Identification of Serum Bridging Molecules that Mediate Human Endothelial Cell Invasion by *Candida* species

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Abstract

During hematogenously disseminated candidiasis, blood borne fungi must invade the endothelial cells that line the blood vessels to infect the deep tissues. Although *Candida albicans*, which forms hyphae, readily invades endothelial cells, other medically important species of *Candida* are poorly invasive in standard in vitro assays. Here, we show that *Candida glabrata, Candida tropicalis, Candida parapsilosis,* and *Candida krusei* can bind to vitronectin and high molecular weight kininogen present in human serum. Acting as bridging molecules, vitronectin and kininogen bind to αv integrins and the globular C1q receptor (gC1qR), inducing human endothelial cells to endocytose the fungus. This mechanism of endothelial cell invasion is poorly supported by mouse endothelial cells, but can be restored when mouse endothelial cells are engineered to express human gC1qR or αv integrin. Overall, these data indicate that bridging molecule-mediated endocytosis is a common pathogenic strategy used by many medically important *Candida spp.* to invade human vascular endothelial cells.

Significance

The invasion of vascular endothelial cells is a key step in the pathogenesis of hematogenously disseminated candidiasis. How species of *Candida* other than *C. albicans* invade endothelial cells is poorly understood because these fungi are weakly invasive in serum-free media. Here, we demonstrate that *C. glabrata* and other *Candida* spp. bind to the serum proteins kininogen and vitronectin, which act as bridging molecules and mediate the adherence and endocytosis of the organisms by endothelial cells. These serum proteins induce endocytosis when they interact with the globular C1q receptor and αv integrins on human, but not mouse endothelial cells. Thus, bridging molecule-mediated endocytosis is a common mechanism by which medically important *Candida* spp. invade human endothelial cells.

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1   Despite the widespread use of antifungal agents, disseminated candidiasis continues to

2   be a serious problem in hospitalized patients. Previously, *Candida albicans* was the most

3   common cause of candidemia (1). However, the epidemiology of this disease has changed, and

4   *C. albicans* now causes less than half of these infections. In fact, the combined incidence of

5   infections caused by *Candida glabrata*, *Candida parapsilosis*, and *Candida tropicalis* now

6   exceeds the incidence of infections caused by *C. albicans* (2, 3). Even though the causative

7   agents of candidemia have changed, this infection remains highly lethal. Approximately 40% of

8   patients with candidemia die, even with currently available therapy (2, 3). A deeper

9   understanding of the pathogenesis of this disease is essential for developing new strategies to

10   prevent and treat invasive infections caused by multiple species of *Candida*.

11   During hematogenously disseminated candidiasis, blood-borne organisms must invade

12   the endothelial cell lining of the vasculature to reach the target organs (4). A number of hyphal-

13   associated factors have been found to participate in the pathogenic interactions of *C. albicans*

14   with endothelial cells. *C. albicans* hyphae express invasins such as Als3 and Ssa1 that interact

15   with specific host cell receptors and stimulate fungal endocytosis by endothelial cells in vitro (5-

16   9). Organisms that do not form true hyphae on endothelial cells, such as *C. albicans* mutants

17   with defects in hyphal formation, and *C. glabrata* and *C. tropicalis*, have greatly impaired

18   capacity to invade these cells in standard assays (10, 11) and have highly attenuated virulence

19   in immunocompetent mice (12-14). In patients, organisms that grow only as yeast in vivo are

20   still able to cross the endothelial cell lining of the vasculature and infect target organs during

21   disseminated infections (2, 3). Indeed, patients with candidemia caused by *C. glabrata*, which

22   grows only in the yeast form in vivo, have at least as high mortality as those with candidemia

23   due to *C. albicans* (2, 15). Thus, in humans, afilamentous *C. glabrata* is as virulent as hypha-

24   forming *C. albicans*.

25   These data suggest that yeast-phase *Candida* spp. must be able to penetrate

26   endothelial cells in vivo by a mechanism that is not evident in standard in vitro invasion assays.

27   Most assays of *Candida* invasion are performed using media that contain either heat-inactivated

28   serum or no serum at all. Here we demonstrate that when yeast-phase *Candida* spp. are

29   incubated with either fresh human serum or plasma, two proteins, high molecular weight

30   kininogen and vitronectin bind to the fungal surface. Acting as bridging molecules, these serum

31   proteins interact with the globular C1q receptor (gC1qR; also known as p33/HABP) and αv

32   integrins on the surface of human endothelial cells and induce the adherence and endocytosis

33   of the organism. When *C. glabrata* is coated with either human or mouse serum, there is

34   minimal endocytosis by mouse endothelial cells, suggesting a key limitation of the mouse model

35   to study vascular invasion by yeast phase *Candida* spp. This defect in endocytosis can be

36   rescued in vitro by expressing either human gC1qR or human αv integrin in mouse endothelial

37   cells. Thus, we delineate a previously unexplored mechanism by which fungi can invade human

38   endothelial cells.

39

40   **Results**

41   **Serum and plasma enhance the endocytosis of *Candida spp.*** Previously, we found that

42   yeast-phase *C. albicans*, such as live *efg1*Δ/Δ *cph1*Δ/Δ mutant cells or killed wild-type yeast,

43   are very poorly endocytosed by human endothelial cells in vitro (11). Although it has been

44   determined that yeast-phase *C. parapsilosis* cells are endocytosed by endothelial cells in vitro,

45   this process is much slower and less efficient than the endocytosis of hyphal-phase *C. albicans*

46   (16). A limitation of these previous experiments is that they were performed in serum-free

47   media. It is known that serum proteins can act as bridging molecules and mediate the

48   adherence of bacteria to endothelial cells (17, 18). Therefore, we investigated whether serum

49   components could act as bridging molecules between *Candida* spp. and endothelial cells. Live

50   *C. glabrata* yeast and methanol killed, yeast phase *C. albicans* cells were incubated in 20%

51   pooled human serum that was either fresh or heat-inactivated. Killed *C. albicans* cells were

52   used in these experiments because live organisms germinate when exposed to serum (19). The

53   fungal cells were then rinsed and incubated with human umbilical vein endothelial cells. When

54   the organisms were incubated with heat-inactivated serum, few cells were endocytosed,

55   similarly to control organisms that had been incubated in serum-free medium (Fig. 1A and B).

