

Mucor circinelloides induces platelet aggregation through integrin α IIb β 3 and Fc γ RIIA

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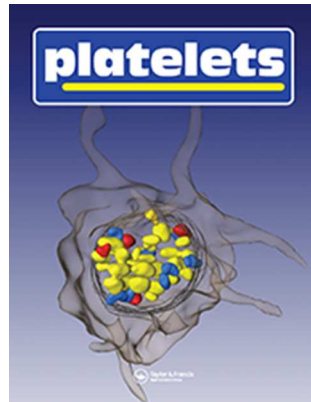
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Mucor circinelloides induces platelet aggregation through integrin α IIb β 3 and Fc γ RIIA

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3 1 **Title:** *Mucor circinelloides* induces platelet aggregation through integrin $\alpha\text{IIb}\beta\text{3}$ and Fc γ RIIA

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14 6 **Short title:** Platelet signaling during mucormycosis

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44 19 **Key words:** mucormycetes, mucormycosis, *Mucor circinelloides*, platelets, thrombosis,
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3 25 **Abstract**
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5 26 Thrombosis is a hallmark of the fatal fungal infection mucormycosis. Yet, the platelet
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7 27 activation pathway in response to mucormycetes is unknown. In this study we determined
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9 28 the platelet aggregation potential of *Mucor circinelloides* (*M. circinelloides*) NRRL3631,
10
11 29 characterized the signaling pathway facilitating aggregation in response to fungal spores,
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14 30 and identified the influence of the spore developmental stage upon platelet aggregation
15
16 31 potential. Using impedance and light-transmission aggregometry, we showed that *M.*
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18 32 *circinelloides* induced platelet aggregation in whole blood and in platelet-rich plasma,
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20 33 respectively. The formation of large spore-platelet aggregates was confirmed by light-sheet
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22 34 microscopy, which showed spores dispersed throughout the aggregate. Aggregation
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24 35 potential was dependent on the spore's developmental stage, with the strongest platelet
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26 36 aggregation by spores in mid-germination. Inhibitor studies revealed platelet aggregation
27
28 37 was mediated by the low affinity IgG receptor FcγRIIA and integrin αIIbβ3; Src and Syk
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30 38 tyrosine kinase signaling; and the secondary mediators TxA₂ and ADP. Flow cytometry of
31
32 39 antibody stained platelets showed that interaction with spores increased expression of
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34 40 platelet surface integrin αIIbβ3 and the platelet activation marker CD62P. Together, this is
35
36 41 the first elucidation of the signaling pathways underlying thrombosis formation during a
37
38 42 fungal infection, highlighting targets for therapeutic intervention.
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49 Introduction

50 The incidence of invasive fungal infections is on the rise, and this is attributed to the
51 increasing population of immunosuppressed individuals under the influence of modern
52 medicine [1-4]. Mucormycosis - a previously uncommon infection - has grown in occurrence
53 to become the third most common invasive fungal infection after aspergillosis and
54 candidiasis, respectively [5]. Infection is caused by species of the Mucorales order, with
55 *Rhizopus* spp. and *Mucor* spp. being the most common causative agents [1-4]. Prognosis for
56 this severe fungal infection is poor, with studies reporting mortality rates in the range of 60-
57 100% [4]. This staggeringly high mortality rate is reflective of the aggressive nature of
58 infection, and the poor efficacy of antifungal therapeutics currently employed [2, 3]. Risk
59 factors identified for mucormycosis include uncontrolled diabetes mellitus, diabetes
60 mellitus with ketoacidosis, organ transplantation and neutropenia [3, 4, 6].

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

68 The platelet IgG receptor FcγRIIA and integrin αIIbβ3 have been highlighted as crucial to
69 platelet activation in response to both Gram-negative and Gram-positive bacteria [7-9].

70 While the proteins that give rise to bacterial-platelet interaction are strain-specific, platelet
71 activation is mediated by a common pathway consisting of FcγRIIA, Src and Syk tyrosine
72 kinase activation, αIIbβ3 engagement and the secondary mediators thromboxane A₂ (TxA₂)

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3 73 and adenosine 5'-diphosphate (ADP) [8, 9]. There is currently little information on the
4
5 74 interaction between platelets and mucormycetes under physiological conditions and the
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7 75 molecular signaling pathways underlying this interaction. An improved understanding of the
8
9 76 signaling underlying thrombus formation during mucormycosis might offer novel
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11 77 therapeutic targets to improve current treatment approaches and thus patient outcome.
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14 78 Hence, we investigated the interaction between the clinical isolate *Mucor circinelloides*
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16 79 NRRL3631 and platelets. We show that platelets form aggregates with fungal spores
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18 80 dependent on the spore developmental stage. Spore aggregation is mediated through the
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20 81 platelet IgG receptor FcγRIIA, integrin αIIbβ3 and Src and Syk tyrosine kinases, and
21
22 82 secondary mediators TxA₂ and ADP. Platelet activation is also associated with increased
23
24 83 expression of platelet surface integrin αIIbβ3 and the platelet activation marker CD62P.
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26 84 Together, this provides the first elucidation of the signaling pathways underlying thrombosis
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28 85 formation during mucormycosis highlighting potential strategies to interfere with thrombus
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30 86 formation.
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37 88 **Methods**

39 89 *Fungal strains and growth conditions*

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42 90 The *Mucor circinelloides* strain used was *Mucor circinelloides* f. sp. *lusitanicus* strain
43
44 91 NRRL3631, a clinical isolate [10]. The strain was grown on Sabouraud dextrose agar (Merck-
45
46 92 Millipore, Billerica, MA, USA) at room temperature for 7 days prior to use.
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51 94 *Spore preparation for aggregation assays*

53
54 95 Spores were collected in phosphate buffered saline (PBS) and centrifuged at 1811 x g for
55
56 96 three min. The spore pellet was washed twice with PBS and then re-suspended in Sabouraud
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3 97 broth (Sigma-Aldrich, St. Louis, MO, USA). Spores were cultured at 37°C with shaking at 45
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5 98 rpm, over 0, 3, 6 and 48 hours. Following incubation, spore suspensions were centrifuged at
6
7 99 1811 x g for three min. The spore pellet was washed twice, and re-suspended in PBS at
8
9
10 100 concentrations to allow for 1:10, 1:20, 1:100 and 1:500 spore:platelet ratios. Spore
11
12 101 suspensions were kept on ice until used for aggregometry.

