MAJOR ARTICLE

# *Candida albicans* Biofilms Do Not Trigger Reactive Oxygen Species and Evade Neutrophil Killing

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**Neutrophils are found within *Candida albicans* biofilms in vivo and could play a crucial role in clearing the pathogen from biofilms forming on catheters and mucosal surfaces. Our goal was to compare the antimicrobial activity of neutrophils against developing and mature *C. albicans* biofilms and identify biofilm-specific properties mediating resistance to immune cells. Antibiofilm activity was measured with the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)2H-tetrazolium-5-carboxanilide assay and a molecular *Candida* viability assay. Reactive oxygen species generation was assessed by measuring fluorescence of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester in preloaded neutrophils. We found that mature biofilms were resistant to leukocytic killing and did not trigger reactive oxygen species, even though neutrophils retained their viability and functional activation potential. Beta-glucans found in the extracellular matrix negatively affected antibiofilm activities. We conclude that these polymers act as a decoy mechanism to prevent neutrophil activation and that this represents an important innate immune evasion mechanism of *C. albicans* biofilms.**

A prominent feature of biofilm infections is infiltration of adjacent tissues by neutrophils [1, 2]. Neutrophils swarm into the biofilm mass in mucosal surface biofilms formed by *Candida albicans* [3]; however, their activation state within biofilms is unknown. Functional activation within bacterial biofilms varies, with active phagocytosis detected within biofilms of certain bacterial species but not others [1, 4, 5]. Currently there is a paucity of information on the functional status of immune cells, including neutrophils, interacting with *Candida* biofilms in vivo or in vitro, and the fungal biofilm factors affecting antimicrobial effector functions are completely unknown.

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Bacterial biofilm resistance to immune cell attack is well studied [1, 5–8]. A few studies thus far have examined the interactions of phagocytic cells with *Candida* biofilms [9–12]. In early biofilms, mononuclear cells enhance biofilm thickness [9], whereas the interaction between mature biofilms and monocytes or polymorphonuclear cells results in reduced antifungal activity compared with planktonic cells [10]. No study to date has compared developing and/or early vs mature biofilms in terms of their interactions with polymorphonuclear cells, and, although mature biofilms were found to be resistant to phagocytic killing [10–12], there is currently no understanding of the mechanism (s) that render fungal cells within biofilms resistant.

In this study, we sought to systematically characterize the antimicrobial activity of neutrophils against developing or mature *C. albicans* biofilms and determine the biofilm-specific properties that mediate resistance. We hypothesized that, relative to early biofilms, mature biofilms trigger a lower oxidative response by neutrophils and that this is associated with reduced susceptibility to neutrophil-inflicted damage. We also

designed experiments to test the hypothesis that biofilm structure and the abundance of β-glucans in the extracellular matrix may be responsible for the reduced functional activation of neutrophils.

## METHODS

### *Candida albicans* Planktonic and Biofilm Growth

Strain SC5314 [13] and its green fluorescent protein–tagged derivative (strain MRL51, kindly provided by Dr Aaron Mitchell, Carnegie Mellon University) were used in all experiments. Strains were maintained on yeast extract peptone dextrose agar. Yeast were grown overnight in yeast extract peptone dextrose broth supplemented with 2% dextrose at 24° C. Biofilms were grown in flat bottom 96-well plates ($1 \times 10^5$ cells per well) in Roswell Park Memorial Institute 1640media (RPMI 1640) with 10% fetal bovine serum and incubated at 37°C in 5% carbon dioxide for up to 48 hours.

In preliminary experiments, 3-, 24-, or 48-hour biofilm organisms were mechanically detached from the wells, sonicated at 20% amplitude for 10 minutes, and counted using a hemocytometer. Each filamentous organism was counted as 1 multicell unit, and the cell unit ratio in 3-hour:24-hour:48-hour biofilms was 1:24:35. Ratios were confirmed with the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)2H-tetrazolium-5-carboxanilide (XTT) assay. Effector concentrations were adjusted accordingly, to reflect similar effector to target (E:T) ratios in early (3-hour) and late (24-hour, 48-hour) biofilms. To disrupt biofilm architecture in some experiments, biofilms were treated by sonication as above and washed 3 times in phosphate-buffered saline. This sonication triggers <10% loss of viability and removes some of the soluble matrix [14, 15].

Planktonic cultures were grown in glass flasks on a shaker under otherwise identical growth conditions to biofilms. Cells grown in this manner formed increasingly larger aggregates of yeasts and filaments in suspension. In preliminary work, aggregates were disrupted by sonication and quantified as above to normalize E:T ratios between planktonic and biofilm cultures. Three-hour planktonic cultures did not differ from 3-hour biofilms, whereas 24-hour planktonic cultures contained 1.7-fold more cell units than 24-hour biofilm cultures, and 48-hour planktonic cultures contained 2-fold more cell units than 48-hour biofilm cultures.

To test the effect of mature biofilm-secreted factors on leukocytic killing, biofilms were grown for 48 hours as above and supernatants were collected, filter sterilized, and stored at −80°C prior to use.

### Leukocytes

Neutrophils were isolated from anticoagulated blood of healthy donors following informed consent (institutional review board 02-288-2) using dextran T-500 (Sigma-Aldrich) sedimentation followed by density gradient centrifugation [16]. To address donor–donor variability, which often hinders statistical significance in neutrophil experiments [17], when possible, experiments were run in triplicate (ie, on 3 different days) with cells from a single donor. When a donor was not available for repeated blood donations, the experiment was repeated with cells from different donors. In some experiments, a neutrophil-like cell line (HL-60 cells, American Type Culture Collection [ATCC]) was used to assess anti-*Candida* activity [16]. These cells were driven to granulocyte differentiation and are an excellent model for the study of neutrophil–*C. albicans* interactions [16, 17].