56   When the organisms were incubated in fresh serum, the number of endocytosed cells increased

57   by 8- to 9-fold. Incubating *C. glabrata* and *C. albicans* with fresh serum also increased the

58   number of cell-associated organisms, a measure of adherence (Fig. 1C and D). To verify that

59   serum could enhance the endothelial cell interactions of live *C. albicans*, we tested an *efg1*Δ/Δ

60   *cph1*Δ/Δ mutant strain that remains in the yeast phase when exposed to serum (14). The

61   endocytosis and adherence of this strain were increased when it was incubated in fresh serum

62   as compared to heat-inactivated serum (SI Appendix, Fig. S1A and B). Fresh serum also

63   significantly enhanced the endothelial cell endocytosis and adherence of live, yeast-phase *C.*

64   *parapsilosis*, *Candida krusei*, and *Candida auris*, but not *C. tropicalis* (Fig. 1E and F). Fresh

65   human plasma was at least as effective as fresh human serum at enhancing the endocytosis

66   and adherence of *C. glabrata* (SI Appendix, Fig. S1C and D), indicating that both plasma and

67   serum contain factors that strongly enhance the endothelial cell interactions of yeast-phase

68   organisms.

69   Although fresh serum significantly enhanced the endocytosis and adherence of multiple

70   species of *Candida,* it only increased the adherence of *S. cerevisiae* but had no effect on

71   endocytosis (SI Appendix, Fig. S1E and F). Thus, the bridging molecules that bind to *Candida*

72   spp*.* appear to be non-functional after they bind to *S. cerevisiae*.

73   To verify that the serum-coated organisms were being endocytosed, endothelial cells

74   were infected with serum-coated *C. glabrata*, fixed and then stained for actin. We observed that

75   actin microfilaments coalesced around *C. glabrata* cells, a hallmark of endocytosis (Fig. 1G).

76   When endothelial cells were treated with cytochalasin D to depolymerize actin, the endocytosis

77   of serum-coated organisms was significantly decreased (Fig. 1H). Cytochalasin D also reduced

78   the number of adherent organisms (Fig. 1I). Collectively, these data suggest the model that

79   heat-labile serum proteins function as bridging molecules that induce endothelial cells to

80   endocytose yeast-phase *Candida* spp.

81

82   **The globular C1q receptor (gC1qR) and αv integrins are endothelial cell receptors for**

83   **serum-coated yeast-phase *C. glabrata*.** To identify potential endothelial cell receptors for

84   serum-coated organisms, we employed an affinity-purification approach (6-8) using intact *C.*

85   *glabrata* cells that had been coated with either fresh or heat-inactivated serum. Among the

86   endothelial cell membrane proteins that were found to bind to serum-coated *C. glabrata* (SI

87   Appendix, Table S1), gC1qR was selected for in-depth analysis because it is known to bind to

88   several different serum proteins (20). Of note, we did not detect binding of serum-coated *C.*

89   *glabrata* to N-cadherin, which we have found previously to be an endothelial cell receptor for *C.*

90   *albicans* Als3 and Ssa1, invasins that are expressed by hyphae (5, 6, 8). Thus, serum-coated *C.*

91   *glabrata* cells interact with different endothelial cell receptors than do *C. albicans* hyphae.

92   By immunoblotting with an anti-gC1qR monoclonal antibody, we verified that gC1qR was

93   indeed bound by serum-coated *C. glabrata* cells (Fig. 2A). To determine the functional

94   significance of this binding, we used siRNA to knockdown gC1qR. We found the gC1qR siRNA

95   significantly inhibited the endocytosis of serum-coated *C. glabrata* (Fig. 2B and SI Appendix,

96   Fig. S2). The gC1qR siRNA also slightly inhibited *C. glabrata* adherence (Fig. 2C). Because

97   gC1qR is known to be expressed both intracellularly and on the cell surface (21, 22), siRNA

98   knockdown likely depleted both pools of this protein. To verify that surface-expressed gC1qR

99   was required for the endocytosis of serum-coated *C. glabrata,* we tested two different anti-

100   gC1qR monoclonal antibodies for their capacity to inhibit endothelial cell interactions of serum-

101   coated *C. glabrata.* Antibody 74.5.2, which recognizes the high molecular weight kininogen

102   binding site in the C-terminus of the gC1qR (23, 24), reduced endocytosis by 45% but did not

103   significantly affect adherence (Fig. 2D and E). By contrast, antibody 60.11, which is directed

104   against the C1q binding site in the N-terminus of the gC1qR, had no effect on either endocytosis

105   or adherence. Collectively, these data suggest that the gC1qR functions as an endothelial cell

106   receptor for serum-coated *C. glabrata*.

107   The finding that blocking gC1qR resulted in incomplete inhibition of endocytosis

108   prompted us to search for additional endothelial cell receptors for serum-coated *C. glabrata*.

109   Because integrins bind to serum proteins that could potentially act as bridging molecules, we

110   screened a panel of anti-integrin monoclonal antibodies for their capacity to block the endo-

111   cytosis of serum-coated *C. glabrata*. We found that antibodies against integrins αvβ3 and αvβ5

112   inhibited endocytosis and adherence by approximately 45% (Fig. 3A-D). Although some

113   antibodies against integrins αvβ3 and αvβ5 also bind to the surface of *C. albicans* (25, 26), flow

114   cytometry confirmed that the monoclonal antibodies used in our experiments did not bind to *C.*

115   *glabrata* (SI Appendix, Fig. S3). Endocytosis and adherence of *C. glabrata* was similarly

116   inhibited by siRNA knockdown of integrin αv, but not by knockdown of the unrelated integrin α5

117   (Fig. 3E and F, SI Appendix, Fig. S4). Notably, blocking gC1qR and integrins αvβ3 and αvβ5

118   simultaneously resulted in near maximal reduction in endocytosis, but did not further decrease

119   adherence (Fig. 3G and H), indicating that gC1qR and integrins αvβ3 and αvβ5 make additive

120   contributions to endocytosis.

