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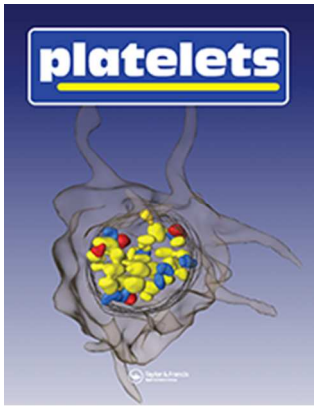

Mucor circinelloides induces platelet aggregation through integrin αIIbβ3 and FcγRIIA

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Title: *Mucor circinelloides* induces platelet aggregation through integrin αIIbβ3 and FcγRIIA

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Abstract

Thrombosis is a hallmark of the fatal fungal infection mucormycosis. Yet, the platelet activation pathway in response to mucormycetes is unknown. In this study we determined the platelet aggregation potential of *Mucor circinelloides* (*M. circinelloides*) NRRL3631, characterized the signaling pathway facilitating aggregation in response to fungal spores, and identified the influence of the spore developmental stage upon platelet aggregation potential. Using impedance and light-transmission aggregometry, we showed that *M. circinelloides* induced platelet aggregation in whole blood and in platelet-rich plasma, respectively. The formation of large spore-platelet aggregates was confirmed by light-sheet microscopy, which showed spores dispersed throughout the aggregate. Aggregation potential was dependent on the spore's developmental stage, with the strongest platelet aggregation by spores in mid-germination. Inhibitor studies revealed platelet aggregation was mediated by the low affinity IgG receptor FcγRIIA and integrin αIIbβ3; Src and Syk tyrosine kinase signaling; and the secondary mediators TxA_2 and ADP. Flow cytometry of antibody stained platelets showed that interaction with spores increased expression of platelet surface integrin αIIbβ3 and the platelet activation marker CD62P. Together, this is the first elucidation of the signaling pathways underlying thrombosis formation during a fungal infection, highlighting targets for therapeutic intervention.

Introduction

The incidence of invasive fungal infections is on the rise, and this is attributed to the increasing population of immunosuppressed individuals under the influence of modern medicine [1-4]. Mucormycosis - a previously uncommon infection - has grown in occurrence to become the third most common invasive fungal infection after aspergillosis and candidiasis, respectively [5]. Infection is caused by species of the Mucorales order, with *Rhizopus* spp. and *Mucor* spp. being the most common causative agents [1-4]. Prognosis for this severe fungal infection is poor, with studies reporting mortality rates in the range of 60-100% [4]. This staggeringly high mortality rate is reflective of the aggressive nature of infection, and the poor efficacy of antifungal therapeutics currently employed [2, 3]. Risk factors identified for mucormycosis include uncontrolled diabetes mellitus, diabetes mellitus with ketoacidosis, organ transplantation and neutropenia [3, 4, 6].

The hallmarks of mucormycosis are considered to be angioinvasion, tissue necrosis and thrombosis, the latter indicating a potential role of platelets [6]. Platelets have been identified as major players of the innate immune system with the onset of thrombocytopenia common during infectious diseases. Infective endocarditis and septicemia in particular have sparked interest into the interaction between pathogens and platelets [7-9]. It is unknown if thrombus formation protects from mucormycosis or exaggerates symptoms by inducing excessive inflammation and tissue necrosis.

The platelet IgG receptor FcγRIIA and integrin αIIbβ3 have been highlighted as crucial to platelet activation in response to both Gram-negative and Gram-positive bacteria [7-9]. While the proteins that give rise to bacterial-platelet interaction are strain-specific, platelet activation is mediated by a common pathway consisting of FcγRIIA, Src and Syk tyrosine kinase activation, αIIbβ3 engagement and the secondary mediators thromboxane A_2 (TxA_2)

and adenosine 5'-diphosphate (ADP) [8, 9]. There is currently little information on the interaction between platelets and mucormycetes under physiological conditions and the molecular signaling pathways underlying this interaction. An improved understanding of the signaling underlying thrombus formation during mucormycosis might offer novel therapeutic targets to improve current treatment approaches and thus patient outcome. Hence, we investigated the interaction between the clinical isolate *Mucor circinelloides* NRRL3631 and platelets. We show that platelets form aggregates with fungal spores dependent on the spore developmental stage. Spore aggregation is mediated through the platelet IgG receptor FcγRIIA, integrin αIIbβ3 and Src and Syk tyrosine kinases, and secondary mediators TxA_2 and ADP. Platelet activation is also associated with increased expression of platelet surface integrin αIIbβ3 and the platelet activation marker CD62P. Together, this provides the first elucidation of the signaling pathways underlying thrombosis formation during mucormycosis highlighting potential strategies to interfere with thrombus formation.

Methods

Fungal strains and growth conditions

The *Mucor circinelloides* strain used was *Mucor circinelloides* f. sp. *lusitanicus* strain NRRL3631, a clinical isolate [10]. The strain was grown on Sabouraud dextrose agar (Merck-Millipore, Billerica, MA, USA) at room temperature for 7 days prior to use.

Spore preparation for aggregation assays

Spores were collected in phosphate buffered saline (PBS) and centrifuged at 1811 *x g* for three min. The spore pellet was washed twice with PBS and then re-suspended in Saboraud

broth (Sigma-Aldrich, St. Louis, MO, USA). Spores were cultured at 37°C with shaking at 45 rpm, over 0, 3, 6 and 48 hours. Following incubation, spore suspensions were centrifuged at 1811 *x g* for three min. The spore pellet was washed twice, and re-suspended in PBS at concentrations to allow for 1:10, 1:20, 1:100 and 1:500 spore:platelet ratios. Spore suspensions were kept on ice until used for aggregometry.