### Leukocyte Viability Assays

To assess leukocyte viability microscopically, differentiated HL-60 cells were labeled with CellTracker Orange (Molecular Probes) [1, 18]. A Live/Dead Viability/Cytotoxicity assay (Molecular Probes) was used to quantify viability after 3 hours or 24 hours of incubation with mature biofilms using a fluorescence scanner. Results were expressed as percentage viability (ie, fluorescence units in the presence of biofilm divided by fluorescence in the absence of biofilm × 100).

### Assessment of Antibiofilm Activity of Leukocytes

Susceptibility of biofilms to HL-60 cells or to human peripheral blood neutrophils was determined by a modified XTT assay, which measures residual metabolic activity in biofilms after exposure to leukocytes [16]. Leukocytes were added to *C. albicans* at E:T ratios ranging from 1:1 to 15:1. Antifungal activity was calculated according to the following formula: % fungal damage = $(1-x/n)*100$, where $x$ is the absorbance of experimental wells (*C. albicans* with effectors) and $n$ is the absorbance of control wells (*C. albicans* only).

To confirm biofilm damage by immune effectors, a molecular viability assay was also used in some experiments [19]. Briefly, after lysis of effectors with diethylpyrocarbonate (DEPC)-treated sterile water, total RNA from biofilms was isolated using the RiboPure yeast kit (Ambion), reversed transcribed, and amplified using primer sequences for the *C. albicans* translation elongation factor-1β (*EFB1)* gene. Transcript numbers were quantified based on standard curves generated using a known amount of a plasmid containing the EFB1 sequence, using the iCycler iQ real-time polymerase chain reaction detection system and software (Bio-Rad). Antibiofilm activity was calculated according to the following formula: % fungal damage (or percentage reduction in *EFB1* transcript number) = $(1-x/n)*100$, where $x$ is the *EFB1* transcript copy number of experimental wells (*C. albicans* with effectors) and $n$ is the *EFB1* transcript copy number of control wells (*C. albicans* only).

### Reactive Oxygen Species (ROS) Assay

To assess intracellular ROS generation, freshly isolated neutrophils were preloaded with CM-H2DCFDA, (8 μM, Molecular Probes), washed with Hanks Balanced Salt Solution (HBSS) without calcium/magnesium, and resuspended in RPMI 1640 with 10% fetal bovine serum at 37°C for 15–30 minutes. Biofilm media were removed, and neutrophils were added to plates. Formyl-methionyl-leucil-phenylalanine (fMLP) or phorbol myristoyl acetate (PMA) was used to trigger an ROS response in the presence or absence of biofilms (1 and 0.1 μM, respectively; Sigma). Reactive oxygen species were quantified using a fluorescence reader. After correcting for background, results were calculated by subtracting fluorescence at time 0 from that at 1 hour and were expressed as relative fluorescence units. When different donors were used, results were expressed as ROS-fold over basal (ie, as the ratio of fluorescence in the presence and/or absence of stimulus) to adjust for variable baseline activation.

### Biofilm Matrix Experiments

Biofilm matrix was isolated from organisms growing in polystyrene flasks for 48 hours at 37°C, according to previously published protocols [14, 15, 20]. Briefly, biofilms were collected with a cell scraper and sonicated at 30% amplitude for 5 minutes, followed by centrifugation at $12\,000 \times g$ for 20 minutes. Supernatants were carefully removed and transferred to a new tube, and this process was repeated twice. Extracts of planktonic cells (negative control) were prepared using a roughly equivalent biomass to biofilms. Prior to use in experiments, extracts (prepared in sterile water or RPMI 1640) were concentrated 6-fold and plated on Sabouraud dextrose agar to ensure absence of growth. DNA content in extracts was assessed using a fluorescence DNA quantitation assay (Bio-Rad), whereas the Glucatell kit was used to quantify (1→3)-β-D-glucans (Associates of Cape Cod).

To visualize matrix polysaccharides, strain MRL51 was incubated with biofilm or planktonic extracts for 30 minutes and washed with phosphate-buffered saline. Organisms were subsequently stained with either ConA-Alexa Fluor 633 or a monoclonal antibody highly specific for (1→6) branched, (1→3)-β-D-glucans (BFDiv, Biothera), followed by a Cy-3-conjugated secondary antibody. Mouse immunoglobulin M was used as an isotype control [3]. Cells were washed and observed with a fluorescence (Imager M1, Carl Zeiss MicroImaging) or confocal microscope (Zeiss LSM 510 NLO/FSM).

To assess the effect of matrix on fMLP-triggered ROS generation, CM-H2DCFDA–labelled polymorphonuclear (PMN) cells were exposed to 1 μM fMLP (100 μL) in the presence or absence of extract prepared in RPMI 1640 (100 μL) for 1 hour, and ROS were measured as above. To test whether matrix extract from 48-hour biofilms influences ROS activation, yeast cells were seeded in 96-well plates ($10^5$ cells/well, in RPMI 1640 with 10% fetal bovine serum) and incubated at 37°C for 3 hours. Subsequently, media were discarded and the cells were coated with matrix extracts for 30 minutes at 37°C. Extracts were discarded from the wells and CM-H2DCFDA–labelled PMN were added. In some experiments, extracts were pretreated with glucanase or DNAse I (both at 200 μg/mL; Sigma) for 30 minutes prior to coating, whereas in others, in lieu of extracts, 3-hour biofilms were coated with laminarin (150 μg/mL; Sigma).

