Anidulafungin increases the antibacterial activity of tigecycline in polymicrobial Candida albicans/Staphylococcus aureus biofilms on intraperitoneally implanted foreign bodies

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Objectives: We aimed to establish a novel murine intra-abdominal foreign body infection model to study the activity of anidulafungin and tigecycline against dual species Candida albicans/Staphylococcus aureus biofilms.

Methods: In vitro and in vivo single and dual species biofilms were developed inside serum-coated triple-lumen catheters placed in 24-well plates or implanted intraperitoneally in BALB/c mice. The effect of tigecycline and anidulafungin alone and in combination was tested using clinically relevant concentrations. Scanning electron microscopy was used to visualize the mature biofilm structure developed intraperitoneally. Flow cytometry was used to determine the immunological response upon infection. Immunoblot analysis allowed us to determine the effect of anidulafungin on poly-β-(1,6)-N-acetylglucosamine in in vitro-grown S. aureus biofilms.

Results: We determined the MIC, MBC and in vitro susceptibility profile for anidulafungin and tigecycline against C. albicans and S. aureus in mixed and single species biofilms. We demonstrated that anidulafungin acts synergistically when combined with tigecycline against in vivo intra-abdominal biofilms. Moreover, we reveal that anidulafungin reduces the abundance of S. aureus poly-β-(1,6)-N-acetylglucosamine. The influx of neutrophils is much increased when infected with mixed biofilms compared with single species biofilms.

Conclusions: Currently, treatment of intra-abdominal infections, in particular polymicrobial catheter-associated peritonitis, is ineffective. To the best of our knowledge, this is the first study that provides insight into new possible options for treatment of C. albicans/S. aureus biofilms present in the abdominal cavity.

Introduction

Candida albicans is a major human fungal pathogen causing a variety of infections. The second most common type of invasive infection caused by C. albicans is intra-abdominal candidiasis, which has mortality rates up to 65%.1–3 Intra-abdominal infections (IAIs) involve inflammation of single organs, primary, secondary or tertiary peritonitis (often as a result of laparotomy surgery) and intestinal hernias; they are most commonly caused by insertion of peritoneal dialysis catheters.3 During IAIs, bacterial coinfections occurred in 67% of the patients suffering from intra-abdominal candidiasis.4 In ~15%–50% of IAIs caused by C. albicans, coinfection with Staphylococcus aureus is observed.4,5 The relationship between S. aureus and C. albicans has been shown to be mutually beneficial and associated with enhanced pathogenicity, disease severity and morbidity.5,7 Peters and Noverr7 demonstrated that coinfection with C. albicans and S. aureus in a murine model of peritonitis resulted in 40%–60% mortality by 48 h post-inoculation and higher morbidity as evidenced by an increased microbial burden within tissues and an amplified local inflammatory response. It has been shown that S. aureus preferentially binds to C. albicans hyphae,8 however, more recent studies demonstrate that
morphogenesis is not required for \textit{C. albicans}/\textit{S. aureus} IAI-mediated dissemination and lethal sepsis.\textsuperscript{6,9} Surprisingly, a yeast-locked strain resulted in mortality rates, inflammation and microbial burdens equivalent to those seen with the WT strain, suggesting that the inflammatory response is primarily responsible for mortality during polymicrobial infection.\textsuperscript{9} Today’s increased usage of medical devices generates an elevated occurrence of biofilm-related infections, which are associated with the emergence of resistance to antimicrobials of microorganisms embedded within biofilms. Biofilm infections are difficult to eradicate and current treatment of IAI, and catheter-associated peritonitis in particular, is ineffective.\textsuperscript{2} Echinocandins show activity against in vitro and in vivo \textit{C. albicans} biofilms.\textsuperscript{10,11} however, there is evidence of increasing resistance to these drugs.\textsuperscript{12} Clinical factors that contribute to decreased activity of echinocandins are prophylaxis, host reservoirs including biofilms in the gastrointestinal tract and IAIs.\textsuperscript{12} Along with existing antifungals, the number of antibiotics that show activity against single species \textit{S. aureus} biofilms is limited. The glycyccline antibiotic tigecycline is a derivative of minocycline, which exhibits potent activity against a broad spectrum of bacteria including MRSA and shows significant activity against \textit{S. aureus} biofilms.\textsuperscript{13} Moreover, tigecycline was potent against biofilm-related staphylococcal infections developed in various animal models.\textsuperscript{14–16} Today, this drug is the first-line agent used to treat severe IAI, complicated skin infections and community-acquired bacterial pneumonia.\textsuperscript{17}