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15 16 103 *Blood preparation*

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19 104 Blood samples were collected from healthy human donors into 4% (w/v) citrate (Sigma-
20
21 105 Aldrich, St. Louis, MO, USA). The study design was approved by the University of
22
23 106 Birmingham's research ethics committee (ERN_11-0175). Blood was centrifuged at 200 x g
24
25
26 107 for 20 min and platelet-rich plasma (PRP) collected. For light-transmission aggregometry, a
27
28 108 PRP platelet count was taken using Coulter® Z2 Particle Counter in triplicate and averaged.
29
30 109 For multiple-electrode aggregometry, a whole blood platelet count was taken using Sysmex
31
32 110 XN-1000 Hematology Analyzer.

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36 37 112 *Platelet aggregometry in PRP*

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39 113 PRP was incubated at 37°C for 1 min and then stirred for 1 min. *M. circinelloides* NRRL3631
40
41 114 suspension containing appropriate spore numbers for 1:10, 1:20, 1:100 and 1:500
42
43 115 spore:platelet ratios was added to the PRP, and platelet aggregation recorded over 30 min
44
45 116 using light-transmission aggregometer PAP-8E (Bio/Data Corporation, Horsham, PA, USA).
46
47 117 As a positive control, Thrombin Receptor-Activating Peptide (TRAP; Severn Biotech,
48
49 118 Kidderminster, UK) (100 µM) was added to PRP, and as a negative control PBS was added.

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53 54 120 *Platelet aggregometry in whole blood*

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3 121 Whole blood was added to sodium chloride and incubated for 3 min at 37°C. *M.*
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5 122 *circinelloides* NRRL3631 at 3 hours germination, and at a spore concentration allowing for a
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7 123 spore:platelet ratio of 1:10, was added and platelet aggregation recorded over 30 min using
8
9 124 multiple electrode aggregometer, Multiplate® Analyzer (Roche, Basel, Switzerland) . TRAP
10
11 125 was added to PRP and used as a positive control, and PBS was used as a negative control.
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16 127 *Cell staining and microscopy*

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18 128 Platelets were stained with CellMask Deep Red Plasma Membrane Stain (1:2000, Thermo
19
20 129 Fisher Scientific, Waltham, MA, USA), and *M. circinelloides* NRRL3631 spores with
21
22 130 Concanavalin A, Alexa fluor™ 488 conjugate (300 µg ml⁻¹, Thermo Fisher Scientific, Waltham,
23
24 131 MA, USA). Formed aggregates were immobilized on poly-L-lysine (Sigma) coated rectangular
25
26 132 coverslips (25x50 mm), fixed using 4% PFA, washed and placed in the imaging chamber filled
27
28 133 with PBS. Orthogonal views of the aggregates were acquired on a Marianas LightSheet
29
30 134 (Intelligent Imaging Innovations, Denver, CO, USA), a dual inverted Selective Plane
31
32 135 Illumination Microscope (diSPIM) which uses two perpendicular 0.8 NA, 40x water
33
34 136 immersion objectives to excite and detect fluorescence in an alternating duty cycle.
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36 137 Volumes of 200 image planes were captured using both arms sequentially in slice scan mode,
37
38 138 with a step size of 0.5 µm, for both 488 nm and 640 nm excitation wavelengths, on ORCA-
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40 139 Flash4.0 V3 sCMOS cameras (Hamamatsu), driven by SlideBook 6.0 software (Intelligent
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42 140 Imaging Innovations, Denver, CO, USA).
43
44 141 Image analysis was performed using Fiji software [11], and a maximal intensity projection
45
46 142 was made of a single volume view. For the supplementary movie, the two orthogonal
47
48 143 volumes were registered and deconvolved (joint-deconvolution) using the multiview
49
50 144 Reconstruction plugin [12] and visualized by rotating the 3D volume around the y-axis.
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5 146 *Inhibitor treatments*6
7 147 Inhibition of $\alpha\text{IIb}\beta\text{3}$ was achieved by pre-incubating platelets with 9 μM eptifibatide8
9 148 (GlaxoSmithKline, Coventry, UK) for 1 min at room temperature. Fc γ RIIA was blocked with10
11 149 10 μM mAbIV.3 (hybridoma from American Tissue Culture Corporation (Manassas, Virginia),12
13 150 Src and Syk tyrosine kinases with 4 μM dasatinib (D-3307, LC Laboratories, Woburn, MA,14
15 151 USA) and 10 μM PRT-060318 (AdipoGen Life Sciences, Liestal, Switzerland) respectively, and16
17 152 secondary mediators TxA₂ and ADP with 30 μM Indomethacin (I7378, Sigma-Aldrich, St.18
19 153 Louis, MO, USA) and 2 U Apyrase (A6535, Sigma-Aldrich, St. Louis, MO, USA) respectively.20
21 154 Platelet aggregation was assessed by light-transmission in a PAP-8E aggregometer over 3022
23 155 min.24
25 15626
27 157 *Platelet receptor labeling*28
29 158 Conjugated anti-human CD32 (#60012; mouse; StemCell Technologies, Vancouver, Canada)30
31 159 and anti-mouse 2° Alexa Fluor® 488 conjugate (A10680; goat; Invitrogen, Carlsbad, CA, USA)32
33 160 was used to label Fc γ RIIA. Anti-Human CD41a-APC (BD559777; mouse; BD Biosciences, San34
35 161 Jose, CA, USA) was used to label $\alpha\text{IIb}\beta\text{3}$. CD62P-FITC (BC A07790; mouse; Beckman Coulter,36
37 162 Brea, CA, USA) was used to label CD62P. Platelet aggregation was assessed by light-38
39 163 transmission in a PAP-8E aggregometer over 30 min. Receptor antibody labels were added40
41 164 to samples for 30 min prior to flow cytometry assays. Flow cytometry analysis was42
43 165 conducted using BD Accuri™ C6 Plus (BD Biosciences, Oxford, UK). Samples were run for44
45 166 10000 events, and cell count vs fluorescence recorded.46
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49 168 *Statistical analysis*

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3 169 Statistical analysis was conducted using GraphPad Prism 6.0. Data is presented as mean \pm
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5 170 SEM, unless stated otherwise. Data was analysed using the one-way analysis of variance
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7 171 (ANOVA) with post hoc Dunnett's multiple comparison test, Mann-Whitney *U*-test or
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9 172 Kruskal-Wallis test with post hoc Dunn's multiple comparison test, as indicated in the figure
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11 173 legends. $P < 0.05$ was deemed to be statistically significant. Technical repeats were
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13 174 conducted at $n=3$, and biological repeats at $n=5$ unless otherwise stated.
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19 176 **Results**

21 177 ***Mucor circinelloides* NRRL3631 induces platelet aggregation in whole human blood and**