To test the effect of specific matrix components on leukocytic killing, mature biofilms were treated with glucanase (4.5 U/mL) [15], heat-inactivated glucanase (negative control), alpha mannosidase (4.5 U/mL; from Jack beans, Sigma), or DNAse I (353 U/mL) [21] for 30 minutes and washed with phosphate-buffered saline before leukocytes were added. The effects of glucanase treatment on Con-A- and BFDiv-reactive polysaccharides in biofilms were examined by staining, as described above. Preliminary experiments showed that enzymatic treatment had a negligible effect on cell viability (data not shown).

## RESULTS

### Biofilms Are Resistant to Leukocyte Killing

Mature *Candida* biofilms are resistant to several pharmacological agents [22–26]; we thus hypothesized that they are also resistant to killing by innate immune effector cells. To test this hypothesis, we compared the susceptibility of 3-hour, 24-hour, and 48-hour biofilms to leukocytes. Twenty-four-hour and 48-hour biofilms were remarkably resistant to HL-60 cells, with overall reduction in biofilm metabolic activity of <30%. In comparison, 3-hour biofilms lost >80% of their metabolic activity at the higher E:T ratio tested. Resistance increased as biofilms matured, and results were similar regardless of the number of effectors added or the assay used to determine biofilm damage (Figure 1*A* and *B*). Planktonic cultures also became more resistant to leukocytes over time because these growth conditions favored formation of increasingly large cell aggregates. However, when compared with planktonic cultures, biofilm cultures were more resistant to leukocytes at all growth times and E:T ratios tested (Figure 1). Similar results were obtained with peripheral blood neutrophils (not shown). These findings support the hypothesis that the biofilm growth state confers increased resistance to leukocyte killing.

### Biofilms Fail to Trigger an ROS Response but Do Not Compromise Leukocyte Viability or Function

Because ROS are responsible for a large proportion of neutrophil anti-*Candida* activity [27, 28], we hypothesized that mature biofilms fail to trigger an effective ROS response in

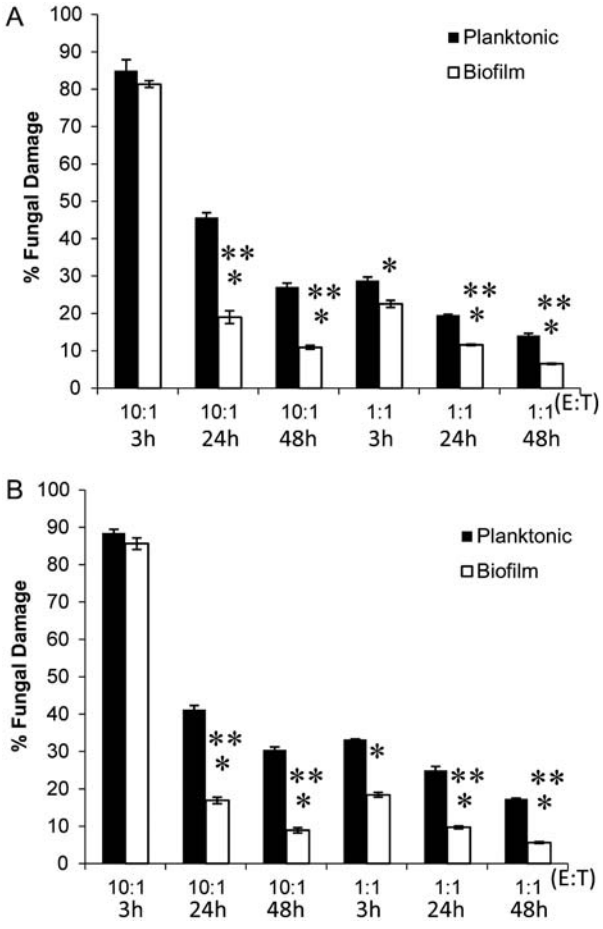


**Figure 1.** *Candida albicans* mature biofilms are resistant to leukocytic killing compared with early biofilms and planktonic cells. Killing was assessed by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide (XTT) assay (*A*) or a real-time reverse-transcription polymerase chain reaction assay (*B*). Planktonic cultures, early (3-hour) biofilms, and mature (24-hour, 48-hour) biofilms were tested at 2 effector-to-target (E:T) ratios (10:1, 1:1). Results from triplicate experiments with HL-60 cells are shown/ $^{**}P < .005$ compared with 3-hour biofilm and $^{*}P < .05$ for a *t*-test comparison between planktonic cultures and mature biofilms.