In the search for an effective treatment strategy, we investigated the activity of the antifungal drug anidulafungin and the antibiotic tigecycline against dual species \textit{C. albicans}/\textit{S. aureus} biofilms developed in a novel murine foreign body IAI model. We demonstrate that anidulafungin acts synergistically when combined with tigecycline against in vivo biofilms. Moreover, we reveal that anidulafungin reduces the abundance of \textit{S. aureus} poly-β-(1,6)-N-acetylg glucosamine (PNAG), which is an essential building block of the bacterial biofilm matrix.

**Materials and methods**

**Strains, media and therapeutics**

MRSA USA300 standard strain\textsuperscript{18} was grown on Trypticase Soy Agar plates (Becton Dickinson, Benelux) at 37°C. \textit{C. aureus} overnight cultures were prepared in Trypticase Soy Broth (TSB) supplemented with 0.2% glucose (TSBg) at 37°C. \textit{C. albicans} SC5314\textsuperscript{19} WT strain was streaked on YPD and incubated at 30°C. Overnight culture of \textit{C. albicans} was prepared in liquid YPD medium at 30°C. RPMI 1640 medium (with -l-glutamine and phenol red) without bicarbonate was buffered with MOPS (Sigma, USA), pH 7.0. Pure substances of anidulafungin and tigecycline were kindly provided by Pfizer (Brussels, Belgium). The stock solution of anidulafungin was prepared in 100% DMSO, and tigecycline was diluted in TSBg medium and allowed to propagate at 37°C until mid-log phase was reached (3 h). For single species biofilms, 500 μL of \textit{C. albicans} (5×10^5 cells/mL) or \textit{S. aureus} cells (5×10^6 cells/mL) were added separately to each device. To this amount, 500 μL of fresh RPMI 1640 medium was added. For polymicrobial biofilms, 500 μL of each microorganism was added to each catheter. Samples were incubated for 90 min at 37°C (period of adhesion). Afterwards, devices were washed twice with PBS and placed on ice. Animals were anaesthetized as previously described.\textsuperscript{17} The surgery is shown in Figure S1 (available as Supplementary data at JAC Online). Briefly, the lower left side of the abdomen was shaved and disinfected with 0.5% chlorhexidine in 70% alcohol. A 5 mm incision was made longitudinally and the subcutis was carefully cut to access the intraperitoneal cavity. Subsequently, two devices were implanted between intestines in the intra-abdominal cavity through the groin area (Figure S2). The incision was closed with surgical sutures and lidocaine was applied directly on to the wound to prevent pain. Last, we administered reversal of anaesthesia (100 μL per 10 g of body weight) consisting of atipamezole (Antiserad, 5 mg/mL). For catheter explant, animals were sacrificed by cervical dislocation. The skin was disinfected and catheters were removed from the abdominal cavity, washed twice with PBS and placed in a micro-centrifuge tube containing 1 mL of PBS. Catheters were sonicated, vortexed and plated to enumerate the cfu, as described above. During kinetics experiments, biofilms were studied for 24 h, 4 days, 7 days, 9 days and 21 days post-implant.

Furthermore, liver, spleen and kidneys were harvested and submerged in PBS. Organs were homogenized and 100 μL of homogenized tissue was plated on chromogenic medium for quantification of \textit{S. aureus} (CHROMagar Staph aureus, TA672, France) and \textit{C. albicans} (CHROMagar Candida, CA222, France) cells by cfu counting.