23 178 **PRP**

25 179 Thrombus formation during mucormycosis might be induced by fungal invasion of blood
26
27 180 vessels, and thus formation of a platelet-reactive surface, or direct interaction between
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29 181 platelets and the fungus. Therefore, we investigated the potential of the clinical isolate
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31 182 *Mucor circinelloides* NRRL3631 to induce platelet aggregation qualitatively and
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33 183 quantitatively.
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37 184 Initially, we visualized spore-platelet interaction by dual inverted Selective Plane
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39 185 Illumination fluorescence microscopy (diSPIM), which gives isotropic resolution in 3
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41 186 dimensions. *M. circinelloides* spores were stained with Concanavalin A, Alexa Fluor™ 488
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43 187 conjugate and platelets in plasma-rich platelets (PRP) with CellMask Deep Red Plasma
44
45 188 Membrane Stain. *M. circinelloides* spores were incubated in PRP at a 1:10 spore:platelet
46
47 189 ratio under stirring conditions in the PAP-8E aggregometer and formed aggregates were
48
49 190 fixed onto glass coverslips. Fluorescence microscopy revealed *M. circinelloides* NRRL3631
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51 191 spores to be contained within large platelet aggregates with individual spores being
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53 192 surrounded by platelets (Figure 1Ai & ii and Supplementary Movie 1).
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3 193 We then quantified this interaction by light transmission aggregometry. Experiments were
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5 194 performed in PRP using 1:10, 1:20, 1:100 and 1:500 spore:platelet ratios. The agonist
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7 195 thrombin-related-activating peptide (TRAP) induced maximal platelet aggregation ($91.8 \pm$
8
9
10 196 2.0%). *M. circinelloides* NRRL3631 induced platelet aggregation in a concentration-
11
12 197 dependent manner, with significant platelet aggregation occurring at 1:10 and 1:20
13
14 198 spore:platelet ratios (1:10: $56.0 \pm 12.3\%$ and 1:20: $52.8\% \pm 14.6\%$ platelet aggregation;
15
16 199 $P < 0.01$) (Figure 1B).

18
19 200 To examine the physiological relevance of platelet aggregation in response to
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21 201 mucormycetes, we determined the platelet aggregation potential of *M. circinelloides*
22
23 202 NRRL3631 in whole blood using impedance aggregometry. *M. circinelloides* NRRL3631 was
24
25 203 added to whole blood at a spore:platelet ratio of 1:10, and platelet aggregation assessed. *M.*
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27 204 *circinelloides* NRRL3631 induced significant platelet aggregation ($180 \pm 16U$; $P < 0.05$) in
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29 205 comparison to PBS ($81 \pm 16U$) (Figure 1C).

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33 206 In summary, these data show that platelets interact with mucormycete spores to form large
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35 207 platelet-spore aggregates. This indicates that thrombus formation during mucormycosis can
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37 208 be mediated by a direct platelet response to fungal spores.

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42 210 **Platelet aggregation in response to *Mucor circinelloides* NRRL3631 is supported by the**
43
44 211 **FcγRIIA receptor and integrin αIIbβ3**

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46 212 Having shown that platelets form aggregates with mucormycete spores, we next wanted to
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48 213 identify the receptor(s) and downstream signaling components mediating this interaction.
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50 214 Aggregation studies with washed platelets did not induce aggregation suggesting the
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52 215 requirement of a serum factor. Therefore, this study performed aggregations in PRP with
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54 216 the focus to elucidate the receptors and downstream signaling mediators. The platelet low

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3 217 affinity immune receptor, FcγRIIA has been identified as a key receptor in platelet activation
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5 218 in response to bacterial pathogen through the result of antibody-pathogen interaction [8, 9,
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7 219 13, 14]. We hypothesized that FcγRIIA plays a key role in the induction of platelet
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9 220 aggregation by *M. circinelloides* NRRL3631 in view of the dependency on plasma. In addition,
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11 221 platelet integrin αIIbβ3, the most abundant platelet surface glycoprotein, has been shown
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13 222 to be essential for platelet activation by bacteria [13-16]. To investigate whether platelet
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15 223 aggregation in response to *M. circinelloides* NRRL3631 is supported by FcγRIIA and αIIbβ3
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17 224 engagement, platelets were treated with the FcγRIIA blocking mAb IV.3 or αIIbβ3 inhibitor
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19 225 eptifibatide, prior to *M. circinelloides* NRRL3631 exposure. Blocking FcγRIIA significantly
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21 226 decreased platelet aggregation in response to *M. circinelloides* NRRL3631 (1:10 (-)mAb IV.3:
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23 227 $48.8 \pm 9.58\%$; (+) mAb IV.3: $12.8 \pm 10.57\%$; $P < 0.05$) (Figure 2A). Platelet aggregation was
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25 228 abrogated by eptifibatide (1:10 (-)eptifibatide: $58.0 \pm 7.46\%$; (+)eptifibatide: $0.60 \pm 0.25\%$;
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27 229 $P < 0.01$) and (Figure 2B).
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32 230 Upon platelet activation, expression of surface αIIbβ3 is upregulated, further supporting
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34 231 platelet-platelet interaction and therefore aggregation [7, 13, 15], including during bacterial
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36 232 infection [8, 9]. Platelet surface integrin αIIbβ3 expression levels are enhanced as a result of
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38 233 platelet activation by means of α-granule release [17]. We thus investigated the expression
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40 234 patterns of FcγRIIA and αIIbβ3 by flow cytometry after antibody labeling. Platelet expression
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42 235 levels of FcγRIIA were not altered in response to *M. circinelloides* NRRL3631 spores under
43
44 236 aggregating conditions (Figure 2C). In contrast, surface αIIbβ3 levels were increased in
45
46 237 response to *M. circinelloides* NRRL3631 spores under the same conditions (Figure 2D).
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48
49 238 Thus, this shows that platelets respond to fungal spores through interaction with the IgG
50
51 239 receptor FcγRIIA and integrin αIIbβ3. Subsequently, αIIbβ3 surface levels are upregulated to
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53 240 further support platelet aggregation.
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241

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

244 The two identified interaction platelet receptors Fc γ RIIA and $\alpha\text{IIb}\beta 3$ both activate
245 downstream Src and Syk tyrosine kinases [16]. To test if the interaction of *M. circinelloides*
246 NRRL3631 with platelets also results in the activation of Src and Syk, platelets were treated
247 with Src inhibitor Dasatinib, and Syk inhibitor PRT-060318 prior to *M. circinelloides*
248 NRRL3631 exposure. Dasatinib and PRT-060318 inhibited platelet aggregation in response to
249 *M. circinelloides* NRRL3631 (Figure 3A).