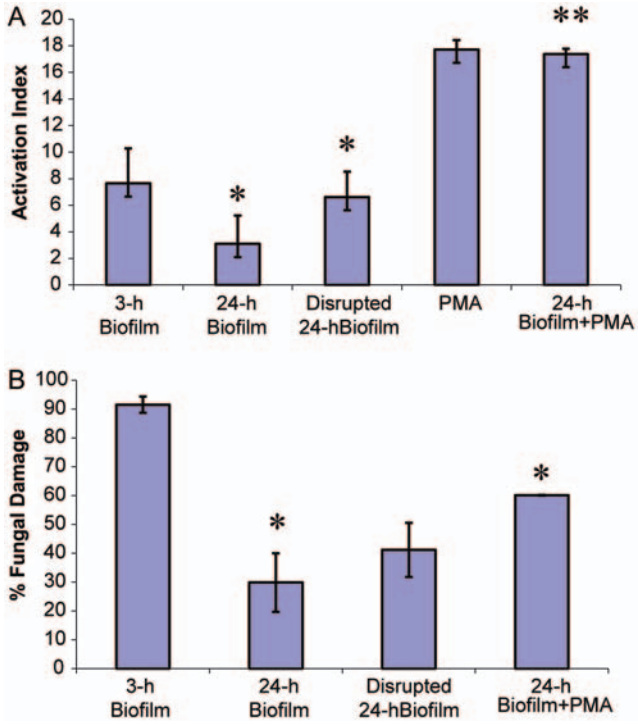


**Figure 2.** Biofilms fail to trigger a reactive oxygen species (ROS) response but do not compromise leukocyte function. *A*, The ROS production was measured after 1 hour of incubation of neutrophils with early (3-hour) biofilms, 24-hour biofilms, phorbol myristoyl acetate (PMA) (0.1 μM), or 24- hour biofilms plus PMA (0.1 μM). $^{*}P < .05$ for *t*-test comparisons between 3-hour biofilms and 24-hour biofilms and between 24-hour biofilms and disrupted 24-hour biofilms. $^{**}P < .005$ for a *t*-test comparison with 24-hour biofilms. *B*, Three-hour biofilms , 24-hour biofilms, 24-hour biofilms plus PMA (0.1 μM), or disrupted 24-hour biofilms were exposed to neutrophils at a 5:1 E:T ratio, and fungal damage was assessed by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide (XTT) assay. $^{*}P < .05$ for *t*-test comparisons between 3-hour biofilms and 24-hour biofilms and between 24-hour biofilms and 24-hour biofilms plus PMA. Results represent means and standard deviations of triplicate experiments with neutrophils from 2 human donors.

neutrophils. Consistent with this hypothesis, we discovered that 24-hour biofilms did not activate a robust oxidative response, in sharp contrast witb 3-hour biofilm organisms (Figure 2*A*).

We then questioned whether the late biofilm environment is toxic to leukocytes, which would explain their reduced function. To explore this possibility, we compared the viability of immune effectors during their interaction with 3-hour or 24-hour biofilms. After 3 hours of interaction with 3-hour or 24-hour biofilms, leukocytes retained their viability and interacted closely with biofilm cells (Figure 3*A*). Using a quantitative assay, we confirmed that leukocytes retain their viability within the late biofilm environment after 3 hours of interaction (Figure 3*B*). Because our ROS and killing assays are 1 hour and 3 hours in duration, respectively, this finding argues against the possibility that loss of viability is responsible for lower leukocyte activation. However, as expected [17], prolonged (24 hours) interaction with late biofilms significantly ($P < .005$) compromised the viability of these cells (Figure 3*B*).

The 24-hour biofilm environment did not suppress ROS generation in response to fMLP (data not shown) or PMA (Figure 2*A*), consistent with the idea that neutrophils retain their functional activation potential. Importantly, PMA-triggered ROS generation was followed by significant biofilm damage ($P < .05$, Figure 2*B*). Collectively, these data show the oxidative response is not activated by mature biofilms and this is associated with reduced killing.

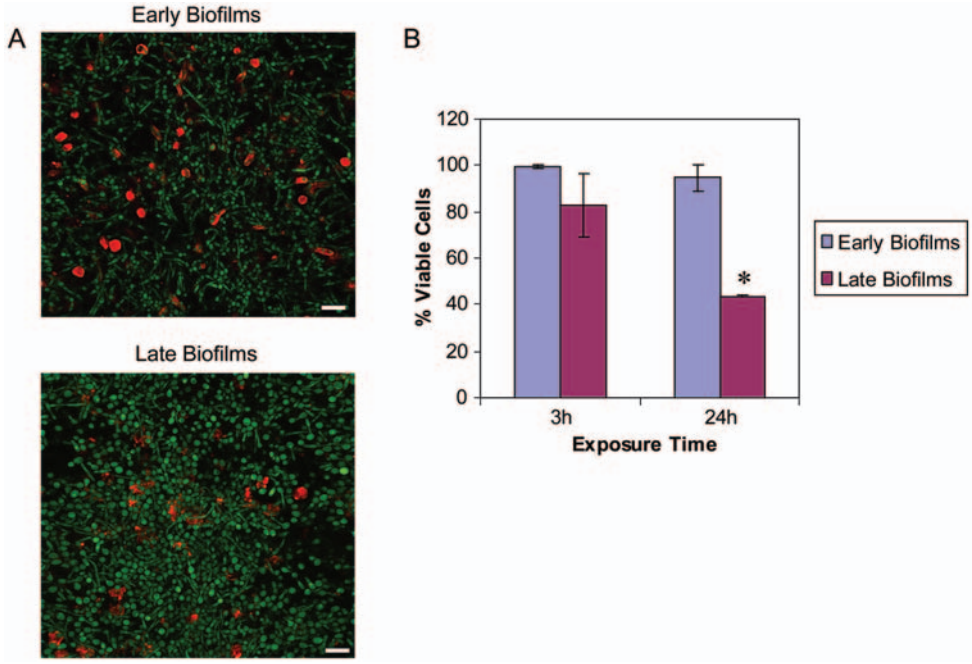


**Figure 3.** Leukocytic cells retain their viability after interacting with biofilms for up to 3 hours. *A,* Live HL-60 cells (red) labeled with Cell Tracker Orange after 3 hours of contact with early (3-hour) or late (24-hour) biofilms. Bars = 50 μm. *B.* Quantification of viability of HL-60 cells after 3 hours and 24 hours of contact with early (3-hour) and late (48-hour) biofilms using a Live/Dead cytotoxicity assay. Results represent means and standard deviations of triplicate experiments. **P* < .005 for a *t*-test comparison between early and late biofilms.