121   To further explore the relationship among the gC1qR and the integrins αvβ3 and αvβ5,

122   we infected endothelial cells with serum-coated *C. glabrata*, stained them with antibodies

123   against the three receptors, and then imaged them with confocal microscopy. We observed that

124   all three receptors accumulated around endocytosed organisms (Fig. 3I). Collectively, these

125   results support the model that when serum proteins bind to a *C. glabrata* cell, they interact with

126   gC1qR and integrins αvβ3 and αvβ5, which causes the fungus to adhere to endothelial cells and

127   induce its own endocytosis.

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129   **High molecular weight kininogen and vitronectin are bridging molecules that mediate the**

130   **endocytosis of serum-coated organisms.** Next, we sought to identify potential bridging

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131   molecules that mediate the binding of serum-coated organisms to gC1qR and integrins αvβ3

132   and αvβ5. After incubating *C. glabrata* cells in fresh serum, we rinsed them extensively and

133   eluted the bound serum proteins with HCl followed by Tris neutralization. The eluted proteins

134   were separated by SDS-PAGE and analyzed by Western blotting to detect proteins that are

135   known to bind to these receptors. Two proteins, high molecular weight kininogen and

136   vitronectin, were identified. These proteins could be eluted from *C. glabrata* when the cells were

137   incubated in fresh serum, but not heat-inactivated serum (Fig. 4A). To determine whether the

138   binding of these proteins to *C. glabrata* was functionally significant, we analyzed the effects of

139   antibodies against kininogen and vitronectin on the endocytosis of serum-coated *C. glabrata*.

140   We found that antibodies against each protein significantly inhibited endocytosis, but had no

141   effect on adherence (Fig. 4B, SI Appendix, Fig. S5A), suggesting that these proteins may

142   function as bridging molecules that induce endothelial cells to endocytose *C. glabrata.*

143   High molecular weight kininogen is cleaved by kallikrein and other proteases, releasing

144   bradykinin from the larger protein. The remaining protein, called HKa, consists of a 62 kDa

145   heavy chain that is linked by a disulfide bond to a 56 kDa light chain (27). By immunoblotting

146   with specific monoclonal antibodies and looking for bands of the appropriate molecular mass,

147   we found that both the heavy and light chains of HKa bound to *C. glabrata* (Fig. 4A and C). Both

148   of these chains were bound by *C. glabrata* when the cells were incubated in fresh human

149   serum, but not with heat-inactivated serum. Collectively, these results suggest that high

150   molecular weight kininogen is cleaved to HKa, which then binds to *C. glabrata*.

151   Using flow cytometry, we analyzed the relationship between the binding of kininogen and

152   vitronectin to *C. glabrata*. We found that when the organisms were incubated with kininogen

153   alone, very little protein bound to them (Fig. 4D and E). When the organisms were incubated

154   with kininogen in the presence of vitronectin, kininogen binding increased significantly. By

155   contrast, vitronectin bound to the organisms both in the presence and absence of kininogen

156   (Fig. 4D and F). These results suggest the model that vitronectin binds to the organism and

157   facilitates the binding of kininogen.

158   To determine if kininogen and vitronectin could function as bridging molecules in the

159   absence of other serum proteins, we incubated *C. glabrata* cells with these proteins, either

160   alone or in combination, and then measured their endocytosis by endothelial cells. When the

161   organisms were incubated with kininogen alone, few organisms were endocytosed, similarly to

162   control organisms that had been incubated in BSA (Fig. 4G). When the organisms were

163   incubated in vitronectin alone, endocytosis increased significantly, and it increased even more

164   when the organisms were incubated in both kininogen and vitronectin. The combination of

165   kininogen and vitronectin also significantly increased the adherence of the organisms, while

166   kininogen and vitronectin alone had no effect (SI Appendix, Fig. S5B). Collectively, these data

167   indicate that the human serum proteins kininogen and vitronectin function as bridging molecules

168   that enhance the adherence and induce endocytosis of *C. glabrata* by human endothelial cells.

169   Next, we investigated the endothelial cell interactions of *C. albicans* cells that had been

170   incubated in kininogen and vitronectin prior to being added to these host cells. These

171   experiments were feasible because, unlike serum, kininogen and vitronectin did not induce

172   significant filamentation. We found that preincubating organisms with these proteins significantly

173   enhanced the endocytosis and adherence of the *C. albicans efg1Δ/Δ cph1Δ/Δ* mutant, which

174   remained in the yeast phase while in contact with the endothelial cells (Fig. 5A and SI Appendix,

175   Fig. S6A). Also, kininogen and vitronectin sightly enhanced the endocytosis of wild-type *C.*

176   *albicans*, which formed hyphae on the endothelial cells, and largely rescued the endocytosis

177   and adherence defects of the invasin-deficient *als3Δ/Δ ssa1Δ/Δ* mutant (Fig. 5B and SI

178   Appendix, Fig. S6B).

179   When wild-type *C. albicans* is endocytosed by endothelial cells, it damages these cells,

180   likely by releasing candidalysin into the invasion pocket (6, 29-31). We tested whether coating

181   the *als3Δ/Δ ssa1Δ/Δ* mutant with kininogen and vitronectin would restore is capacity to damage

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182   endothelial cells. While organisms coated with BSA caused minimal endothelial cell damage,

183   cells coated with kininogen and vitronectin induced significantly greater damage (Fig. 5C).

184   These results indicate that in the absence of invasins, bridging molecules can enhance the

185   endocytosis of *C. albicans* hyphae, leading to subsequent endothelial cell damage.

186   As we had observed that fresh serum increased the endocytosis of species of *Candida*,

187   other than *C. albicans*, we investigated whether human kininogen and vitronectin functioned as

188   bridging molecules for these organisms. We found that these proteins significantly increased the

189   endocytosis of *C. parapsilosis, C. tropicalis,* and *C. krusei*, but not *C. auris*. (Fig. 5D). Kininogen

190   and vitronectin also increased the endothelial cell adherence of *C. parapsilosis* and *C. krusei* (SI

191   Appendix, Fig. S6C). Next, we tested whether the enhanced endocytosis of these organisms by

192   kininogen and vitronectin would result in endothelial cell damage. To increase the sensitivity of

193   the experiment, we increased the inoculum and extended the incubation period to 6 hr. None of

194   these organisms caused detectable damage to the endothelial cells (SI Appendix, Fig. S6D),

195   indicating that induction of endocytosis alone is not sufficient for these species of *Candida* to

196   cause significant endothelial cell damage.