250 The platelet α -granules activation marker CD62P (P-selectin) is expressed on the platelet
251 surface upon platelet activation [18]. Platelets were antibody-labeled for P-selectin, and
252 expression levels measured pre- and postexposure to *M. circinelloides* NRRL3631 spores
253 using flow cytometry. P-selectin expression levels were markedly increased following
254 exposure to *M. circinelloides* spores indicating that platelets are activated by *M.*
255 *circinelloides* NRRL3631 under aggregating conditions (Figure 3Bi). Under non-aggregating
256 conditions by means of eptifibatide platelet pre-treatment, P-selectin expression was
257 negligible, suggesting that $\alpha\text{IIb}\beta 3$ activation is crucial to platelet activation by
258 *M. circinelloides* NRRL3631 interaction (3Bii).

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

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3 265 inhibitor, indomethacin, and ADP inhibitor, apyrase, prior to *M. circinelloides* NRRL3631
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5 266 spore exposure. TxA₂ and ADP inhibition independently (e.g. at 1:10 spore:platelet ratio:
6
7 267 70.0 ± 6.0%; (+)indomethacin: 24.3 ± 6.8%; (+)apyrase: 37.3 ± 12.2%) and in combination
8
9 268 (e.g. at 1:10 spore: platelet ratio (+)indomethacin(+apyrase: 18.3 ± 6.1%) reduced *M.*
10
11 269 *circinelloides* NRRL3631-induced platelet aggregation (Figure 3D).

12
13
14 270 In summary, these data demonstrate that spore interaction with platelet receptors activates
15
16 271 downstream Src and Syk tyrosine kinases leading to platelet activation including the release
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18 272 of α-granules shown by increased surface expression of the activation marker P-selectin and
19
20 273 by increased expression of integrin αIIbβ3. Activation is supported by secondary mediators
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22 274 TxA₂ and ADP.
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26 275 27 28 276 ***Mucor circinelloides* NRRL3631 spore developmental stage impacts on platelet** 29 30 277 **aggregation**

31
32 278 Mucormycete spores undergo both metabolic and structural changes during development
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34 279 from spore to hyphae [21]. The host encounters these different developmental stages
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36 280 during infection, thus requiring a dynamic response. We hypothesized that the
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38 281 developmental stage of the *M. circinelloides* NRRL3631 spore would influence its platelet
39
40 282 aggregation potential.

41
42 283 We first identified stages of germination at which a significant increase in spore size occurs.
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44 284 Resting spores at the start of germination, displayed a surface area of 19.8 ± 3.0 μm² (Figure
45
46 285 4A). Spores significantly increased in size at 3 hours germination (34.1 ± 6.8μm²; P<0.05),
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48 286 and furthermore at 6 hours germination (49.9 ± 15.4 μm²; P<0.05). Hyphae formation was
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50 287 observed on spores at 48 hours germination (hyphae formation efficiency: 27.5 ± 8.5%)
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52 288 (Figure 4B).
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3 289 We investigated *M. circinelloides* NRRL3631 spores at 0, 3 and 6 hours germination, and
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5 290 hyphae at 48 hours germination in PRP, and showed that the developmental stage of *M.*
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7 291 *circinelloides* NRRL3631 influences its platelet aggregation potential (Figure 4C).

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10 292 At the beginning of germination, *M. circinelloides* NRRL3631 induced significant platelet
11
12 293 aggregation (1:10: $15.6 \pm 10.2\%$; $P < 0.05$), however to a lesser degree than spores at 3 hours
13
14 294 germination (1:10: $56.0 \pm 12.3\%$; $P < 0.01$). As *M. circinelloides* NRRL3631 spore development
15
16 295 progressed beyond 3 hours germination, platelet aggregation potential declined (6 hours
17
18 296 germination; 1:10: $32.6 \pm 14.3\%$). Moreover, *M. circinelloides* NRRL3631 spores exhibiting
19
20 297 hyphae formation at 48 hours germination induced negligible platelet aggregation (1:10:
21
22 298 $2.80 \pm 1.11\%$).

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26 299 Together, this shows that the platelet response to *M. circinelloides* varies with the spore
27
28 300 developmental stage, indicating a dynamic interaction pattern that needs to be considered
29
30 301 for mucormycosis treatment approaches.

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34 303 **Discussion**

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37 304 Clinical management of mucormycosis remains a challenge whilst the disease incidence is
38
39 305 on the rise. Many of our antifungal agents are ineffective against mucormycetes and
40
41 306 associated with toxic side effects. The gold standard therapy still is surgical debridement
42
43 307 often leading to long-term disability. Together, this results in extremely high mortality in
44
45 308 patients with mucormycosis and highlights the need for more effective therapeutic
46
47 309 strategies.

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51 310 Thrombosis is a hallmark of mucormycosis. It is currently not clear if thrombus formation is
52
53 311 beneficial for the patient by containing the fungus or detrimental due to reduced oxygen
54
55 312 supply and subsequent tissue necrosis. Ability to manipulate the platelet – spore interaction,

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2
3 313 either by enhancing or inhibiting, might be a promising medical approach to improve
4
5 314 patient outcome.

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7 315 To identify potential targets for medical intervention, we here elucidated components of
8
9 316 the signaling pathway underlying platelet – spore interaction. Our data demonstrate that
10
11 317 platelets form aggregates surrounding mucormycete spores, the infectious agent of
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13 318 mucormycosis. This aggregation is dose-dependent. Platelet activation by spores is
14
15 319 mediated through the platelet receptors FcγRIIA and αIIbβ3 and, the Syk/Src signaling
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17 320 cascade to induces α-granules release. This activation is supported by the secondary
18
19 321 mediators TxA₂ and ADP. The dose-dependent nature of platelet aggregation (Figure 1B)
20
21 322 mimics the all-or-nothing response previously described for *E. coli* and Gram-positive
22
23 323 bacteria [8, 9] suggesting a positive feedback mechanism supporting platelet aggregation.
24
25 324 Inhibitor studies suggest that FcγRIIA is an essential receptor for platelet activation in
26
27 325 response to fungal spores (Figure 2A). As no aggregation was observed in washed platelets,
28
29 326 this suggests that one or more plasma factor(s) mediate this interaction. The current
30
31 327 literature suggests these are IgG, by forming an immune complex with spores, to interact
32
33 328 with FcγRIIA and fibrinogen as a bridging molecule to interact with αIIbβ3 [9]. Whilst we
34
35 329 currently do not know the fungal cell wall components interacting with platelets and
36
37 330 whether this interaction is direct or indirect, binding of fibrinogen has been reported to
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39 331 *Candida albicans* cell wall [22]. Due to the constant exposure of humans to these
40
41 332 environmentally ubiquitous fungal spores, it is also highly likely that humans have
42
43 333 circulating antibodies to support the platelet-spore interaction. Research on bacterial
44
45 334 interaction with platelets has shown that several platelet receptors are required for efficient
46
47 335 platelet activation [8, 9, 23-25]. Similarly, platelet activation after spore encounter also
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49 336 requires integrin αIIbβ3 (Figure 2B) and secondary mediators TxA₂ and ADP supporting the
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3 337 notion of positive feedback mechanisms reinforcing initial FcγRIIA activation. Inside-out
4
5 338 signaling has previously been reported for αIIbβ3 to induce secondary platelet-platelet
6
7 339 aggregation after initial activation in response to a stimulus through FcγRIIA [8, 26, 27]. Thus
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9
10 340 both receptors might have dual functionality in initiating as well as amplifying thrombus
11
12 341 formation. Together, these signaling events correspond to those reported previously for a
13
14 342 range of bacterial interaction with platelets [8, 9] and thus indicate that the platelet
15
16 343 response to infectious particles is conserved.