Blood preparation

Blood samples were collected from healthy human donors into 4% (w/v) citrate (Sigma-Aldrich, St. Louis, MO, USA). The study design was approved by the University of Birmingham's research ethics committee (ERN_11-0175). Blood was centrifuged at 200 x *g* for 20 min and platelet-rich plasma (PRP) collected. For light-transmission aggregometry, a PRP platelet count was taken using Coulter® Z2 Particle Counter in triplicate and averaged. For multiple-electrode aggregometry, a whole blood platelet count was taken using Sysmex XN-1000 Hematology Analyzer.

Platelet aggregometry in PRP

PRP was incubated at 37°C for 1 min and then stirred for 1 min. *M. circinelloides* NRRL3631 suspension containing appropriate spore numbers for 1:10, 1:20, 1:100 and 1:500 spore:platelet ratios was added to the PRP, and platelet aggregation recorded over 30 min using light-transmission aggregometer PAP-8E (Bio/Data Corporation, Horsham, PA, USA). As a positive control, Thrombin Receptor-Activating Peptide (TRAP; Severn Biotech, Kidderminster, UK) (100 µM) was added to PRP, and as a negative control PBS was added.

Platelet aggregometry in whole blood

Whole blood was added to sodium chloride and incubated for 3 min at 37°C. *M. circinelloides* NRRL3631 at 3 hours germination, and at a spore concentration allowing for a spore:platelet ratio of 1:10, was added and platelet aggregation recorded over 30 min using multiple electrode aggregometer, Multiplate® Analyzer (Roche, Basel, Switzerland) . TRAP was added to PRP and used as a positive control, and PBS was used as a negative control.

Cell staining and microscopy

Platelets were stained with CellMask Deep Red Plasma Membrane Stain (1:2000, Thermo Fisher Scientific, Waltham, MA, USA), and *M. circinelloides* NRRL3631 spores with Concanavalin A, Alexa fluor™ 488 conjugate (300 µg ml^{-1}, Thermo Fisher Scientific, Waltham, MA, USA). Formed aggregates were immobilized on poly-L-lysine (Sigma) coated rectangular coverslips (25x50 mm), fixed using 4% PFA, washed and placed in the imaging chamber filled with PBS. Orthogonal views of the aggregates were acquired on a Marianas LightSheet (Intelligent Imaging Innovations, Denver, CO, USA), a dual inverted Selective Plane Illumination Microscope (diSPIM) which uses two perpendicular 0.8 NA, 40x water immersion objectives to excite and detect fluorescence in an alternating duty cycle.

Volumes of 200 image planes were captured using both arms sequentially in slice scan mode, with a step size of 0.5 µm, for both 488 nm and 640 nm excitation wavelengths, on ORCA-Flash4.0 V3 sCMOS cameras (Hamamatsu), driven by SlideBook 6.0 software (Intelligent Imaging Innovations, Denver, CO, USA).

Image analysis was performed using Fiji software [11], and a maximal intensity projection was made of a single volume view. For the supplementary movie, the two orthogonal volumes were registered and deconvolved (joint-deconvolution) using the multiview Reconstruction plugin [12] and visualized by rotating the 3D volume around the y-axis.

Inhibitor treatments

Inhibition of αIIbβ3 was achieved by pre-incubating platelets with 9 μM eptifibatide (GlaxoSmithKline, Coventry, UK) for 1 min at room temperature. FcγRIIA was blocked with 10 μM mAbIV.3 (hybridoma from American Tissue Culture Corporation (Manassas, Virginia), Src and Syk tyrosine kinases with 4 μM dasatinib (D-3307, LC Laboratories, Woburn, MA, USA) and 10 μM PRT-060318 (AdipoGen Life Sciences, Liestal, Switzerland) respectively, and secondary mediators TxA_2 and ADP with 30 μM Indomethacin (I7378, Sigma-Aldrich, St. Louis, MO, USA) and 2 U Apryase (A6535, Sigma-Aldrich, St. Louis, MO, USA) respectively. Platelet aggregation was assessed by light-transmission in a PAP-8E aggregometer over 30 min.

Platelet receptor labeling

Conjugated anti-human CD32 (#60012; mouse; StemCell Technologies, Vancouver, Canada) and anti-mouse 2° Alexa Fluor® 488 conjugate (A10680; goat; Invitrogen, Carlsbad, CA, USA) was used to label FcγRIIA. Anti-Human CD41a-APC (BD559777; mouse; BD Biosciences, San Jose, CA, USA) was used to label αIIbβ3. CD62P-FITC (BC A07790; mouse; Beckman Coulter, Brea, CA, USA) was used to label CD62P. Platelet aggregation was assessed by light-transmission in a PAP-8E aggregometer over 30 min. Receptor antibody labels were added to samples for 30 min prior to flow cytometry assays. Flow cytometry analysis was conducted using BD Accuri™ C6 Plus (BD Biosciences, Oxford, UK). Samples were run for 10000 events, and cell count vs fluorescence recorded.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 6.0. Data is presented as mean ± SEM, unless stated otherwise. Data was analysed using the one-way analysis of variance (ANOVA) with post hoc Dunnett's multiple comparison test, Mann-Whitney *U*-test or Kruskal-Wallis test with post hoc Dunn's multiple comparison test, as indicated in the figure legends. $P<0.05$ was deemed to be statistically significant. Technical repeats were conducted at $n=3$, and biological repeats at $n=5$ unless otherwise stated.

Results

Mucor circinelloides NRRL3631 induces platelet aggregation in whole human blood and PRP

Thrombus formation during mucormycosis might be induced by fungal invasion of blood vessels, and thus formation of a platelet-reactive surface, or direct interaction between platelets and the fungus. Therefore, we investigated the potential of the clinical isolate *Mucor circinelloides* NRRL3631 to induce platelet aggregation qualitatively and quantitatively.