### Biofilm Structure and Extracellular Matrix, and Not a Biofilm-Secreted Factor, Play a Role in Biofilm Resistance

Bacterial biofilm-secreted products can modulate neutrophil antimicrobial activities [29, 30]. Thus, to further explore the mechanism(s) of reduced antibiofilm activity of immune effectors, we hypothesized that mature biofilms secrete a soluble factor(s) that inhibits neutrophil killing. To test this hypothesis, supernatants from 48-hour biofilms were added to 3-hour biofilms, and leukocyte-inflicted damage was assessed. Contrary to our hypothesis, 48-hour biofilm supernatants did not inhibit neutrophil killing but increased the ability of neutrophils to damage early biofilms by 15%–30% (Figure 4).

We then questioned whether biofilm architecture and the presence of extracellular matrix may be responsible for the reduced antibiofilm effector function. To begin to test this hypothesis, we subjected 24-hour biofilms to gentle sonication to disperse biofilm cells and washed the cells to remove some of the soluble extracellular matrix [14, 15]. We then tested the dispersed biofilm ROS generation potential as well as their susceptibility to killing. Dispersed 24-hour biofilms triggered an oxidative response comparable with that of 3-hour biofilms (Figure 2*A*), arguing that the biofilm architecture is contributing to the inability of mature biofilms to trigger a robust ROS

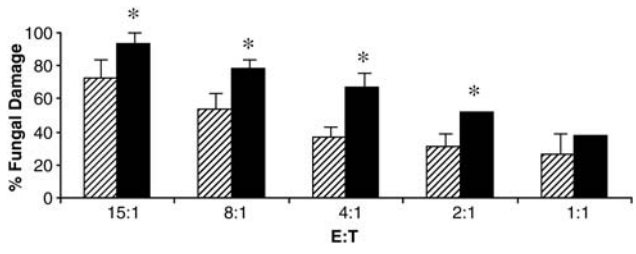


**Figure 4.** Mature biofilm supernatants do not inhibit killing of early biofilms. Early (3-hour) biofilms were exposed to HL-60 cells in the presence (dark bars) or absence (light bars) of supernatants from late biofilms at 5 different effector-to-target (E:T) ratios. Forty-eight-hour biofilm supernatants were added at 1:2 dilution. Killing of early biofilms was assessed by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide (XTT) assay, and results represent means and standard deviations of triplicate experiments. **P* < .05 for a *t*-test comparison between the presence and absence of supernatants.

**Table 1. Beta Glucan and DNA Content of 3-Hour and 48-Hour Biofilm Extracts and Overnight Planktonic Culture Controls**

| | 3-Hour Biofilm Extract | 48-Hour Biofilm Extract | Planktonic Cell Extract (control) |
|---|---|---|---|
| DNA, μg/mL | 0.00 ± 0.00 | 2.86 ± 0.17* | 0.61 ± 0.10 |
| Glucan, ng/mL | 0.04 ± 0.004 | 0.12 ± 0.002** | 0.05 ± 0.006 |

**P* < .00001 and ***P* < .005 for a *t*-test comparison between early and mature biofilms.

response. Dispersed biofilms were also more susceptible to neutrophil damage than intact biofilms, thus linking the ROS response to killing (Figure 2*B*).

To further assess the effect of biofilm matrix on neutrophil activation, we extracted matrix from biofilms and analyzed its content. The 48-hour biofilm matrix contained significantly higher amounts of β-glucan ($P < .005$) and DNA ($P < .00001$) than 3-hour biofilm or planktonic extracts (Table 1), consistent with prior reports [15, 21, 31, 32]. Staining the biofilm matrix-coated organisms with the BFDiv antibody confirmed the presence of (1→3)-β-D-glucans (Figure 5*A*). ConA-Alexa Fluor 633 stained these cells yellow with a red halo, suggesting the presence of polymers with α-linked mannose residues [33, 34]). Neither BFDiv nor ConA reactivity were altered by planktonic culture extracts, suggesting they were devoid of high amounts of polysaccharides (Figure 5*A*).

Biofilm matrix and planktonic cell extracts were subsequently tested for their effect on leukocytes. When added to neutrophils, biofilm matrix alone did not trigger an oxidative response and did not affect fMLP-triggered activation (not shown), in line with our previous finding that the matrix-rich late biofilm environment does not compromise ROS