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18
19 344 The conserved nature of the platelet activation pathway in response to pathogens might be
20
21 345 indicative of a protective innate immune function performed by platelets. Attachment of
22
23 346 platelets to fungal hyphae has shown to result in hyphal damage and reduced viability [6,
24
25 347 28]. Yet, a potential detrimental outcome due to excessive platelet aggregation causing
26
27 348 tissue necrosis, similar to exaggerated inflammatory responses needs to be considered. The
28
29 349 release of immune-stimulatory effectors such as pro-inflammatory and pro-necrotic factor
30
31 350 TNF-α and phagocyte chemoattractant TGF-β in α-granules [29] would support this idea. In
32
33 351 the context of mucormycosis, thrombocytopenia has been suggested as factor for severe
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35 352 disease and poor patient outcome [30, 31] suggesting that thrombus formation contributes
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37 353 to disease pathology.

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42 354 During filamentous fungal infections, platelets encounter a range of morphological
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44 355 structures. Whilst initial infection often occurs through inhalation of spores, the propagules
45
46 356 then undergo a developmental program of metabolic and physiological changes to form
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48 357 invasive hyphae. During this germination process, water uptake causes the spore to 'swell'
49
50 358 and undergo a change in both size and structural composition. As mucormycete spores
51
52 359 germinate, there is a depletion of melanin from the outer surface exposing a glucan-rich
53
54 360 outer wall [32]. In the latter stages of germination hyphae are formed, the outer wall of

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3 361 which is chitosan-rich and formed by the germinating spore's inner wall [32]. During the
4
5 362 preliminary stages of *M. circinelloides* NRRL3631 germination we saw a stark increase in
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7 363 platelet aggregation potential, reaching a peak at 3 hours germination, where spores are
8
9 364 between resting and maximal swelling stage. Surprisingly, platelet aggregation potential of
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11 365 *M. circinelloides* NRRL3631 declined towards the latter stages of germination as spores
12
13 366 reach their maximal swelling stage, and *M. circinelloides* hyphal structures appeared to
14
15 367 induce negligible platelet aggregation altogether. Two plausible explanations for the change
16
17 368 in mucormycete spore platelet aggregation potential during germination are: (I)
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19 369 compositional changes of the germinating mucormycete spore, and (II) the secretion of
20
21 370 platelet inhibitory fungal secretory factors. The secretion of platelet inhibitory fungal
22
23 371 secretory factor has been shown in *Candida albicans* [33]. *C. albicans* activates platelets but
24
25 372 inhibits aggregation to fibrinogen, in part via the fungal secretory factor gliotoxin [33].
26
27 373 In summary, this is the first analysis of the signaling underlying platelet aggregation in
28
29 374 response to fungi and thus providing a better understanding of this interaction. The
30
31 375 interaction with fungi is dependent on the developmental stage of the fungus, which might
32
33 376 lead to different outcomes of this interaction that can be beneficial as well as detrimental to
34
35 377 the host. This needs to be carefully considered for the clinical management of patients.
36
37 378 During mucormycosis, platelets have the potential to inhibit the germination process of
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39 379 mucormycetes [6]. We identify several receptors and the downstream signaling
40
41 380 components that could be targeted with already available medical interventions as
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43 381 preventative measures inhibiting disease onset (i.e. spore germination) or by targeting
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45 382 thrombus formation to improve current disease outcome.
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53 383

54 384 **Acknowledgements**

1
2
3 385 We would like to thank Soo Chan Lee for providing the mucormycete strain NRRL3631 used
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5 386 in this study.
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9
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11
12 389 Conceived and designed the experiments: KV, SPW, HG, MZ. Performed the experiments:
13
14 390 HG, AS-R, MZ. Analyzed the data: HG, SW, KV. Wrote the paper: HG, KV. Critically reviewed
15
16 391 the manuscript: KV, SPW.
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21 393 **Declaration of Interests**

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23 394 **Conflict of Interest**

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25 395 The authors report no declarations of interest.
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51 503 **Figure Legends**

52
53 504 **Figure 1 *Mucor circinelloides* NRRL3631 induces platelet aggregation in whole blood and**
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55 505 **PRP. (A) *M. circinelloides* NRRL3631 spores (green) interact with human platelets (magenta)**