To test the activity of drugs on in vivo biofilms, intraperitoneal administration of anidulafungin (10 mg/kg/day)\textsuperscript{13} and tigecycline (1 or 10 mg/kg/day)\textsuperscript{14} was initiated 24 h post-implant. Drugs were prepared in sterile water and injected once daily for 7 days intraperitoneally. In the case of anidulafungin, the concentration of DMSO used for the injection did not exceed 1%.

**Statistical analysis and reproducibility of results**

Statistical analyses were performed using Student’s t-test and one-way ANOVA with post hoc Tukey HSD test (GraphPad Prism Software).
Results

C. albicans and S. aureus planktonic cells are susceptible to anidulafungin and tigecycline, respectively

The MICs of anidulafungin and tigecycline were 0.0625 and 0.125 mg/L against C. albicans and S. aureus, respectively, and were not modified by the presence of the other organism in mixed species cultures. Conversely, neither of these two drugs had an inhibitory activity on the planktonic growth of the other species even at the highest concentration (16 mg/L) tested in this study.

Anidulafungin and tigecycline are active against in vitro C. albicans and S. aureus single and dual species biofilms, respectively

A significant difference between the cfu attached on catheters after 90 min (adhesion period) and 24 h was found, indicating that fungal and bacterial cells proliferated and formed biofilms in vitro (Figure S3). When added to 24 h biofilms, anidulafungin significantly reduced the number of C. albicans cells in both single species and mixed species models (for concentrations ≥1–2 mg/L) (Figure 1a), but lacked any activity against single and dual species S. aureus biofilms (Figure 1b). Tigecycline was not active on C. albicans even at the highest concentration (16 mg/L) tested (Figure 1c). However, S. aureus cells within single and mixed species biofilms were susceptible to low concentrations (<0.5 mg/L) of tigecycline (Figure 1d). Together, these data demonstrate the activity of anidulafungin and tigecycline on in vitro C. albicans and S. aureus cells in mono and dual species biofilms, respectively.

In vivo single and dual species C. albicans/S. aureus biofilm formation on foreign bodies implanted intraperitoneally

Single and dual species C. albicans biofilms formed 24 h post-implant were characterized by a significantly increased number of cfu recovered from catheters in comparison with the adhesion period (Figure S4a, b). Similarly, the number of bacterial biofilm-forming cells significantly increased 24 h after the...
operation in single and mixed biofilms (Figure S4c, d). The amount of fungal cfu retrieved remained stable until 21 days. However, bacterial biofilm-associated cells continued to proliferate over a prolonged period of time compared with C. albicans. Simultaneously, we studied the dissemination of fungal and bacterial cells into the spleen, kidneys and liver (Figure S5e, f and g, respectively). Animals carrying catheters infected with both pathogens exhibited increased numbers of bacterial cells in the spleen and liver but this difference was not significant. Daily monitoring of host organisms showed no signs of the presence of S. aureus in any vital organs for 21 days. C. albicans did not disseminate into any organ over the period of 21 days (data not shown). Although specific pathogen-free BALB/c mice were used, animals were confirmed to be negative for possible contamination of organs by S. aureus originating from the gut flora of the host. Therefore, a group of animals containing two non-infected catheters was included in each experiment. Catheters and organs retrieved from these animals remained pathogen-free, supporting our finding that S. aureus cells present in organs during biofilm formation originated from infected catheters (data not shown). Scanning electron microscopy images revealed that 24 h old in vivo biofilm architecture of single species C. albicans was characterized by a dense network of hyphal and yeast cells. S. aureus biofilms (24 h old) were formed alongside the catheter lumen and consisted of bacterial cells embedded within a material that resembled an extracellular matrix. Dual species biofilms consisted of C. albicans hyphae creating a net with patches of bacterial cells (Figure 2).

