies that the signal triggered by Saps is via activation of PIP3, which binds to and activates the phosphoinositide-dependent protein kinase 1. This, in turn, phosphorylates and activates the downstream target Akt, which can then activate the NF- $\kappa$ B pathway. Therefore, Saps trigger the Akt/NF- $\kappa$ B pathway, which is known to regulate the gene transcription of inflammatory cytokines. This assumption was corroborated by the capacity of wortmannin, a PIP3-Akt pathway inhibitor, to completely block the Sap-induced production of cytokines such as IL-1 $\beta$ , TNF, and IL-6.

Taken together, these results reveal that the Sap-induced inflammatory response is independent from both enzymatic activity and PAR activation. In addition, Sap-induced inflammation is dependent on the antigenic structure and is triggered by the Akt/NF- $\kappa$ B activation pathway. This newly discovered immunoregulatory aspect sheds light on *C. albicans* virulence traits.

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