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3 506 to form large complex structures. We imaged a total of 17 aggregates and show here images
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5 507 for two of those. These images show (i) an optical section through a platelet and spore
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7 508 aggregate and (ii) the maximal intensity projection of the (single view diSPIM) 3D volume of
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9
10 509 the spore-induced platelet aggregate. Images are representative of 17 aggregates from two
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12 510 independent experiments. Complexes were visualized by dual inverted Selective Plane
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14 511 Illumination Microscopy (diSPIM) using 40x objectives and analysed on Image J (FIJI); scale
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16 512 bar: 10 μ m. **(B)** Platelet aggregation in response to TRAP, PBS and *M. circinelloides* NRRL3631
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18 513 over increasing spore:platelet ratios was measured in PRP using light-transmission
19
20 514 aggregometry over 30 min. Significant platelet aggregation was induced by *M. circinelloides*
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22 515 NRRL3631 at a 1:10 and 1:20 spore:platelet ratio. Notably *M. circinelloides* NRRL3631
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24 516 induced platelet aggregation in a concentration-dependent manner. Data shown are
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26 517 mean \pm SEM of five independent experimental repeats; **P<0.01, Mann-Whitney U test. **(C)**
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28 518 Platelet aggregation in response to TRAP, PBS and *Mucor circinelloides* NRRL3631 spores
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30 519 was measured in whole blood using multiple-electrode aggregometry over 30 min. *M.*
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32 520 *circinelloides* NRRL3631 spores induced significant platelet aggregation in whole blood. Data
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34 521 shown are mean \pm SEM of five independent experimental repeats; *P<0.05, Mann-Whitney U
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36 522 test.
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44 524 **Figure 2 Platelet aggregation in response to *Mucor circinelloides* NRRL3631 is supported**
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46 525 **by the Fc γ RIIA receptor and integrin α IIb β 3 (A) *Mucor circinelloides* NRRL3631 is recognized**
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48 526 **by the Fc γ RIIA receptor. Platelet aggregation in response to *M. circinelloides* NRRL3631 in**
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50 527 **the presence and absence of Fc γ RIIA blocking mAb IV.3 was measured in PRP using light-**
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52 528 **transmission aggregometry over 30 min. mAb IV.3 significantly inhibited platelet**
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54 529 **aggregation in response to *M. circinelloides* NRRL3631. Data shown are mean \pm SEM of five**
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3 530 independent experimental repeats; **P<0.01, Mann-Whitney U test. **(B)** Platelet
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5 531 aggregation in response to *Mucor circinelloides* NRRL3631 is supported by the α IIb β 3
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7 532 integrin. Platelet aggregation in response to *M. circinelloides* NRRL3631 spores in the
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9 533 presence and absence of α IIb β 3 inhibitor, eptifibatide, was measured in PRP using light-
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11 534 transmission aggregometry over 30 min. Eptifibatide significantly inhibited platelet
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13 535 aggregation in response to *M. circinelloides* NRRL3631. Data shown are mean \pm SEM of five
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15 536 independent experimental repeats; *P<0.05, **P<0.01, Mann-Whitney U test. **(C)** *Mucor*
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17 537 *circinelloides* NRRL3631-platelet interaction does not affect Fc γ RIIA platelet surface
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19 538 expression but does **(D)** increase α IIb β 3 platelet surface expression. Platelets were labelled
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21 539 for Fc γ RIIA, using conjugated anti-human CD32 + anti-mouse 2^o Alexa 488, and α IIb β 3, using
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23 540 APC-Mouse Anti-Human CD41a. Platelet surface expression of Fc γ RIIA and α IIb β 3 were
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25 541 read using flow cytometry.
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33 **Figure 3 *Mucor circinelloides* NRRL3631 activates Src and Syk signaling cascades and**
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35 **induces platelet activation supported by secondary mediators TxA₂ and ADP (A) *Mucor***
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37 545 *circinelloides* NRRL3631 activates the Src and Syk signaling cascades. Platelet aggregation in
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39 546 response to *M. circinelloides* NRRL3631 in the presence and absence of Src receptor
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41 547 inhibitor, dasatinib (4 μ M), and Syk inhibitor, PRT-060318 (10 μ M), was measured in PRP
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43 548 using light-transmission aggregometry over 30 min. Dasatinib significantly inhibited platelet
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45 549 aggregation in response to *M. circinelloides* NRRL3631. PRT-060318 also significantly
46
47 550 inhibited platelet aggregation in response to *M. circinelloides* NRRL3631. Data shown are
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49 551 mean \pm SEM of five independent experimental repeats; **P<0.01, Mann-Whitney U test. **(B)**
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51 552 *M. circinelloides* NRRL3631 activates platelets under (i) aggregating conditions but not
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53 553 under (ii) non-aggregating conditions. Platelet aggregation in response to *M. circinelloides*