**Significantly greater neutrophil influx upon polymicrobial device-associated infection**

Flow cytometry data revealed significant changes in the number of immune cells that were retrieved at the infection locus 24 h post-surgery. The abdominal cavity of control mice (non-operated) harboured primarily resident monocytes, naive macrophages and B cells, whereas neutrophils represented <5% of the resident immune cell population. As a response to the surgery alone (operated animals carrying non-infected devices), the neutrophil subset within the total immune cell population shifted towards an average 55%, which recalculates to the recruitment of \( \approx 1.75 \times 10^6 \) neutrophils. Interestingly, the nature of infection (single species versus polymicrobial) significantly contributed to the altered immune profile (Figure 3a). A significantly higher number of neutrophils was recruited to the peritoneum of mice infected with mixed species \( \approx 4.55 \times 10^6 \) neutrophils. During polymicrobial infection, we observed a large increase in the number of peritoneal cells retrieved from lavage 24 h post-surgery (Figure 3b). These data suggest a rapid response and mobilization of neutrophils during polymicrobial infection compared with single species infection.

**Anidulafungin is effective against in vivo C. albicans in single, as well as in dual species biofilms, whereas tigecycline is less potent against S. aureus in mixed species than in single species biofilms**

Twenty-four hours after catheter implant, mice were treated with anidulafungin (10 mg/kg/day) or tigecycline (1 or
10 mg/kg/day). Drugs were injected intraperitoneally daily for 7 days. Control mice were injected with sterile water. Anidulafungin significantly reduced *C. albicans* biofilms grown as single species (Figure 4a) or when combined with *S. aureus* (Figure 4b). Tigecycline significantly decreased the number of bacterial cells retrieved from mono species biofilms regardless of the dosage used (Figure 4c). However, daily injection of 1 mg/kg of body weight/day of tigecycline failed to be active on bacterial biofilm-forming cells found in the mixed species population (Figure 4d). Notwithstanding, upon administration of a higher dose of tigecycline (10 mg/kg of body weight/day), the bacterial device-associated counts were reduced (Figure 4d). Importantly, anidulafungin did not modulate the amount of *S. aureus* cells recovered from single and mixed species biofilms in vivo (data not shown). Tigecycline did not inhibit the number of *C. albicans* biofilm-forming cells retrieved from mono and dual species in vivo biofilms (data not shown).

**Anidulafungin and tigecycline (1 mg/kg of body weight/day) act synergistically against in vivo *S. aureus* biofilm-forming cells in mixed species device-associated infections**

Mice implanted with mixed species catheter-associated biofilms were treated after 24 h with anidulafungin (10 mg/kg/day) combined with tigecycline at two different dosages (1 and 10 mg/kg/day). Therapeutics were injected intraperitoneally simultaneously daily for 7 days. Anidulafungin combined with two different dosages of tigecycline did not improve the effect on *C. albicans* biofilm-forming cells in comparison with anidulafungin monotherapy (Figure 5a). However, increased efficacy was observed upon combinational therapy against *S. aureus* biofilm-forming cells retrieved from polymicrobial biofilms. Although tigecycline (1 mg/kg of body weight/day) was unable to diminish *S. aureus* biofilm-associated cfu in mixed biofilms (Figure 4c), it became effective when combined with anidulafungin (Figure 5b). This finding points to a previously unidentified synergism.
between these two drugs. Nonetheless, this synergy was not observed when anidulafungin was combined with 10 mg/kg/day of tigecycline (Figure 5c).

Anidulafungin impairs PNAG synthesis of *S. aureus*

Previous work demonstrated that caspofungin, another echinocandin, inhibits the activity of IcaA in *S. aureus*, an enzyme sharing homology with the fungal glucan synthase and involved in the synthesis of PNAG, a major constituent of the *S. aureus* biofilm matrix. This effect resulted in an increase in fluoroquinolone activity against *S. aureus* biofilms. We therefore asked whether anidulafungin could also act upon the staphylococcal biofilm matrix.

Hence, we compared the abundance of PNAG in *S. aureus* biofilms incubated in the absence (untreated sample) or in the presence of increasing concentrations of anidulafungin. We observed a concentration-dependent reduction in the amount of PNAG detected in the presence of anidulafungin (Figure 6).