197   To investigate which endothelial cell receptor was responsible for interacting with each

198   bridging molecule, we tested the inhibitory effects of specific antibodies directed against gC1qR

199   and αv integrins. When *C. glabrata* cells were incubated with vitronectin alone, endocytosis was

200   significantly inhibited by antibodies against integrins αvβ3 and αvβ5, but not by the anti-gC1qR

201   antibody (Fig. 5E). None of these antibodies significantly reduced the adherence of vitronectin-

202   coated organisms (SI Appendix, Fig. S6E). When the organisms were incubated with vitronectin

203   and kininogen, endocytosis was inhibited by both the anti-gC1qR antibody and the anti-αv

204   integrin antibodies (Fig. 5F). The combination of all 3 antibodies inhibited endocytosis in an

205   additive manner and also inhibited adherence (SI Appendix, Fig. S6F). Taken together, these

206   data support the model that vitronectin likely binds first to the fungal surface where it is

207   recognized mainly by integrins αvβ3 and αvβ5 (Fig. 6). Binding of vitronectin enables kininogen

208   to bind to fungal cell surface, and the vitronectin-kininogen complex is recognized by both

209   gC1qR and the αv integrins, leading to the strong adherence and subsequent endocytosis of the

210   organism.

211

212   **Expression of human gC1qR and αv integrins on mouse endothelial cells enhances**

213   **bridging molecule mediated endocytosis.** Next, we investigated whether mouse serum

214   bridging molecules also mediated the endocytosis of *C. glabrata* by comparing the capacity of

215   mouse and human serum to mediate endocytosis by human endothelial cells. To maximize

216   endocytosis, we incubated the organisms in 100% serum. We observed that after 45 min,

217   mouse serum enhanced the endocytosis of *C. glabrata* by human endothelial cells, but to a

218   lesser extent than human serum (Fig. 7A). Mouse serum also increased adherence to human

219   endothelial cells, but not as much as human serum (SI Appendix, Fig. S7A). These differences

220   in endocytosis and adherence persisted even when the incubation period was increased to 3 h

221   (Fig. 7B and SI Appendix, Fig. S7B). These results indicate that while mouse serum proteins

222   can function as bridging molecules between *C. glabrata* and human endothelial cells, they are

223   less effective than human serum proteins.

224   To investigate whether serum bridging molecules could mediate the endocytosis of *C.*

225   *glabrata* by mouse endothelial cells, we obtained primary mouse kidney and liver endothelial

226   cells and tested their capacity to endocytose *C. glabrata* cells that had been coated with either

227   human or mouse serum. We found that there was minimal endocytosis and adherence of

228   organisms coated with human or mouse serum by mouse endothelial cells after both 45 min and

229   180 min (Fig. 7A and B, SI Appendix, Fig. S7A and B). To verify that human endothelial cells

230   other than those obtained from umbilical cord veins were able to endocytose serum coated

231   organisms, we tested a Tert-immortalized human microvascular endothelial (TIME) cell line. *C.*

232   *glabrata* cells coated with human serum were endocytosed by and adhered to the TIME cells

233   more than human umbilical vein endothelial cells (SI Appendix, Fig. S7C and D). Collectively,

234   these data indicate that while both mouse and human serum proteins can function as bridging

235   molecules between *C. glabrata* and human endothelial cells, mouse endothelial cells have very

236   limited capacity to endocytose organisms coated with serum from either mice or humans.

237   We considered the possibility that the inability of mouse endothelial cells to endocytose

238   serum coated *C. glabrata* was due to difference between the receptors on mouse vs. human

239   endothelial cells. To evaluate the possibility, we used lentivirus to transduce primary mouse liver

240   endothelial cells with human *C1QBP* (gC1qR), *ITGAV* (integrin αv), or *ITGB5* (integrin β5).

241   Control cells were transduced with lentivirus encoding GFP. The expression of the human

242   proteins by the transduced endothelial cells was verified by Western blotting (SI Appendix, Fig.

243   7E). Endothelial cells that expressed human gC1qR and integrin αv endocytosed significantly

244   more serum-coated organisms than did the control endothelial cells (Fig. 7C). *C. albicans* also

245   had enhanced adherence to the cells that expressed human gC1qR, integrin αv, and integrin β5

246   (SI Appendix, Fig. S7F). These data demonstrate that human gC1qR and integrin αv mediate

247   the endocytosis and adherence of serum-coated *C. glabrata.* They also suggest that these

248   human receptors are functionally different from their mouse counterparts.

249

Discussion

251   In this study, we sought to elucidate how species of *Candida* that do not form true hyphae are

252   able to invade vascular endothelial cells. Using *C. glabrata* as a representative fungus that

253   grows only as yeast within the human host, we determined that proteins present in human

254   serum act as bridging molecules between the fungus and human endothelial cells and induce

255   the adherence and subsequent endocytosis of the organism. The data presented here indicate

256   that binding of vitronectin to the fungal surface facilitates the subsequent binding of kininogen.

257   Vitronectin interacts mainly with the integrins αvβ3 and αvβ5 endothelial cells, and the

258   kininogen-vitronectin complex also interacts with gC1qR. The binding of these serum proteins to

259   their respective receptors causes the organism to adhere to endothelial cells and stimulates its

260   subsequent endocytosis (Fig. 6). Not only did kininogen and vitronectin act as bridging

261   molecules for *C. glabrata*, but they also mediated endothelial cell endocytosis of yeast-locked

262   and invasin-deficient *C. albicans* mutants and other medically important species of *Candida*,

263   including *C. tropicalis, C. parapsilosis,* and *C. krusei.* Although serum enhanced the endocytosis

264   of *C auris,* kininogen and vitronectin did not, suggesting that other serum proteins must function

265   as bridging molecules for this organism. Also, serum bridging molecules did not induce the

266   endocytosis of *S. cerevisiae,* indicating that bridging molecule-mediated endocytosis is not a

267   general property of yeast. Taken together, these results indicate that invasion of vascular

268   endothelial cells via bridging molecule-mediated endocytosis is a pathogenic strategy shared by

269   many medically important *Candida* spp.