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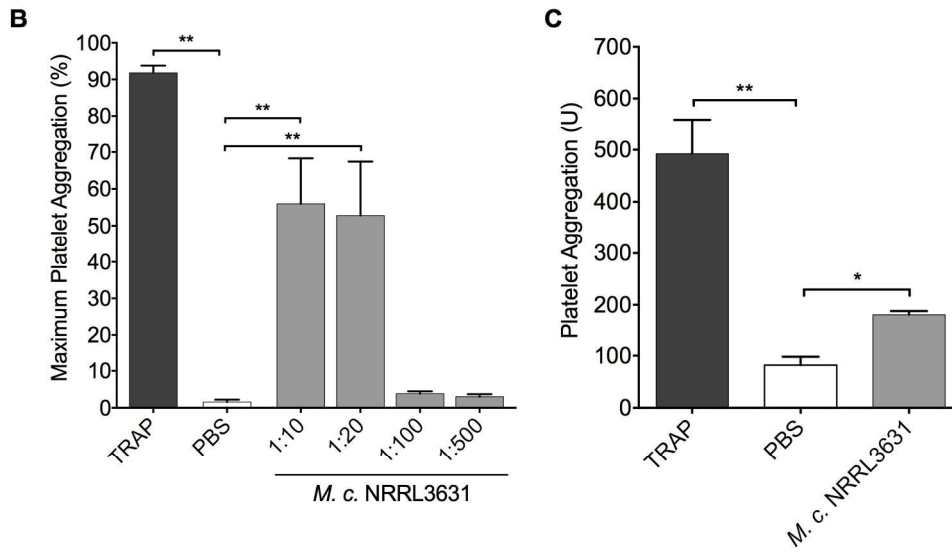
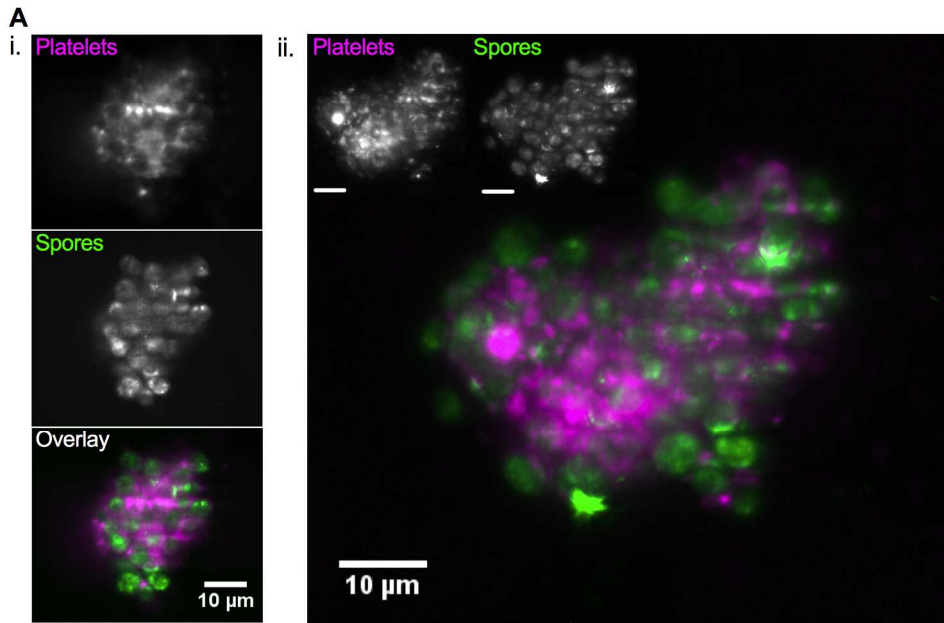
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3 554 NRRL3631 in the presence and absence of $\alpha\text{IIb}\beta\text{3}$ inhibitor, eptifibatide, and was measured
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5 555 in PRP using light-transmission aggregometry over 30 min. Platelets were labelled for CD62P
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7 556 activation marker using CD62P-FITC and surface expression read using flow cytometry. (i)
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9 557 Under aggregating conditions CD62P expression is enhanced following *M. circinelloides*
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11 558 NRRL3631 exposure. (ii) Under non-aggregating conditions, change in CD62P expression is
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13 559 undetectable following *M. circinelloides* NRRL3631 exposure. **(C)** Example aggregation
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15 560 traces showing fast initiation of platelet aggregation in response to the agonist TRAP and a
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17 561 lag phase before induction of platelet activation in response to fungal spores. **(D)** Secondary
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19 562 mediators TxA_2 and ADP play a role in *M. circinelloides* NRRL3631-induced platelet
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21 563 aggregation. Platelet aggregation in response to *M. circinelloides* NRRL3631 in the presence
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23 564 and absence of TxA_2 inhibitor, indomethacin (30 μM), and ADP inhibitor, apyrase (2 U), was
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25 565 measured in PRP using light-transmission aggregometry over 30 min. Indomethacin
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27 566 significantly inhibited platelet aggregation in response to *M. circinelloides* NRRL3631, and
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29 567 apyrase markedly reduced platelet aggregation in response to *M. circinelloides* NRRL3631.
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31 568 Combined indomethacin and apyrase treatment significantly further reduced platelet
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33 569 aggregation in response to *M. circinelloides* NRRL3631. Data shown are mean \pm SEM of three
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35 570 independent experimental repeats; * $P < 0.05$, ** $P < 0.01$, One-way ANOVA with post hoc
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37 571 Dunnett's multiple comparison test.
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46 573 **Figure 4 *Mucor circinelloides* NRRL3631 spore developmental stage impacts on platelet**
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48 574 **aggregation (A) *Mucor circinelloides* spore development.** *M. circinelloides* NRRL3631 spore
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50 575 swelling was investigated over 0, 3 and 6 hours. Spores were visualized by DIC light
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52 576 microscopy at 60x magnification; scale bar: 5 μm . 100 spores from each time point were
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54 577 analysed on ImageJ: a length and width measurement (in μm) was taken from each spore
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3 578 and the spore area calculated $((\text{length}/2) * (\text{width}/2) * \pi)$. *M. circinelloides* NRRL3631 spores
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5 579 exhibited significant increase in size from 0 to 3 hours germination, and from 3 to 6 hours
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7 580 germination. Data shown are pooled data points with mean \pm SD of three independent
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9 581 experimental repeats, each analysing the surface area of 100 spores per incubation period;
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11 582 *** $P < 0.001$, one-way ANOVA with post hoc Dunnett's multiple comparison test. **(B)** *M.*
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13 583 *circinelloides* hyphae formation efficiency. *M. circinelloides* NRRL3631 hyphae formation
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15 584 was studied at 48 hours germination. Spores were visualized by DIC light microscopy at 60x
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17 585 magnification; scale bar: 10 μ m. 100 spores at 48 hours germination were analysed on
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19 586 ImageJ to determine the percentage of hyphae per 100 spores. Data shown are mean \pm SEM
20
21 587 from three independent experimental repeats; scale bar: 10 μ m. **(C)** Platelet aggregation
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23 588 potential is dependent on the developmental stage of *Mucor circinelloides* NRRL3631.
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25 589 Platelet aggregation in response to *M. circinelloides* NRRL3631 spores over progressive
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27 590 developmental stages, was measured in PRP using light-transmission aggregometry over 30
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29 591 min. *M. circinelloides* NRRL3631 spores at time zero germination induced significant platelet
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31 592 aggregation at a 1:10 spore:platelet ratio. *M. circinelloides* NRRL3631 spores at 3 hours
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33 593 germination induced significant platelet aggregation at a 1:10 and 1:20 spore:platelet ratio
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35 594 (data from Figure 2B). *M. circinelloides* NRRL3631 spores at 6 hours induced aggregation to
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37 595 a lesser degree than those at 3 hours germination, and spores at 48 hours germination
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39 596 induced negligible platelet aggregation. Data shown are mean \pm SEM of five independent
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41 597 experimental repeats; * $P < 0.05$, ** $P < 0.01$, Kruskal-Wallis test.
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43 598