Initially, we visualized spore-platelet interaction by dual inverted Selective Plane Illumination fluorescence microscopy (diSPIM), which gives isotropic resolution in 3 dimensions. *M. circinelloides* spores were stained with Concanavalin A, Alexa Fluor™ 488 conjugate and platelets in plasma-rich platelets (PRP) with CellMask Deep Red Plasma Membrane Stain. *M. circinelloides* spores were incubated in PRP at a 1:10 spore:platelet ratio under stirring conditions in the PAP-8E aggregometer and formed aggregates were fixed onto glass coverslips. Fluorescence microscopy revealed *M. circinelloides* NRRL3631 spores to be contained within large platelet aggregates with individual spores being surrounded by platelets (Figure 1Ai & ii and Supplementary Movie 1).

We then quantified this interaction by light transmission aggregometry. Experiments were performed in PRP using 1:10, 1:20, 1:100 and 1:500 spore:platelet ratios. The agonist thrombin-related-activating peptide (TRAP) induced maximal platelet aggregation (91.8 ± 2.0%). *M. circinelloides* NRRL3631 induced platelet aggregation in a concentration-dependent manner, with significant platelet aggregation occurring at 1:10 and 1:20 spore:platelet ratios (1:10: 56.0 ±12.3% and 1:20: 52.8% ±14.6% platelet aggregation; $P<0.01$) (Figure 1B).

To examine the physiological relevance of platelet aggregation in response to mucormycetes, we determined the platelet aggregation potential of *M. circinelloides* NRRL3631 in whole blood using impedance aggregometry. *M. circinelloides* NRRL3631 was added to whole blood at a spore:platelet ratio of 1:10, and platelet aggregation assessed. *M. circinelloides* NRRL3631 induced significant platelet aggregation (180 ± 16U; $P<0.05$) in comparison to PBS (81 ± 16U) (Figure 1C).

In summary, these data show that platelets interact with mucormycete spores to form large platelet-spore aggregates. This indicates that thrombus formation during mucormycosis can be mediated by a direct platelet response to fungal spores.

Platelet aggregation in response to *Mucor circinelloides* NRRL3631 is supported by the FcγRIIA receptor and integrin αIIbβ3

Having shown that platelets form aggregates with mucormycete spores, we next wanted to identify the receptor(s) and downstream signaling components mediating this interaction. Aggregation studies with washed platelets did not induce aggregation suggesting the requirement of a serum factor. Therefore, this study performed aggregations in PRP with the focus to elucidate the receptors and downstream signaling mediators. The platelet low

affinity immune receptor, FcγRIIA has been identified as a key receptor in platelet activation in response to bacterial pathogen through the result of antibody-pathogen interaction [8, 9, 13, 14]. We hypothesized that FcγRIIA plays a key role in the induction of platelet aggregation by *M. circinelloides* NRRL3631 in view of the dependency on plasma. In addition, platelet integrin αIIbβ3, the most abundant platelet surface glycoprotein, has been shown to be essential for platelet activation by bacteria [13-16]. To investigate whether platelet aggregation in response to *M. circinelloides* NRRL3631 is supported by FcγRIIA and αIIbβ3 engagement, platelets were treated with the FcγRIIA blocking mAb IV.3 or αIIbβ3 inhibitor eptifibatide, prior to *M. circinelloides* NRRL3631 exposure. Blocking FcγRIIA significantly decreased platelet aggregation in response to *M. circinelloides* NRRL3631 (1:10 (-)mAb IV.3: 48.8 $\pm$ 9.58%; (+) mAb IV.3: 12.8 $\pm$ 10.57%; $P<0.05$) (Figure 2A). Platelet aggregation was abrogated by eptifibatide (1:10 (-)eptifibatide: 58.0 $\pm$ 7.46%; (+)eptifibatide: 0.60 $\pm$ 0.25%; $P<0.01$) and (Figure 2B).

Upon platelet activation, expression of surface αIIbβ3 is upregulated, further supporting platelet-platelet interaction and therefore aggregation [7, 13, 15], including during bacterial infection [8, 9]. Platelet surface integrin αIIbβ3 expression levels are enhanced as a result of platelet activation by means of α-granule release [17]. We thus investigated the expression patterns of FcγRIIA and αIIbβ3 by flow cytometry after antibody labeling. Platelet expression levels of FcγRIIA were not altered in response to *M. circinelloides* NRRL3631 spores under aggregating conditions (Figure 2C). In contrast, surface αIIbβ3 levels were increased in response to *M. circinelloides* NRRL3631 spores under the same conditions (Figure 2D).

Thus, this shows that platelets respond to fungal spores through interaction with the IgG receptor FcγRIIA and integrin αIIbβ3. Subsequently, αIIbβ3 surface levels are upregulated to further support platelet aggregation.

Mucor circinelloides NRRL3631 activates Src and Syk signaling cascades and induces platelet activation supported by secondary mediators TxA_2 and ADP.

The two identified interaction platelet receptors FcγRIIA and αIIbβ3 both activate downstream Src and Syk tyrosine kinases [16]. To test if the interaction of *M. circinelloides* NRRL3631 with platelets also results in the activation of Src and Syk, platelets were treated with Src inhibitor Dasatinib, and Syk inhibitor PRT-060318 prior to *M. circinelloides* NRRL3631 exposure. Dasatinib and PRT-060318 inhibited platelet aggregation in response to *M. circinelloides* NRRL3631 (Figure 3A).

The platelet α-granules activation marker CD62P (P-selectin) is expressed on the platelet surface upon platelet activation [18]. Platelets were antibody-labeled for P-selectin, and expression levels measured pre- and postexposure to *M. circinelloides* NRRL3631 spores using flow cytometry. P-selectin expression levels were markedly increased following exposure to *M. circinelloides* spores indicating that platelets are activated by *M. circinelloides* NRRL3631 under aggregating conditions (Figure 3Bi). Under non-aggregating conditions by means of eptifibatide platelet pre-treatment, P-selectin expression was negligible, suggesting that αIIbβ3 activation is crucial to platelet activation by *M. circinelloides* NRRL3631 interaction (3Bii).