270   Vitronectin, which is bound by integrins αvβ3 and αvβ5, is known to function as a

271   bridging molecule that mediates adherence to respiratory epithelial cells of a variety of bacteria,

272   including nontypeable *Haemophilus influenzae*, *Moraxella catarrhalis,* group A streptococci, and

273   *Pseudomonas aeruginosa* (reviewed in (32)). In addition to mediating adherence, vitronectin

274   induces the internalization of *Neisseria gonorrhoeae* and *Pseudomonas fluorescens* by

275   epithelial cells (33, 34). *C. albicans*, C. *parapsilosis* and *C. tropicalis* have been shown to bind to

276   fluid phase vitronectin (35), and the binding of *C. albicans* to vitronectin mediates adherence to

277   keratinocytes (36). Our findings demonstrate that *C. glabrata* and *C. krusei* are additional

278   *Candida* spp. that bind to vitronectin. More importantly, we show that vitronectin acts as a

279   bridging molecule that, in conjunction with kininogen, mediates the endocytosis of these

280   organisms by human endothelial cells.

281   Studies of the interaction of kininogen with microbial pathogens have focused mainly on

282   its proteolytic cleavage to release bradykinin and other fragments with antimicrobial activity.

283   Kininogen has been found to bind to *S. aureus*, *Salmonella typhimurium*, and *Bacteroides* spp.

284   (37, 38). Rapala-Kozik et al., have determined that virtually all medically important *Candida spp.*

285   bind kininogen (39-41). In contrast to the results shown here, they found that kininogen could

286   bind to the fungus in the absence of additional serum proteins, whereas we found that there was

287   minimal binding of kininogen to *C. glabrata* unless vitronectin was present. The likely

288   explanation for these divergent results is that the other investigators used a more sensitive

289   assay that was able to detect the binding of even low amounts of kininogen to the fungal

290   surface. Nevertheless, our results indicate that vitronectin dramatically increases the amount of

291   kininogen that binds to *C. glabrata* and enables kininogen to function as a bridging molecule

292   that enhances fungal endocytosis.

293   Although the function of kininogen as a bridging molecule between microbial pathogens

294   and host cells has not been appreciated previously, it is known that kininogen can bind to

295   glycoprotein 1b on platelets and integrin αMβ2 on neutrophils to enhance the co-adherence of

296   these two cells (42). We determined that unlike platelets and neutrophils, endothelial cells bind

297   kininogen via gC1qR, a result that has been reported by others (43). gC1qR has also been

298   found to be a receptor for *Listeria monocytogenes* that mediates the internalization of this

299   organism. However, this bacterium binds directly to gC1qR, and the interaction can be blocked

300   by both C1q and monoclonal antibody 60.11, which is directed against the C1q binding site of

301   gC1qR (44). By contrast, we found that while monoclonal antibody 60.11 did not inhibit bridging

302   molecule-mediated endocytosis of *C. glabrata,* monoclonal antibody 74.5.2, which is directed

303   against the kininogen binding site of gC1qR, was highly inhibitory. These results support the

304   model that when kininogen is bound to the surface of *Candid* spp*.,* it interacts with gC1qR on

305   endothelial cells and stimulates the endocytosis of the organisms.

306   Patients with hematogenously disseminated candidiasis due to *C. glabrata*, *C. tropicalis*,

307   *C. krusei,* and *C auris* have at least as high mortality as those who are infected with *C.*

308   *albicans*(2, 15, 45). These data suggest that in humans, these different species of *Candida* have

309   similar virulence. In immunocompetent mice, *C. albicans* is highly virulent, and most wild-type

310   strains are capable of causing a lethal infection. By contrast, intravenous infection of

15

311 immunocompetent mice with *C. glabrata*, *C. tropicalis, C. krusei,* and *C. auris* induces minimal

312 mortality even when high inocula are used (46, 47). Thus in mice, these species of *Candida*

313 have greatly attenuated virulence. A possible explanation for this discrepancy is that *C.*

314 *albicans* is able to form hyphae that express invasins such as Als3 and Ssa1 that interact

315 directly with endothelial cell receptors and induce endothelial cell endocytosis. By contrast,

316 other species of *Candida*, such as *C. glabrata* invade endothelial cells by bridging molecule-

317 mediated endocytosis, a process that occurs inefficiently in mice.

318   The results of the in vitro studies supported this concept. *C. albicans* was endocytosed

319 avidly by human endothelial cells in the absence of serum and coating the organism with serum

320 only increased endocytosis slightly. *C. glabrata* was avidly endocytosed by human endothelial

321 cells only when it was coated with serum proteins. Both mouse and human proteins increased

322 endocytosis by human cells, indicating that mouse serum proteins can function as bridging

323 molecules, albeit not as well as human proteins. Importantly, *C. glabrata* was poorly

324 endocytosed by mouse liver and kidney endothelial cells when it was coated with either mouse

325 or human serum. When the mouse endothelial cells were engineered to express human gC1qR

326 or integrin αv, they gained the capacity to endocytose serum-coated *C. glabrata.* These data

327 indicate that a key difference between mice and humans is that mouse gC1qR and integrin αv

328 do not support bridging molecule-mediated endocytosis of *C. glabrata.*

329   Although the mouse model of disseminated candidiasis is an excellent model for

330 investigating antifungal efficacy and many aspects of fungal pathogenicity, our results suggest

331 that this model is not optimal for investigating how *C. glabrata* and possibly other *Candida* spp.

332 other than *C. albicans* disseminate hematogenously because mouse endothelial cells do not

333 support bridging molecule-mediated vascular invasion. Even though mice inoculated

334 intravenously with these organisms still contain some fungal cells in their tissues, we speculate

335 that the organisms must egress from the vasculature by another mechanism(s) that has less

336 pathogenic impact. This possibility is currently being investigated.

16

337   The results presented here indicate that many medically important species of *Candida*

338   can utilize serum proteins as bridging molecules to induce their own endocytosis by human

339   vascular endothelial cells. Because this mechanism is shared by multiple *Candida spp.,* it

340   represents a promising therapeutic target for preventing or ameliorating hematogenously

341   disseminated candidiasis.

342

343   **Methods**

344   **Ethics statement.** All animal work was approved by the Institutional Animal Care and Use

345   Committee (IACUC) of the Lundquist Institute for Biomedical Innovation at Harbor-UCLA

346   Medical Center. The collection of blood from normal human volunteers was performed under

347   protocol 30636-01R, which was approved by the IRB of the Lundquist Institute. Informed

348   consent was obtained prior to phlebotomy.