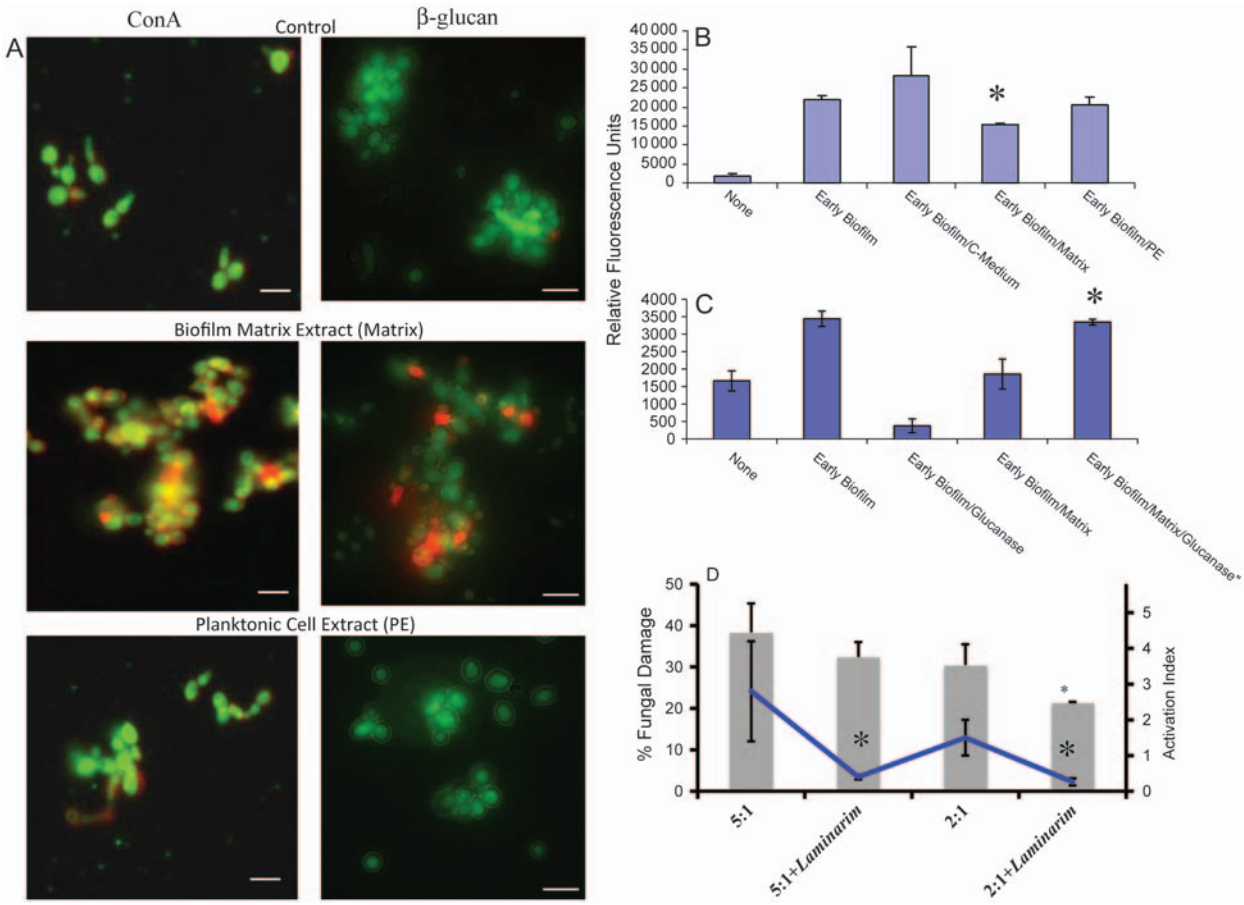


**Figure 5.** The effects of the biofilm matrix on neutrophil activation are β-glucan-dependent. *A*, Matrix from 48-hour biofilms coating green fluorescent protein (GFP)–expressing yeast organisms. To visualize presence of extracellular polysaccharides, a GFP-tagged *Candida albicans* strain (green) was coated with water (control), extracted biofilm matrix (in water), or negative control extract from planktonic cultures (in water). Yeast was stained with ConA-Alexa Fluor 633 or an anti-β-glucan antibody (red). Bars = 10 μm. *B* and *C*, Biofilm matrix extract reduces reactive oxygen species (ROS) activation in response to early biofilms. *B*, Matrix was extracted in Roswell Park Memorial Institute 1640 medium (RPMI 1640), concentrated 6-fold and used to coat 3-hour biofilms (Early Biofilm/Matrix). Concentrated RPMI 1640 (C-Medium) or planktonic extract (PE) was used as negative controls. Results shown are based on triplicate runs with neutrophils from a single human donor. *$P < .05$ for a *t*-test comparison with early biofilms. *C*, Three-hour biofilms were treated with glucanase (Early Biofilm/Glucanase) or coated with glucanase-treated matrix extract (Early Biofilm/Matrix/Glucanase), and ROS production was measured. Controls included heat-inactivated glucanase and DNAse I–treated matrix (not shown). Results shown are based on triplicate runs with HL-60 cells *$P < .05$ for a *t*-test comparison with early biofilms with matrix. *D*, Laminarin inhibits ROS activation and killing of early biofilms by HL-60 cells. Laminarin (150 μg/mL) was used to coat 3-hour biofilms, and HL-60 cells were added at 5:1 or 2:1 effector-to-target ratios. Early biofilm damage (bars) and ROS stimulation (lines) were measured after 3 hours and 1 hour, respectively. The ROS inhibition by laminarin was associated with a reduction in killing. $P < .05$ for a *t*-test comparison between the presence and absence of laminarin in each effector-to-target ratio tested.

generation in response to PMA (Figure 2*A*). However, when 3-hour biofilms were coated with biofilm matrix, but not planktonic cell extract, the oxidative burst activity of neutrophils was significantly ($P < .05$) attenuated (Figure 5*B* and *C*).