Whilst platelet aggregation in response to the agonist TRAP occurs rapidly within minutes, platelet aggregation in response to spores is characterised by a lag-phase (Figure 3C). This long incubation time needed to detect spore-induced platelet aggregation suggests that secondary mediators might be required for the platelet response. The secondary mediators, thromboxane A_2 (TxA_2) and adenosine 5'-diphosphate (ADP), are released upon platelet activation and act to support clot consolidation [19, 20]. Platelets were treated with TxA_2

inhibitor, indomethacin, and ADP inhibitor, apyrase, prior to *M. circinelloides* NRRL3631 spore exposure. TxA_2 and ADP inhibition independently (e.g. at 1:10 spore:platelet ratio: 70.0 ± 6.0%; (+)indomethacin: 24.3 ± 6.8%; (+)apyrase: 37.3 ± 12.2%) and in combination (e.g. at 1:10 spore: platelet ratio (+)indomethacin(+)apyrase: 18.3 ± 6.1%) reduced *M. circinelloides* NRRL3631-induced platelet aggregation (Figure 3D).

In summary, these data demonstrate that spore interaction with platelet receptors activates downstream Src and Syk tyrosine kinases leading to platelet activation including the release of α-granules shown by increased surface expression of the activation marker P-selectin and by increased expression of integrin αIIbβ3. Activation is supported by secondary mediators TxA_2 and ADP.

Mucor circinelloides NRRL3631 spore developmental stage impacts on platelet aggregation

Mucormycete spores undergo both metabolic and structural changes during development from spore to hyphae [21]. The host encounters these different developmental stages during infection, thus requiring a dynamic response. We hypothesized that the developmental stage of the *M. circinelloides* NRRL3631 spore would influence its platelet aggregation potential.

We first identified stages of germination at which a significant increase in spore size occurs. Resting spores at the start of germination, displayed a surface area of 19.8 ± 3.0 μm^2 (Figure 4A). Spores significantly increased in size at 3 hours germination (34.1 ± 6.8μm^2; $P<0.05$), and furthermore at 6 hours germination (49.9 ± 15.4 μm^2; $P<0.05$). Hyphae formation was observed on spores at 48 hours germination (hyphae formation efficiency: 27.5 ± 8.5%) (Figure 4B).

We investigated *M. circinelloides* NRRL3631 spores at 0, 3 and 6 hours germination, and hyphae at 48 hours germination in PRP, and showed that the developmental stage of *M. circinelloides* NRRL3631 influences its platelet aggregation potential (Figure 4C).

At the beginning of germination, *M. circinelloides* NRRL3631 induced significant platelet aggregation (1:10: 15.6 ± 10.2%; $P<0.05$), however to a lesser degree than spores at 3 hours germination (1:10: 56.0 ±12.3%; $P<0.01$). As *M. circinelloides* NRRL3631 spore development progressed beyond 3 hours germination, platelet aggregation potential declined (6 hours germination; 1:10: 32.6 ± 14.3%). Moreover, *M. circinelloides* NRRL3631 spores exhibiting hyphae formation at 48 hours germination induced negligible platelet aggregation (1:10: 2.80 ± 1.11%).

Together, this shows that the platelet response to *M. circinelloides* varies with the spore developmental stage, indicating a dynamic interaction pattern that needs to be considered for mucormycosis treatment approaches.

Discussion

Clinical management of mucormycosis remains a challenge whilst the disease incidence is on the rise. Many of our antifungal agents are ineffective against mucormycetes and associated with toxic side effects. The gold standard therapy still is surgical debridement often leading to long-term disability. Together, this results in extremely high mortality in patients with mucormycosis and highlights the need for more effective therapeutic strategies.

Thrombosis is a hallmark of mucormycosis. It is currently not clear if thrombus formation is beneficial for the patient by containing the fungus or detrimental due to reduced oxygen supply and subsequent tissue necrosis. Ability to manipulate the platelet – spore interaction,

either by enhancing or inhibiting, might be a promising medical approach to improve patient outcome.

To identify potential targets for medical intervention, we here elucidated components of the signaling pathway underlying platelet – spore interaction. Our data demonstrate that platelets form aggregates surrounding mucormycete spores, the infectious agent of mucormycosis. This aggregation is dose-dependent. Platelet activation by spores is mediated through the platelet receptors FcγRIIA and αIIbβ3 and, the Syk/Src signaling cascade to induces α-granules release. This activation is supported by the secondary mediators TxA_2 and ADP. The dose-dependent nature of platelet aggregation (Figure 1B) mimics the all-or-nothing response previously described for *E. coli* and Gram-positive bacteria [8, 9] suggesting a positive feedback mechanism supporting platelet aggregation. Inhibitor studies suggest that FcγRIIA is an essential receptor for platelet activation in response to fungal spores (Figure 2A). As no aggregation was observed in washed platelets, this suggests that one or more plasma factor(s) mediate this interaction. The current literature suggests these are IgG, by forming an immune complex with spores, to interact with FcγRIIA and fibrinogen as a bridging molecule to interact with αIIbβ3 [9]. Whilst we currently do not know the fungal cell wall components interacting with platelets and whether this interaction is direct or indirect, binding of fibrinogen has been reported to *Candida albicans* cell wall [22]. Due to the constant exposure of humans to these environmentally ubiquitous fungal spores, it is also highly likely that humans have circulating antibodies to support the platelet-spore interaction. Research on bacterial interaction with platelets has shown that several platelet receptors are required for efficient platelet activation [8, 9, 23-25]. Similarly, platelet activation after spore encounter also requires integrin αIIbβ3 (Figure 2B) and secondary mediators TxA_2 and ADP supporting the

notion of positive feedback mechanisms reinforcing initial FcγRIIA activation. Inside-out signaling has previously been reported for αIIbβ3 to induce secondary platelet-platelet aggregation after initial activation in response to a stimulus through FcγRIIA [8, 26, 27]. Thus both receptors might have dual functionality in initiating as well as amplifying thrombus formation. Together, these signaling events correspond to those reported previously for a range of bacterial interaction with platelets [8, 9] and thus indicate that the platelet response to infectious particles is conserved.