349

350   **Serum and plasma.** After obtaining informed consent, blood was collected by venipuncture

351   from healthy volunteers. Blood was also collected from anesthetized Balb/C mice by cardiac

352   puncture. To obtain serum, the blood was allowed to clot at room temperature for 30 min and

353   then centrifuged at 2000 rpm for 10 min at 4°C. After collecting the serum, samples from

354   individual donors were pooled and stored in aliquots in liquid nitrogen. To make heat-inactivated

355   serum, the fresh serum was incubated at 56°C for 1 hr and stored in aliquots in liquid nitrogen.

356   To obtain plasma, fresh human blood was transferred to 4 ml vacutainer tubes

357   containing 7.2 mg of K_2EDTA (BD, Inc.). The tubes were then centrifuged at 2000 rpm for 10

358   minutes at 4°C, after which the plasma was collected, pooled, aliquoted, and stored in liquid

359   nitrogen. Heat-inactivated plasma was made by incubating fresh plasma at 56°C for 1 hr.

360

361   **Host cells, fungal strains and growth conditions.** Human umbilical vein endothelial cells

362   were isolated from umbilical cords and grown as described (30, 48). Mouse kidney endothelial

17

363   cells (Cell Biologics), mouse liver endothelial cells (Cell Biologics), and hTert-immortalized

364   human microvascular endothelial cells (American Type Culture Collection) were purchased and

365   grown according to the suppliers' instructions.

366   The fungal strains used in this work are listed in Supplemental Table 2. For use in the

367   experiments, the organisms were grown overnight in yeast extract peptone dextrose (YPD)

368   broth at 30°C in a shaking incubator. They were harvested by centrifugation, washed twice with

369   PBS and enumerated with a hemacytometer as previously described (30). To produce killed

370   organisms, cells of *C. albicans* strain DIC185 were pelleted by centrifugation and then

371   resuspended in 100% methanol for 2 min. The killed organisms were recovered by

372   centrifugation and washed two times with PBS. Fungal killing was verified by plating a sample of

373   the cells onto YPD agar.

374   Strain DSC10 was constructed by plating strain CAN34 (*efg1*Δ/Δ *cph1*Δ/Δ) on minimal

375   medium containing 5-fluororotic acid. The resulting Ura- strain was transformed with a PstI/NotI-

376   digested fragment of pBSK-URA3 (49) to restore the *URA3-IRO1* locus. Proper integration was

377   verified by PCR.

378

379   **Coating fungal cells with bridging molecules.** To coat the organisms with serum or plasma,

380   approximately 5×10^7 fungal cells were mixed with either RPMI 1640 medium alone (Irvine

381   Scientific) or RPMI 1640 medium containing 20% fresh or heat-inactivated human serum and

382   then incubated for 1 hr at 37°C in a shaking incubator. In some experiments, the human serum

383   was replaced with human plasma to which $CaCl_2$ was added to reverse the effects of the EDTA.

384   In experiments comparing mouse with human serum, the organisms were incubated with either

385   100% mouse or human serum. After coating, the fungal cells were washed twice with PBS,

386   diluted, and counted for use in the assays described below.

387   To coat the organisms with bridging molecules, approximately 2×10^7 fungal cells were

388   incubated with human kininogen (10 μg/ml; Molecular Innovations, Inc., Cat. # HK-TC) and/or

18

389   human vitronectin (30 µg/ml; Molecular Innovations Inc., Cat. # HVN-U) in RPMI 1640 medium

390   supplemented with 50 µM $ZnCl_2$ and 3 µM $CaCl_2$. Control cells were incubated with BSA

391   (Sigma-Aldrich). The cells were incubated for 1 hr at 37ºC in a shaking incubator and processed

392   as described above.

393

394   **Confocal microscopy.** The confocal microscopy was performed as previously described (6).

395   Briefly, endothelial cells were grown to confluency on fibronectin-coated glass coverslips and

396   then infected with 3×10^5 *C. glabrata* cells. After 45 min, the cells were fixed with 3%

397   paraformaldehyde and blocked with 5% goat serum containing 0.05% Triton X-100. The cells

398   were incubated with Alexa Fluor 568-labeled phalloidin (Thermo Fisher Scientific, Cat.

399   #A12380), rabbit anti-gC1qR antibody (Santa Cruz Biotechnology, Cat. #sc-48795), anti-integrin

400   αvβ3 monoclonal antibody (Millipore-Sigma, clone LM609, Cat. # MAB1976), or anti-integrin

401   αvβ5 monoclonal antibody (Millipore-Sigma, clone P1f6, Cat. # MAB1961). After extensive

402   rinsing, the cells were incubated with the appropriate Alexa Fluor labeled secondary antibody

403   (Thermo Fisher Scientific, Cat. #A-11031 or A-11034), rinsed, and then imaged by confocal

404   microscopy. The *C. glabrata* cells were viewed by differential interference contrast. Consecutive

405   z-stacks were combined to create the final images.

406

407   **Endocytosis assay.** The endocytosis of the various organisms by endothelial cells was

408   determined by our standard differential fluorescence assay as described previously (6, 50).

409   Briefly, endothelial cells grown on fibronectin-coated glass coverslips were incubated with 10^5

410   fungal cells in 5% CO_2 at 37ºC for 45 or 180 min. Next, the cells were fixed in 3%

411   paraformaldehyde, and the non-endocytosed organisms were stained with an anti-*Candida*

412   antibody (Meridian Life Science, Cat. # B65411R) that had been conjugated with Alexa Fluor

413   568 (Thermo Fisher Scientific, Cat. # A-10235). After rinsing the cells extensively with PBS, the

414   endothelial cells were permeabilized in 0.05% Triton X-100 (Sigma-Aldrich), and the cell-

19

415   associated organisms were stained with the anti-*Candida* antibody conjugated with Alexa Fluor

416   488 (Thermo Fisher Scientific). The coverslips were mounted inverted on microscope slides and

417   viewed with an epifluorescent microscope. The number of endocytosed organisms was

418   determined by scoring at least 100 organisms per slide. Each experiment was performed at

419   least three times in triplicate.