To explore the role of specific matrix components in the inhibition of ROS, we pretreated the extracted matrix with glucanase, heat-inactivated glucanase, or DNAse prior to coating the 3-hour biofilms. Glucanase treatment completely abrogated the matrix ROS-attenuating effect (Figure 5*C*), in contrast with heat-inactivated glucanase and DNAse (data not shown). These results suggest that β-glucans, present in the biofilm matrix, were responsible for attenuating the ROS response. To further prove this point, we coated early biofilms with a soluble β-glucan (laminarin) at a similar concentration as in the 48-hour matrix extract, and its effect on neutrophil function was examined. As expected, laminarin attenuated both ROS and killing of early biofilms, further implicating soluble β-glucans in these processes (Figure 5*D*).

Next, 48-hour biofilms were treated with glucanase, alpha mannosidase, or DNAse to test whether this would render them more susceptible to leukocytic killing. As predicted, only glucanase treatment significantly ($P < .05$) enhanced killing of 48-hour biofilms (Figure 6*D*). Glucanase treatment did not alter the ConA reactivity of the organisms (Figure 6*A* and *B*), but reduced biofilm hyphal reactivity to the BFDiv antibody (Figure 6*C*), suggesting that its effect on biofilm susceptibility is mediated by dissolution of glucans. Taken together, these results indicate that β-glucans in the biofilm matrix hinder neutrophil effector function.

## DISCUSSION

When *Candida* grows in the biofilm state, it displays phenotypic properties that are different from those in planktonic growth, such as enhanced resistance to antifungals [23, 25] and altered gene expression [35, 36]. We hypothesized that

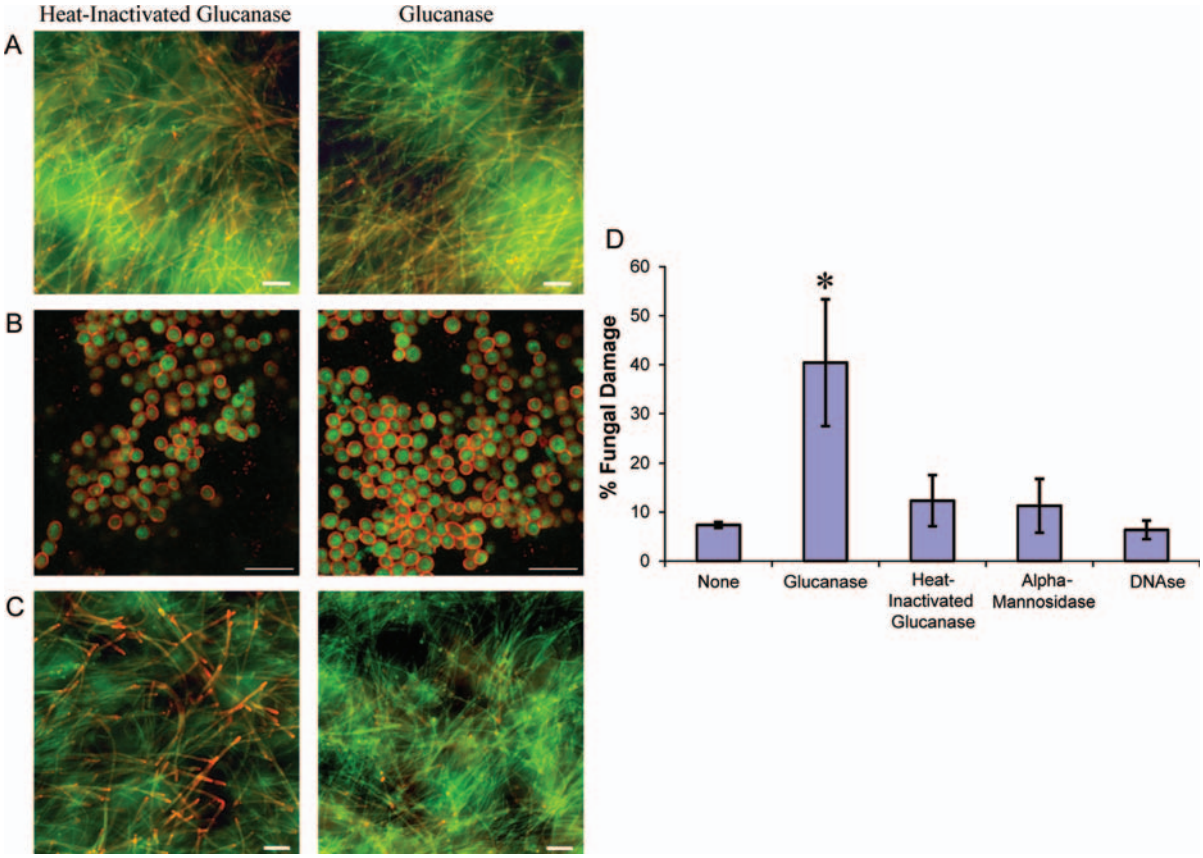


**Figure 6.** Glucanase treatment depletes biofilms of β-glucans and increases their susceptibility to killing. *A* and *B*, Effect of glucanase or heat-inactivated glucanase treatment on biofilm cell ConA-reactivity. Biofilms of a green fluorescent protein (GFP)–tagged *Candida albicans* strain were grown for 48 hours. Cultures were stained with ConA-Alexa Fluor 633 (red). Biofilms were examined using a fluorescence microscope (*A*). Basal yeast cell layer of 48-hour biofilms as seen by confocal microscopy (*B*). *C*, Effect of glucanase treatment on biofilm β-glucans. Biofilms of a GFP-tagged *C. albicans* strain were grown for 48 hours and stained with an anti-β-glucan antibody, followed by a Cy-3-conjugated secondary antibody (red). Bars = 20 μm. *D*, Glucanase treatment of biofilms increases their susceptibility to killing. Forty-eight-hour biofilms were treated with glucanase (4.5 U/mL), heat-inactivated glucanase, alpha-mannosidase (4.5 U/mL), or DNAse (353 U/mL) for 30 minutes, and HL-60 cells were added ($10^5$ cells/well) for 3 hours, followed by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide (XTT) assay. $P < .05$ for a *t*-test comparison with no treatment.