The conserved nature of the platelet activation pathway in response to pathogens might be indicative of a protective innate immune function performed by platelets. Attachment of platelets to fungal hyphae has shown to result in hyphal damage and reduced viability [6, 28]. Yet, a potential detrimental outcome due to excessive platelet aggregation causing tissue necrosis, similar to exaggerated inflammatory responses needs to be considered. The release of immune-stimulatory effectors such as pro-inflammatory and pro-necrotic factor TNF-α and phagocyte chemoattractant TGF-β in α-granules [29] would support this idea. In the context of mucormycosis, thrombocytopenia has been suggested as factor for severe disease and poor patient outcome [30, 31] suggesting that thrombus formation contributes to disease pathology.

During filamentous fungal infections, platelets encounter a range of morphological structures. Whilst initial infection often occurs through inhalation of spores, the propagules then undergo a developmental program of metabolic and physiological changes to form invasive hyphae. During this germination process, water uptake causes the spore to 'swell' and undergo a change in both size and structural composition. As mucormycete spores germinate, there is a depletion of melanin from the outer surface exposing a glucan-rich outer wall [32]. In the latter stages of germination hyphae are formed, the outer wall of

which is chitosan-rich and formed by the germinating spore's inner wall [32]. During the preliminary stages of *M. circinelloides* NRRL3631 germination we saw a stark increase in platelet aggregation potential, reaching a peak at 3 hours germination, where spores are between resting and maximal swelling stage. Surprisingly, platelet aggregation potential of *M. circinelloides* NRRL3631 declined towards the latter stages of germination as spores reach their maximal swelling stage, and *M. circinelloides* hyphal structures appeared to induce negligible platelet aggregation altogether. Two plausible explanations for the change in mucormycete spore platelet aggregation potential during germination are: (I) compositional changes of the germinating mucormycete spore, and (II) the secretion of platelet inhibitory fungal secretory factors. The secretion of platelet inhibitory fungal secretory factor has been shown in *Candida albicans* [33]. *C. albicans* activates platelets but inhibits aggregation to fibrinogen, in part via the fungal secretory factor gliotoxin [33].

In summary, this is the first analysis of the signaling underlying platelet aggregation in response to fungi and thus providing a better understanding of this interaction. The interaction with fungi is dependent on the developmental stage of the fungus, which might lead to different outcomes of this interaction that can be beneficial as well as detrimental to the host. This needs to be carefully considered for the clinical management of patients. During mucormycosis, platelets have the potential to inhibit the germination process of mucormycetes [6]. We identify several receptors and the downstream signaling components that could be targeted with already available medical interventions as preventative measures inhibiting disease onset (i.e. spore germination) or by targeting thrombus formation to improve current disease outcome.

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Author Contributions

Conceived and designed the experiments: KV, SPW, HG, MZ. Performed the experiments: HG, AS-R, MZ. Analyzed the data: HG, SW, KV. Wrote the paper: HG, KV. Critically reviewed the manuscript: KV, SPW.

Declaration of Interests

Conflict of Interest

The authors report no declarations of interest.

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Figure Legends

Figure 1 *Mucor circinelloides* NRRL3631 induces platelet aggregation in whole blood and PRP. (A) *M. circinelloides* NRRL3631 spores (green) interact with human platelets (magenta)

to form large complex structures. We imaged a total of 17 aggregates and show here images for two of those. These images show (i) an optical section through a platelet and spore aggregate and (ii) the maximal intensity projection of the (single view diSPIM) 3D volume of the spore-induced platelet aggregate. Images are representative of 17 aggregates from two independent experiments. Complexes were visualized by dual inverted Selective Plane Illumination Microscopy (diSPIM) using 40x objectives and analysed on Image J (FIJI); scale bar: 10µm. **(B)** Platelet aggregation in response to TRAP, PBS and *M. circinelloides* NRRL3631 over increasing spore:platelet ratios was measured in PRP using light-transmission aggregometry over 30 min. Significant platelet aggregation was induced by *M. circinelloides* NRRL3631 at a 1:10 and 1:20 spore:platelet ratio. Notably *M. circinelloides* NRRL3631 induced platelet aggregation in a concentration-dependent manner. Data shown are mean±SEM of five independent experimental repeats; **P<0.01, Mann-Whitney U test. **(C)** Platelet aggregation in response to TRAP, PBS and *Mucor circinelloides* NRRL3631 spores was measured in whole blood using multiple-electrode aggregometry over 30 min. *M. circinelloides* NRRL3631 spores induced significant platelet aggregation in whole blood. Data shown are mean±SEM of five independent experimental repeats; *P<0.05, Mann-Whitney U test.