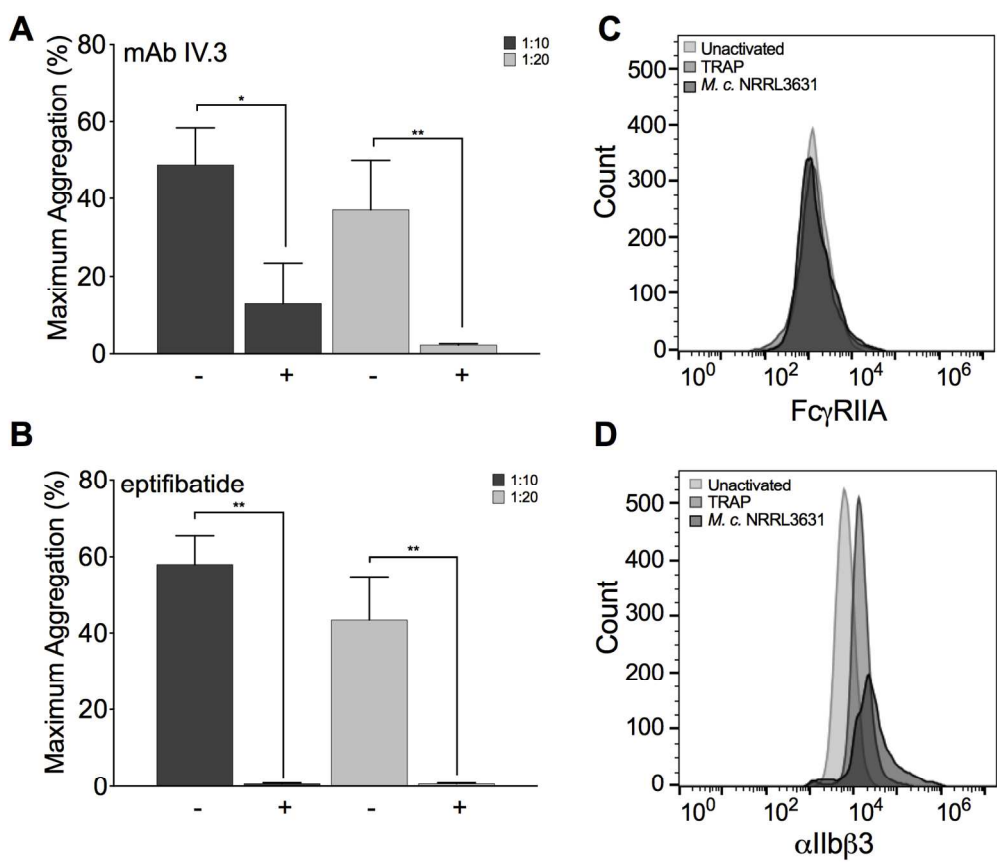
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45 599 **Supplementary Movie 1 Platelet spore aggregate.** 3D visualization of the platelet spore
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47 600 aggregate shown in Figure 1 Aii.
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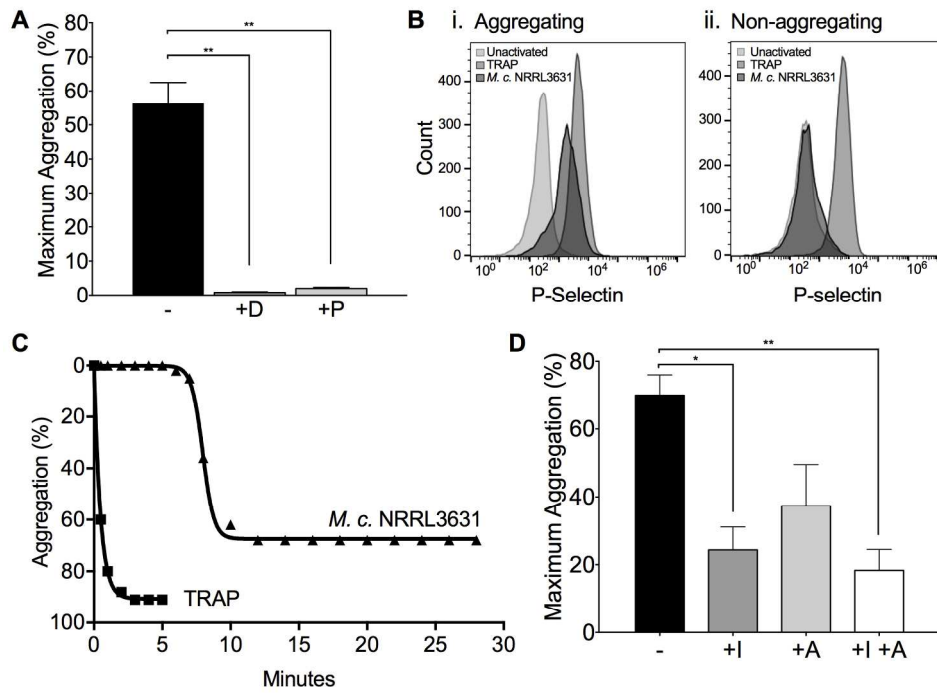
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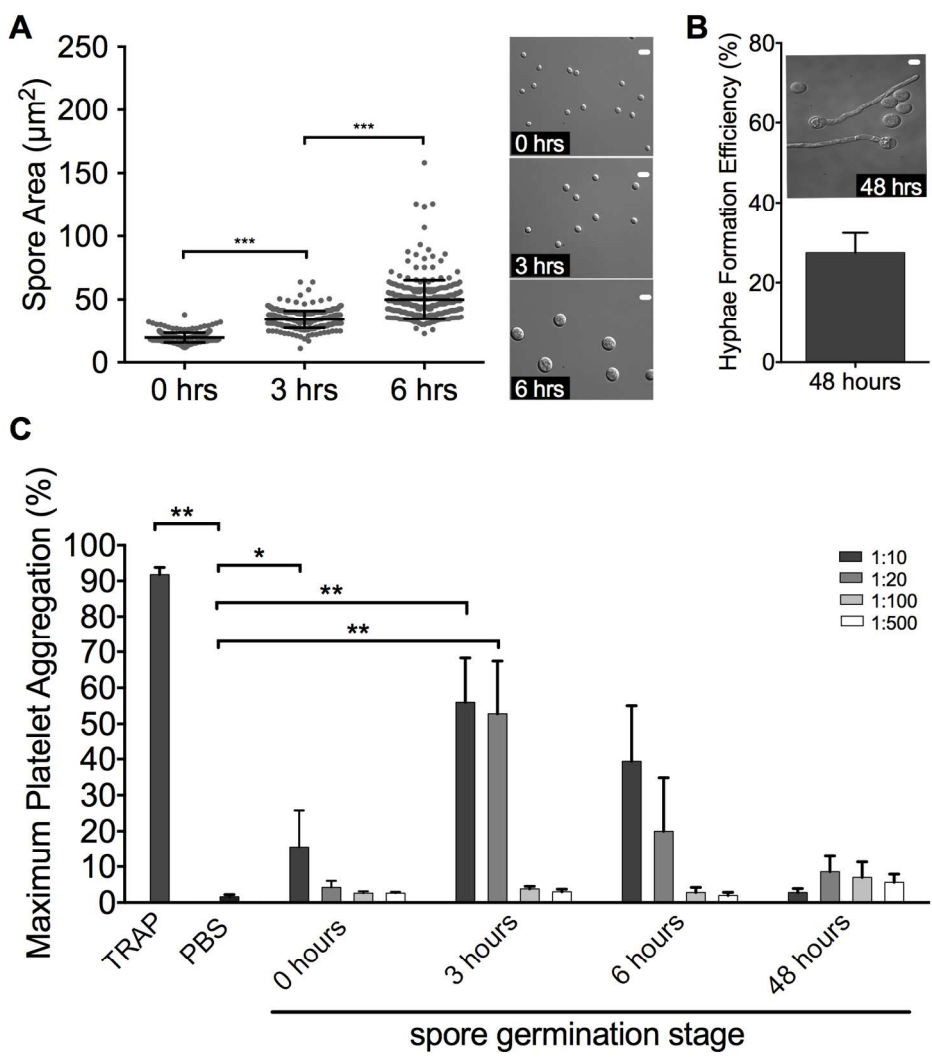
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