420   The effects of depolymerizing microfilaments on endocytosis was determined by

421   incubating the endothelial cells with 0.4 µM cytochalasin D (Sigma-Aldrich) for 45 min prior to

422   infection. Control endothelial cells were incubated in the diluent (0.1% DMSO) in parallel. The

423   cytochalasin D and DMSO remained in the medium for the duration of the infection. To

424   determine the effects of blocking endothelial cell receptors on endocytosis, the endothelial cells

425   were incubated with anti-gC1qR antibodies (Santa Cruz Biotechnology, clone 74.5.2 Cat. #

426   sc-23885 and Abcam, clone 60.11, Cat. # ab24733), anti-αvβ3 antibody (Millipore Sigma, clone

427   LM609, Cat. # MAB1976), αvβ5 (Millipore Sigma, clone P1F6, Cat. # MAB1961), or a

428   combination of antibodies, each at 10 µg/ml. Control cells were incubated in the same

429   concentration of mouse IgG (R&D Systems, clone 11711; # MAB002). The endothelial cells

430   were incubated with the antibodies for 1 hr prior to infection and the antibodies remained in the

431   medium for the duration of infection.

432   To determine the effects of inhibit bridging molecules on endocytosis, *C. glabrata* cells

433   were coated with 20% heat-inactivated or fresh serum in the presence of an anti-kininogen

434   antibody (Santa Cruz Biotechnology, clone 2B5, Cat. # sc-23914), an anti-vitronectin antibody

435   (Millipore Sigma, clone 8E6(LJ8), Cat. # MAB88917), or an isotype control IgG, each at

436   10 µg/ml. The organisms were then washed twice with PBS, counted, and used in the

437   endocytosis assay.

438

439   **Protein purification and Western blotting.** Endothelial cell membrane proteins were isolated

440   using glucopyranoside according to our previously described method[6-8]. To pull down

441 endothelial cell proteins that bound to serum-coated *C. glabrata*, 8×10^8 organisms that had been

442 coated with fresh or heat-inactivated serum were incubated with 1 mg of endothelial cell

443 membrane proteins on ice for 1 hr. Unbound proteins were removed by rinsing with 1.5%

444 glucopyranoside, after which the bound proteins were eluted with 6M Urea. Samples were

445 added to SDS-PAGE sample buffer, heated to 90°C for 5 min, and then separated by SDS-

446 PAGE. After staining the gel with Instant Blue (Expedeon, Cat. #ISB1L), selected bands were

447 excised and the proteins in them were sequenced by MS-MS at the UCLA proteomics core

448 facility. To verify that gC1qR bound to *C. glabrata* cells that had been coated with fresh serum,

449 the pull-down assay was repeated and Western blotting was performed with the anti-gC1qR

450 antibody (clone 74.5.2).

451 To detect serum proteins that bind to *C. glabrata*, 1×10^8 *C. glabrata* cells were incubated

452 with 20% fresh or heat-inactivated or serum in RPMI 1640 medium for 1 hr at 37°C. Unbound

453 serum proteins were removed by rinsing the cells twice with PBS, after which bound serum

454 proteins were eluted with 2M HCl, pH 2.0 and immediately neutralized with Tris buffer, pH 8.0.

455 The proteins were separated by SDS-PAGE and Western blotting using an anti-kininogen heavy

456 chain antibody (Santa Cruz Biotechnology, clone 2B5, Cat. # sc-23914), anti-light chain

457 antibody (Santa Cruz Biotechnology, clone 14J09, Cat. # sc-80524), and anti-vitronectin

458 antibody (clone 8E6(LJ8)) was performed to detect kininogen and vitronectin that had been

459 eluted from *C. glabrata.*

460

461 **siRNA.** Knockdown of endothelial cell surface proteins was accomplished using siRNA. The

462 endothelial cells were transfected with gC1qR siRNA (Santa Cruz Biotechnology, Cat. #

463 sc-42880), integrin α5 siRNA (Santa Cruz Biotechnology, Cat. # sc-29372), integrin αv siRNA

464 (Santa Cruz Biotechnology, Cat. # sc-29373), or scrambled control siRNA (Qiagen, Cat. #

465 1027281) using Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer's

466 instructions. After 48 hr, the transfected endothelial cells were infected with serum coated *C.*

467 *glabrata* and the number of endocytosed organisms was determined. Knockdown of each

468 protein was verified by immunoblotting with antibodies against gC1qR (clone 74.5.2), integrin α5

469 (Millipore-Sigma, Cat. # AB1928), integrin αv (Santa Cruz Biotechnology, clone H-2, Cat # sc-

470 376156), integrin β3 (Santa Cruz Biotechnology, clone B-7, Cat. # sc-46655), integrin β5 (Santa

471 Cruz Biotechnology, clone F-5, Cat. # sc-398214), or actin (Millipore-Sigma, clone C4, Cat. #

472 A5441-100UL)

473

474 **Flow cytometry.** The binding of kininogen and vitronectin to *C. glabrata* was analyzed by a

475 modification of a previously described method (51). *C. glabrata* cells were incubated with

476 kininogen that had been labeled with Alexa Fluor 568 (Thermo Fisher Scientific, Cat. #A20184)

477 and/or unlabeled vitronectin, both at a final concentration of 30 µg/ml, for 1 hr at 37^0C. Control

478 cells were incubated in a similar concentration of BSA. The unbound proteins were removed by

479 washing the cells twice with PBS. Next, the cells were incubated with the anti-vitronectin

480 antibody followed by the Alexa Fluor 488-labeled goat anti-mouse secondary antibody. The

481 fluorescence of the cells was then quantified using a Becton Dickinson FACScalibur flow

482 cytometer, analyzing 10,000 cells per sample using the FlowJo software.

483 The potential binding the anti-αvβ3 and anti-αvβ5 antibodies to *C. glabrata* was

484 determined by incubating *C. glabrata* cells with each antibody at a final concentration of 10

485 µg/ml, followed by the Alexa Fluor 488-labeled goat anti-mouse secondary antibody The

486 fluorescence of the cells was then quantified by flow cytometer, analyzing 10,000 cells per

487 sample.