Figure 2 Platelet aggregation in response to *Mucor circinelloides* NRRL3631 is supported by the FcγRIIA receptor and integrin αIIbβ3 (A) *Mucor circinelloides* NRRL3631 is recognized by the FcγRIIA receptor. Platelet aggregation in response to *M. circinelloides* NRRL3631 in the presence and absence of FcγRIIA blocking mAB IV.3 was measured in PRP using light-transmission aggregometry over 30 min. mAb IV.3 significantly inhibited platelet aggregation in response to *M. circinelloides* NRRL3631. Data shown are mean ±SEM of five

independent experimental repeats; **P<0.01, Mann-Whitney U test. **(B)** Platelet aggregation in response to *Mucor circinelloides* NRRL3631 is supported by the αIIb**β**3 integrin. Platelet aggregation in response to *M. circinelloides* NRRL3631 spores in the presence and absence of αIIbβ3 inhibitor, eptifibatide, was measured in PRP using light-transmission aggregometry over 30 min. Eptifibatide significantly inhibited platelet aggregation in response to *M. circinelloides* NRRL3631. Data shown are mean ±SEM of five independent experimental repeats; **P<0.05, **P<0.01, Mann-Whitney U test. **(C)** *Mucor circinelloides* NRRL3631-platelet interaction does not affect FcγRIIA platelet surface expression but does **(D)** increase αIIbβ3 platelet surface expression. Platelets were labelled for FcγRIIA, using conjugated anti-human CD32 + anti-mouse 2° Alexa 488, and αIIbβ3, using APC-Mouse Anti-Human CD41a. Platelet surface expression of FcγRIIA and αIIbβ3 were read using flow cytometry.

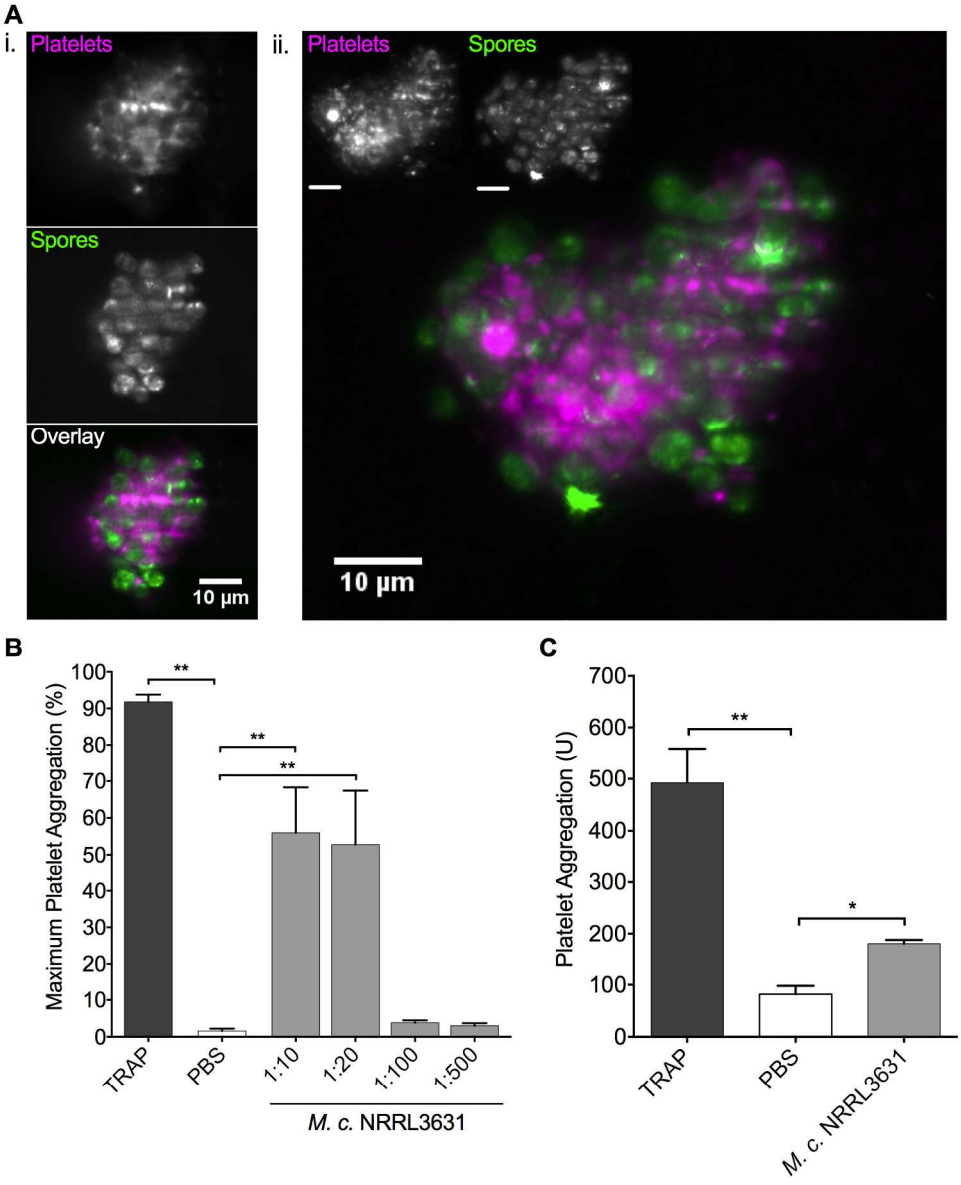
Figure 3 *Mucor circinelloides* NRRL3631 activates Src and Syk signaling cascades and induces platelet activation supported by secondary mediators TxA_2 and ADP (A) *Mucor circinelloides* NRRL3631 activates the Src and Syk signaling cascades. Platelet aggregation in response to *M. circinelloides* NRRL3631 in the presence and absence of Src receptor inhibitor, dasatinib (4 μM), and Syk inhibitor, PRT-060318 (10 μM), was measured in PRP using light-transmission aggregometry over 30 min. Dasatinib significantly inhibited platelet aggregation in response to *M. circinelloides* NRRL3631. PRT-060318 also significantly inhibited platelet aggregation in response to *M. circinelloides* NRRL3631. Data shown are mean ±SEM of five independent experimental repeats; **P<0.01, Mann-Whitney U test. **(B)** *M. circinelloides* NRRL3631 activates platelets under (i) aggregating conditions but not under (ii) non-aggregating conditions. Platelet aggregation in response to *M. circinelloides*