### Discussion

In this study, we introduced a novel model to study polymicrobial *C. albicans/S. aureus* biofilm-associated infection on foreign bodies implanted into the peritoneal cavity. To the best of our knowledge, few studies have characterized *C. albicans/S. aureus* mixed intraperitoneal infections, and such studies most often focused on
infections caused by planktonic cells. In the originally developed model, *P. aeruginosa* biofilms were studied on a silicone tube inserted into the peritoneal cavity of BALB/c mice. In our proposed model, serum-coated polyurethane catheter pieces were challenged with *C. albicans* and/or *S. aureus* during the in vitro adhesion period followed by implant into the peritoneal cavity. During adaptation of our model, no differences in fungal or bacterial biofilm burden were found between the immunocompetent and immunocompromised host (data not shown). *S. aureus* cells disseminated into the spleen, kidneys and liver within the first day of biofilm formation and persisted inside these organs not causing any detrimental changes to the animals. Surprisingly, no Candida cells were recovered from any of these organs. Although it has been previously shown that *C. albicans* hyphae are needed for *S. aureus* attachment and for further dissemination into the bloodstream during oropharyngeal candidiasis, this was not the case.

**Figure 5. In vivo activity of AND and TGC combination on CA and SA cells grown in dual species biofilms.** (a) Activity of AND (10 mg/kg of body weight/day; AND10) combined with TGC at two different dosages, namely 1 (TGC1) and 10 (TGC10) mg/kg/day on CA cells grown in mixed species biofilms intraperitoneally. Activity of (b) 1 mg/kg/day of TGC and (c) 10 mg/kg/day combined with AND on SA cells grown in mixed species biofilms intraperitoneally. Mice carrying catheters with 24 h old biofilms were treated with a drug combination daily for 7 days. Each symbol represents the average log_{10} cfu ± SEM of control (untreated) and treated SA cells, respectively obtained from catheter fragments from a single mouse. Horizontal line represents the mean log_{10} cfu ± SEM of the amount of fungal cells recovered from an untreated and treated group of animals. These experiments were performed twice always using at least six animals per group. Statistical analyses were performed using Student’s t-test. A significant difference (P < 0.05) is shown with an asterisk. AND, anidulafungin; CA, *C. albicans* SC5314; NS, not significant; SA, *S. aureus* USA300; TGC, tigecycline.

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in our model. These findings suggest that the host immune system may contribute to pathogenesis in this model. Flow cytometry data obtained in this work displayed a significantly increased number of neutrophils recruited to the peritoneal cavity 24 h post-surgery in animals infected with mixed species compared with a single species. Although neutrophils are critical for rapid eradication of infection, our experiments demonstrated, in contrast to those of Peters and Noverr,7 the survival of mice for 21 days with stable biofilms and S. aureus colonization. Presumably, our observation of neutrophil influx in mixed infection is an attempt to clear the infection and promote survival of the host during the early stages of mixed species biofilm development.

It is well known that biofilms are very difficult to eradicate and therefore there is a crucial need to investigate the anti-biofilm potential of already existing or novel antimicrobial agents. In our study, we explored for the first time, to the best of our knowledge, the activity of anidulafungin and tigecycline against mono and dual species biofilms developed on foreign bodies implanted into the abdominal cavity. Prior to in vivo biofilm testing, we performed several in vitro tests in which we found that C. albicans and S. aureus planktonic cultures, as well as in vitro mixed biofilms were susceptible to anidulafungin and tigecycline, respectively. In vivo, anidulafungin and tigecycline alone (1 and 10 mg/kg of body weight/day) effectively decreased the number of C. albicans and S. aureus forming biofilms on implanted devices, respectively. However, only the highest dosage of tigecycline (10 mg/kg of body weight/day) was capable of reducing the number of S. aureus cells retrieved from mixed biofilms, suggesting that C. albicans caused protection to bacterial cells. A plausible reason might be the production of an extracellular matrix rich in β-1,3 glucan of fungal origin, which could confer bacterial tolerance to the antibiotic.26 Remarkably, when combined with anidulafungin, tigecycline at the lowest dosage (1 mg/kg of body weight/day) became as effective against mixed species biofilms, as when used alone at the highest dosage.