another phenotypic attribute associated with the mature biofilm state is resistance to neutrophils. We showed that mature *Candida* biofilms were more resistant to killing than early biofilms and did not trigger an ROS response. We also showed that resistance is mediated by the architecture of mature biofilms and the presence of β-glucans in the extracellular matrix. These findings are in line with the observed resistance of mature staphylococcal biofilms to phagocytes, which is matrix mediated [6, 7]. However, unlike certain mature bacterial biofilms that activate neutrophil respiratory burst [29], mature *Candida* biofilms failed to trigger ROS.

Despite the increasing public health significance of *Candida* biofilm infections, interactions of *Candida* biofilms with leukocytes are understudied, and limited mechanistic insights exist on their outcome. This is at least partly due to technical complexities of leukocyte functional assays with high-density population systems such as mature biofilms. Certain leukocyte killing assays are quantitative only within a limited range of effectors and target cells, which may not be attainable with mature biofilms. To overcome this, we used two different assays to assess biofilm damage, and we designed experiments that accounted for the differences in E:T ratios in early vs mature biofilms. Using these approaches, we unequivocally showed a decreased granulocyte antibiofilm activity in mature biofilms. Furthermore, biofilm disruption experiments showed that the physiologic state of biofilm cells is unlikely to be the main reason for innate immune cell resistance because, by disrupting the biofilms and removing some of the extracellular matrix, these cells regained their ability to stimulate an oxidative response and became more susceptible to killing. The fact that killing was not completely restored by dispersion, despite a fully restored ROS response, suggests that ROS are required but not sufficient for optimal *C. albicans* killing, consistent with other reports [37].

Our finding that mature *Candida* biofilms evade neutrophils by not triggering an ROS response is novel. Most of the neutrophil-mediated anti-*Candida* activities depend on oxidative metabolism [27] and release of preformed granule products because no genes involved in antimicrobial function were upregulated in the global transcriptional profiling of *Candida*-challenged PMN [38]. *Candida* genes that govern susceptibility to oxidant stress also determine susceptibility of the organism to neutrophil killing [39]; thus absence of an ROS response upon encounter with mature biofilms may be a biofilm evasion mechanism. This evasion mechanism is particularly important for neutrophils because interaction with neutrophils, but not macrophages, induces a strong oxidative stress response in *C. albicans* [40].

Bacterial quorum-sensing molecules attenuate neutrophil oxidative burst [41]. In *Candida* biofilms, prostaglandins secreted in large amounts by biofilm organisms but not planktonic cells [42] may inhibit neutrophil activation [43]. *Candida* hyphae, abundant in biofilms, are also known to secrete a natural inhibitor of ROS [44]. We thus hypothesized that a secretory product(s) in mature biofilms may be responsible for suppressing the oxidative burst. Two lines of evidence argued against this possibility. First, mature biofilm supernatants did not inhibit killing of early biofilms. Second neither fMLP- nor PMA-mediated activation was inhibited in the mature biofilm environment. The latter finding also supports the hypothesis that lack of ROS production in response to mature biofilms is due to failure of activation via pathogen-specific receptors.

Our data show that the presence of extracellular polysaccharides, such as β-glucans, hinders neutrophil antibiofilm function. Although immunostaining of biofilms with the BFDiv antibody did not stain matrix β-glucans in situ [3, and this work], concentrated matrix extracted from *C. albicans* 48-hour biofilms was immunodetectable and contained high amounts of β-glucan, consistent with other reports [15]. By coating early biofilms with glucanase-treated matrix or laminarin, we showed that some β-glucans interfere with neutrophil–biofilm interactions that activate an ROS response. We also showed that dissolution of glucan, but not mannan or DNA, leads to increased biofilm leukocytic killing. This effect of glucanase could be attributed to solubilization of glucan-linked matrix molecules. However, because laminarin (used at similar concentration as β-glucans in matrix extracts) attenuated ROS and killing of early biofilms, it is more likely that soluble β-glucans in the matrix are responsible for these effects.

Structurally distinct soluble and insoluble β-glucans bind to different glucan receptors on PMN and trigger different biological responses [45–47]. Neither the contribution of structural differences of β-glucans to binding selectivity nor the exact β-glucan structure in the *Candida* biofilm matrix is presently clear. Dectin-1 mediates binding, phagocytosis, and killing of *C. albicans* by human neutrophils, activities that are attenuated by soluble glucans [48]. This is because dectin-1 also binds free glucans and the binding complex is rapidly internalized [49] without forming phagocytic synapses required for dectin-1–mediated activation [50]. Based on the abundance of soluble glucans in the extracellular matrix of *Candida* biofilms [15, 31–32], we propose that during the later stages of biofilm maturation, these glucans bind to dectin-1 and possibly other β-glucan receptors and inhibit neutrophil activation. Our data thus demonstrate a novel role for the glucan-rich matrix as a decoy for neutrophil activation that protects *Candida* biofilms from killing.

## Notes

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***Potential conflicts of interest.*** All authors: No reported conflicts.

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