NRRL3631 in the presence and absence of αIIbβ3 inhibitor, eptifibatide, and was measured in PRP using light-transmission aggregometry over 30 min. Platelets were labelled for CD62P activation marker using CD62P-FITC and surface expression read using flow cytometry. (i) Under aggregating conditions CD62P expression is enhanced following *M. circinelloides* NRRL3631 exposure. (ii) Under non-aggregating conditions, change in CD62P expression is undetectable following *M. circinelloides* NRRL3631 exposure. **(C)** Example aggregation traces showing fast initiation of platelet aggregation in response to the agonist TRAP and a lag phase before induction of platelet activation in response to fungal spores. **(D)** Secondary mediators TxA_2 and ADP play a role in *M. circinelloides* NRRL3631-induced platelet aggregation. Platelet aggregation in response to *M. circinelloides* NRRL3631 in the presence and absence of TxA_2 inhibitor, indomethacin (30 μM), and ADP inhibitor, apyrase (2 U), was measured in PRP using light-transmission aggregometry over 30 min. Indomethacin significantly inhibited platelet aggregation in response to *M. circinelloides* NRRL3631, and apyrase markedly reduced platelet aggregation in response to *M. circinelloides* NRRL3631. Combined indomethacin and apyrase treatment significantly further reduced platelet aggregation in response to *M. circinelloides* NRRL3631. Data shown are mean±SEM of three independent experimental repeats; *P<0.05, **P<0.01, One-way ANOVA with post hoc Dunnett's multiple comparison test.

Figure 4 *Mucor circinelloides* NRRL3631 spore developmental stage impacts on platelet aggregation (A) *Mucor circinelloides* spore development. *M. circinelloides* NRRL3631 spore swelling was investigated over 0, 3 and 6 hours. Spores were visualized by DIC light microscopy at 60x magnification; scale bar: 5μm. 100 spores from each time point were analysed on ImageJ: a length and width measurement (in μm) was taken from each spore

and the spore area calculated ((length/2)*(width/2)*π)). *M. circinelloides* NRRL3631 spores exhibited significant increase in size from 0 to 3 hours germination, and from 3 to 6 hours germination. Data shown are pooled data points with mean±SD of three independent experimental repeats, each analysing the surface area of 100 spores per incubation period; *** P<0.001, one-way ANOVA with post hoc Dunnett's multiple comparison test. **(B)** *M. circinelloides* hyphae formation efficiency. *M. circinelloides* NRRL3631 hyphae formation was studied at 48 hours germination. Spores were visualized by DIC light microscopy at 60x magnification; scale bar: 10µm. 100 spores at 48 hours germination were analysed on ImageJ to determine the percentage of hyphae per 100 spores. Data shown are mean±SEM from three independent experimental repeats; scale bar: 10µm. **(C)** Platelet aggregation potential is dependent on the developmental stage of *Mucor circinelloides* NRRL3631. Platelet aggregation in response to *M. circinelloides* NRRL3631 spores over progressive developmental stages, was measured in PRP using light-transmission aggregometry over 30 min. *M. circinelloides* NRRL3631 spores at time zero germination induced significant platelet aggregation at a 1:10 spore:platelet ratio. *M. circinelloides* NRRL3631 spores at 3 hours germination induced significant platelet aggregation at a 1:10 and 1:20 spore:platelet ratio (data from Figure 2B). *M. circinelloides* NRRL3631 spores at 6 hours induced aggregation to a lesser degree than those at 3 hours germination, and spores at 48 hours germination induced negligible platelet aggregation. Data shown are mean±SEM of five independent experimental repeats; *P<0.05, **P<0.01, Kruskal-Wallis test.

Supplementary Movie 1 Platelet spore aggregate. 3D visualization of the platelet spore aggregate shown in Figure 1 Aii.



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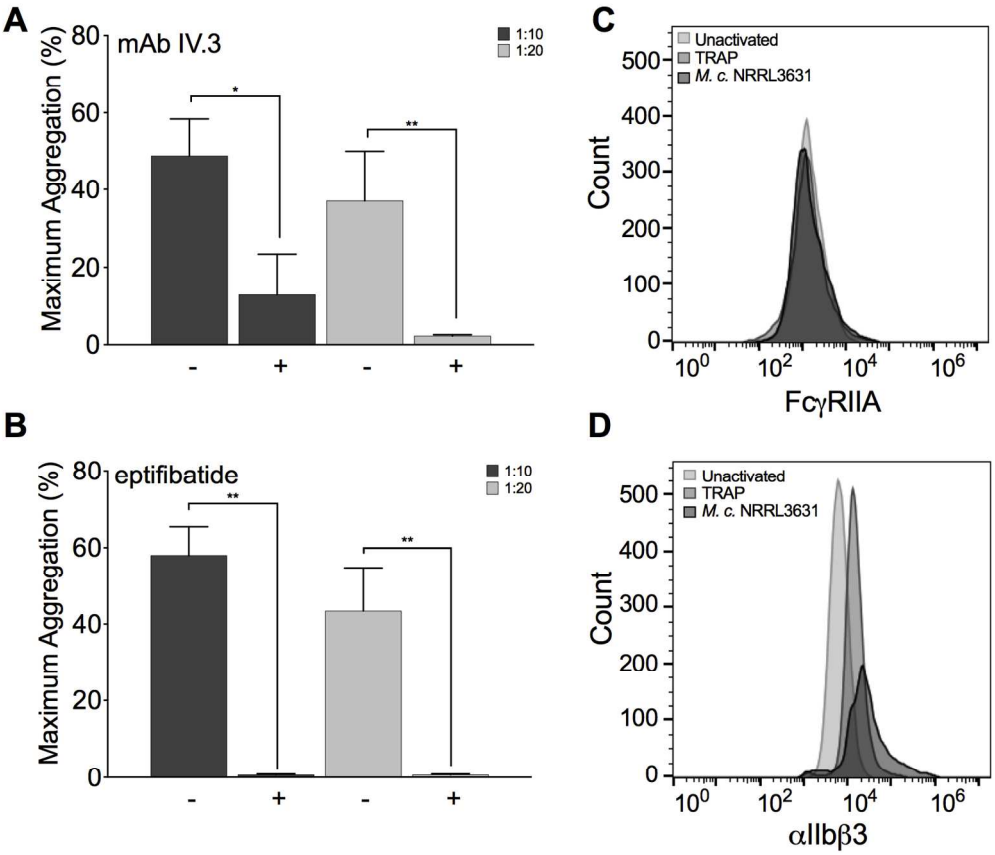