Echinocandins inhibit glucan synthesis, contributing thereby to the destrucutralization of the Candida biofilm matrix. We show here that anidulafungin is also capable of impairing the production of PNAG, a major constituent of the S. aureus biofilm matrix. We can therefore speculate that the combined action of anidulafungin on polysaccharides of both fungal and bacterial origin present in the matrix may contribute to explaining the synergism between these two drugs. Similarly, Siala et al.24 demonstrated that caspofungin impaired PNAG synthesis, which resulted in disruption of the Staphylococcus biofilm matrix and increased penetration of fluoroquinolones into the biofilm. Therefore, our data support the fact that the combination of anidulafungin with tigecycline can be used to treat C. albicans/S. aureus mixed species biofilms developed on foreign bodies implanted intraperitoneally. The synergy between the two drugs was not observed when using 10 mg/kg/day tigecycline, probably because at this high dosage the antibiotic had already reached its maximum effect.

The concentration of drugs at the infection site has not been determined in this study, making direct comparison between in vitro and in vivo data difficult. However, it has been previously shown that anidulafungin was rapidly distributed to clinically relevant tissues, where it reached concentrations higher than those achieved in plasma.27 Although pharmacokinetic and pharmacodynamic data related to tigecycline are very limited, tigecycline had a significantly larger volume of distribution than other tetracyclines and good penetration into tissues.15,28 In clinical practice, anidulafungin,29 as well as tigecycline,26 are administered intravenously. Despite this, it is noteworthy to mention that independent studies have suggested intraperitoneal administration of anidulafungin and tigecycline as a feasible option,30,31 although further in vivo pharmacokinetic studies are required using this route of administration.

In conclusion, this study provides insight into new possible options for treatment of C. albicans/S. aureus biofilms developed on foreign devices implanted into the abdominal cavity. Future studies should focus on the efficacy of such drug combinations against in vivo device-related infections developed in various animal biofilm models, at different sites of infection including different bacterial species and involving resistant bacterial clinical isolates. It would be beneficial to explore the applicability of this combination treatment in clinical practice. Furthermore, later studies should focus on elucidating the specific immune response triggered upon infection, as this is crucial in understanding the pathogenesis of polymicrobial biofilm-related IAI.

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Supplementary data

SUPPLEMENTARY MATERIALS AND METHODS

Anidulafungin increases anti-bacterial activity of tigecycline in polymicrobial *Candida albicans*-*Staphylococcus aureus* biofilms on intraperitoneally implanted foreign bodies

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Anidulafungin and tigecycline activity testing on in vitro biofilms

Overnight culture of *C. albicans* was prepared in liquid YPD medium at 30 °C. Bacterial cells were cultured in Trypticase Soy Broth (TSB) supplemented with 0.2% glucose (TSBg) overnight at 37 °C. Polyurethane catheters (Arrow International Reading, USA) were cut into segments of 1 cm and incubated overnight in Fetal Bovine Serum (FBS) at 37 °C A 1:100 dilution of the *C. albicans* O/N culture was prepared in fresh YPD, whereas *S. aureus* was diluted in TSBg medium and allowed to propagate at 37 °C until mid-log phase was reached (approximately 3 h). For mono-species biofilms, 500 μL of *C. albicans* (5 x 10⁴ cells/mL) or *S. aureus* cells (5 x 10⁴ cells/mL) were added separately to each well. To this amount additional 500 μL of fresh RPMI 1640 medium was given. For polymicrobial biofilms, 500 μL of each microorganism was added to each device. Plates were incubated for 90 min at 37 °C (period of adhesion). Afterwards, each catheter piece was washed twice with PBS and subsequently submerged in 1 mL of RPMI 1640. Biofilms were allowed to form for the next 24 h at 37 °C. Next, non-biofilm-associated cells were removed by two rounds of washing and catheters were submerged in RPMI 1640 containing different concentrations of anidulafungin (0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8 and 16 mg/L) and tigecycline (0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8 and 16 mg/L). Catheters were incubated for 24 h at 37 °C. Afterwards, catheters were washed twice with PBS and transferred to 1 mL of PBS. Cells were detached from biofilms during sonication in a water bath sonicator (40 000 Hz) for 10 min, following vigorous vortexing for 30 s. Subsequently, samples were diluted and plated on
YPD, TSB agar plates or on selective plates, such as YPD agar plates supplemented with 10 mg/L vancomycin (YPD+VAN) and on TSB agar plates supplemented with 8 mg/L of amphotericin B (TSB+AMB).