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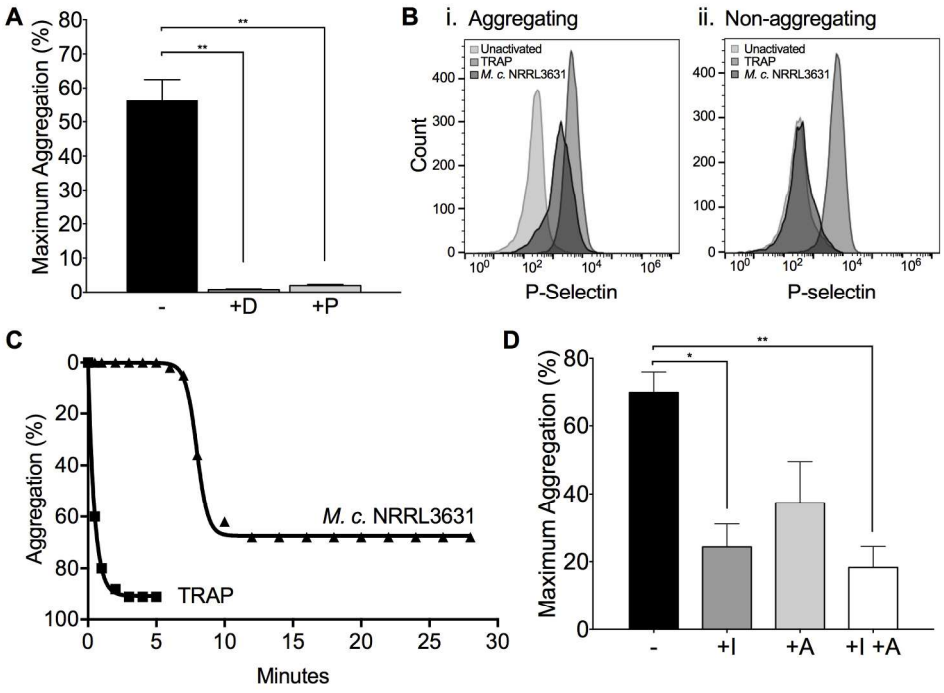

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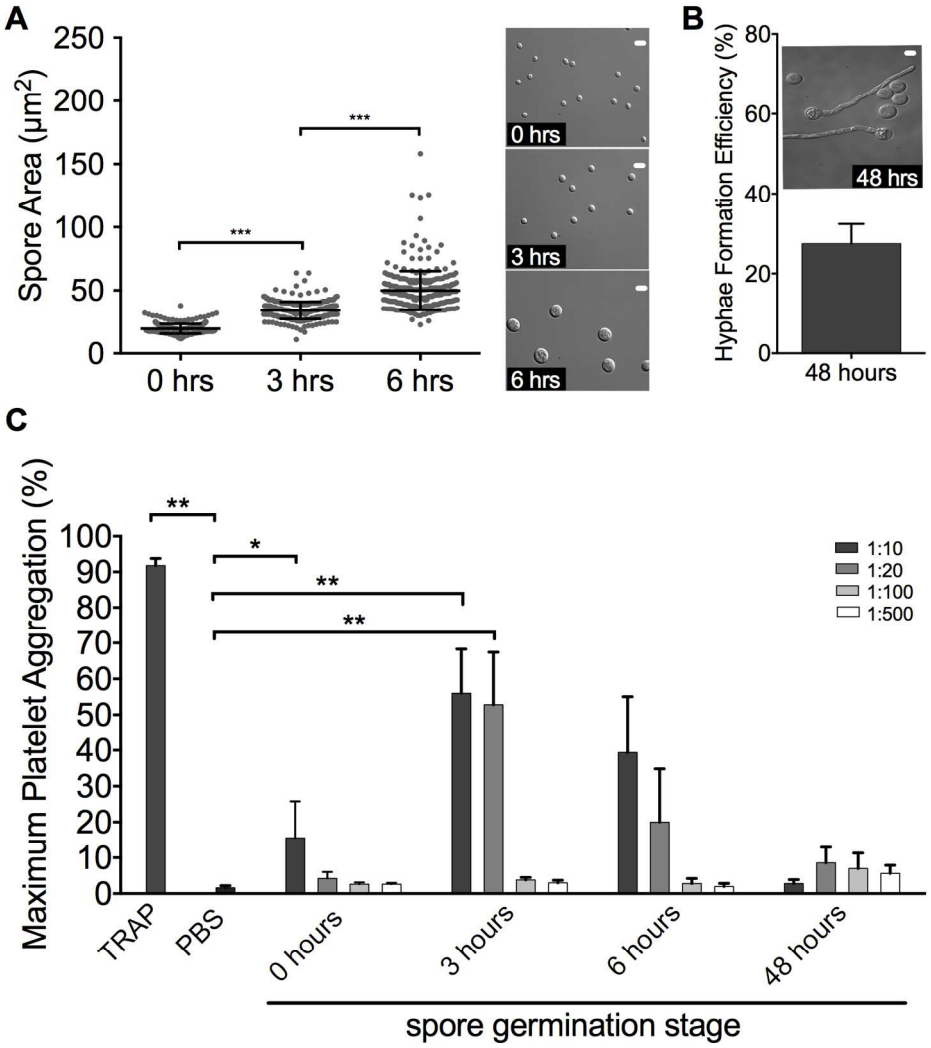

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