**Flow cytometry**

Immune cells were obtained from peritoneal lavage, which was performed according to Ray and Dittel,¹ with few modifications. Mice were implanted with catheters previously infected with: *C. albicans* (6 mice), *S. aureus* (6 mice), *C. albicans – S. aureus* (6 mice). Six mice containing clean catheters were considered as control group and 6 mice were non-operated. Briefly, mice were euthanized by the CO₂ inhalation and disinfected with 70% ethanol. Subsequently, 2 mL of ice cold PBS were injected into the peritoneal cavity and peritoneum was gently massaged to dislodge any attached cells into the PBS solution. Fluid was collected, transferred to microcentrifuge tubes and kept on ice. This procedure was repeated twice. Peritoneal fluid was centrifuged and resuspended in FACS buffer (PBS without Ca and Mg, 3% FBS, 2mM EDTA). Peritoneal cell suspensions were pre-incubated with anti-CD16/CD32, amcyan live-dead stain and F4/80-biotin (BD Bioscience) coupled mAb on ice for 15 min. After washing, cells were stained with fluorochrome-conjugated mAb in an 8-color staining combination: FITC-labeled anti-Ly6G(Biolegend), PE-labeled anti-CD11b (Biolegend), PerCP-Cy5.5 labeled anti-CD115c (Biolegend), PE-Cy7 labeled anti-B220 (Biolegend), Efluor450-labeled anti-Ly6C (eBioscience), streptavidin-coupled BV605 (BD Bioscience) and Alexafluor 647 coupled anti-CD64. Washed cells were fixed for 15 min with 4% paraformaldehyde (PFA). Next, cells were resuspended in 200 μL of FACS buffer. Cells were analyzed and sorted on VIB-Flow core instruments (Becton Dickinson, LSRII). Data were collected for 1 x 10⁶ cells and analyses were carried out using FlowJo software.
PNAG purification and immunoblot analyses

These experiments were performed exactly as previously described. In brief, biofilms were grown for 24 h in 6-well plates in TGN medium (Trypticase Soy Broth supplemented with 2% NaCl and 1% glucose), with a starting inoculum adjusted to an OD$_{620nm}$ = 0.005 in a volume of 2 mL. Biofilms were then exposed for 24 h to increasing concentrations of anidulafungin (10mg/L; 20 mg/L; 40mg/L). Biofilms were washed twice with 2 mL PBS, and sonicated for 1 min in an ultrasonic bath. Then resuspended in PBS, centrifuged and resuspended in 0.5 M EDTA. Further incubated at 100°C for 5 min and at 85 °C for 30 min, and centrifuged again. The resulting supernatant was dialyzed, treated with enzymes (α-amylase, lysozyme, DNase I, RNase A, then proteinase K) and dialyzed again. Polysaccharide preparations were then lyophilized and dissolved in PBS. Aliquots were spotted onto a PVDF membrane. After air drying and overnight blocking with milk, the membrane was incubated overnight at 4 °C with PNAG-antiserum (1:4000; kindly provided by Dr. Gerald B. Pier; Brigham and Women's Hospital, Boston, MA), washed, and probed with 1:5000 goat anti-rabbit HRP for 2 h. Spots were visualized with the SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Fisher Scientific) and analyzed using the FUSION-CAP Software (Analis, Belgium).

Scanning electron microscopy (SEM).

SEM experiments were performed as previously described.
REFERENCES