488

489 **Endothelial cell damage assay.** The capacity of wild-type (CAI4-URA) and *als3Δ/Δ ssa1Δ/Δ*

490 *C. albicans* strains to damage human umbilical vein endothelial cells was determined using our

491 previously described ^{51}Cr release assay (6). Endothelial cells were grown in a 96-well tissue

22

492 culture plate containing detachable wells and loaded with ^{51}Cr. The *C. albicans* were coated

493 with either BSA or kininogen and vitronectin and rinsed, after which 4 x 10^4 fungal cells were

494 added to individual wells of endothelial cells. After incubation for 3 h, the medium above the

495 cells was aspirated and the wells were detached from each other. The amount of ^{51}Cr released

496 into the medium and remaining in the endothelial cells was determined using a gamma counter.

497   When the *C. glabrata, C. tropicalis, C. parapsilosis, C. krusei*, and *C. auris* strains were

498 tested in the damage assay, they were processed similarly to the *C. albicans* cells except that

499 the inoculum was increased to 2 x 10^5 cells per well and the incubation period was increased to

500 6 h.

501

502 **Lentivirus packaging and host cell transduction.** The transfer vectors (pLenti-EF1A-EGFP-

503 Blast, pLenti-EF1A-hITGAV-NEO, pLenti-EF1A-hITGB5-NEO or pLenti-EF1A-hC1QBP-Blast)

504 were constructed by cloning eGFP, hC1QBP [NM_001212.4], hITGAV [NM_002210], or hITGB5

505 [NM_002213] into pLenti-Cas9-Blast (Addgene; # 52962) or pLenti-Cas9-NEO at the BamHI

506 and XbaI sites. The virus was produced by transfecting HEK293T cells with plasmid psPAX2

507 (Addgene; # 12260), plasmid pCMV-VSVG (Addgene; # 8454), and transfer vector at 1:1:1

508 molar ratio using the X-tremeGENE 9 DNA transfection reagent (Sigma-Aldrich; # 6365787001)

509 according to the manufacturer's instructions. The supernatant containing the virus was collected

510 at 60 h post-transfection, passed through a 0.45um PVDF filter and stored at 4℃ (short-term) or

511 -80℃ (long-term).

512   For transduction, mouse primary liver endothelial cells were seeded into a 6-well plate in

513 mouse endothelial cell medium (Cell Biologics, Inc. # M1168). The cells were transduced with

514 lentivirus in the presence of 8 μg/ml polybrene (Santa Cruz Biotechnology; #SC134220),

515 centrifuged at 1000*g* for 30 min, and then incubated at 37℃ in 5% CO$_2$ overnight. The next

516 morning, the cells were transferred to 10 cm diameter tissue culture dishes. For the cells

517 transduced with hC1QBP, 10 μg/ml of blasticidin (Gibco; # A1113903) was added to the

23

518   medium 2 d post transduction to select for transduced cells and selection was maintained for 7

519   d. Expression of eGFP was determined by fluorescent microscopy and expression of gC1qR,

520   integrin αv, and integrin β5 were verified via immunoblotting of whole cell lysates with an anti-

521   gC1qR antibody (clone 60.11), anti- integrin αv antibody (MilliporeSigma; #AB1930), and anti-

522   integrin β5 antibody (My Biosource, Inc; # MBS617750). Total loading was determined by

523   immunoblotting with an anti-GAPDH antibody (Cell Signaling; # 5174).

524

525   **Statistical analysis.** All data were analyzed using Prism (GraphPad). Differences among

526   experimental groups were analyzed by one-way analysis of variance followed by pair-wise

527   analysis with Dunnett's multiple comparison test. When a single pair of data was analyzed, the

528   2-way student's t-test assuming unequal variance was used. P values $\leq$ 0.05 were considered

529   to be significant.

530

531   **Data availability**

532   The raw data that support the findings of this study are available from the corresponding author

533   upon request.

534

535

24

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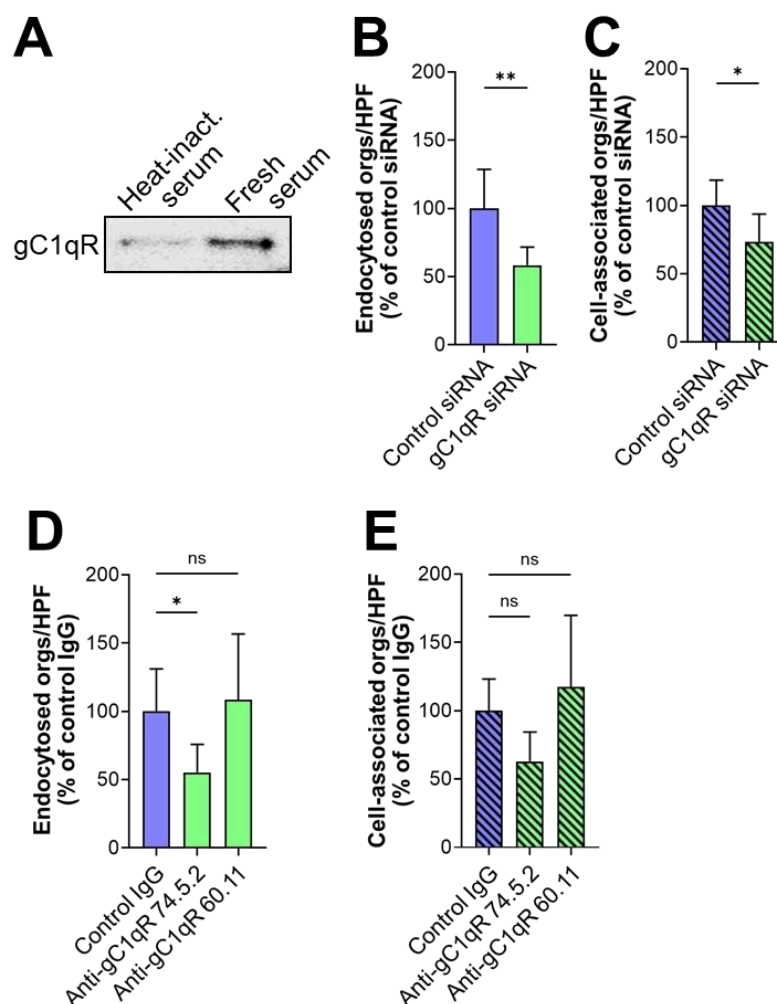
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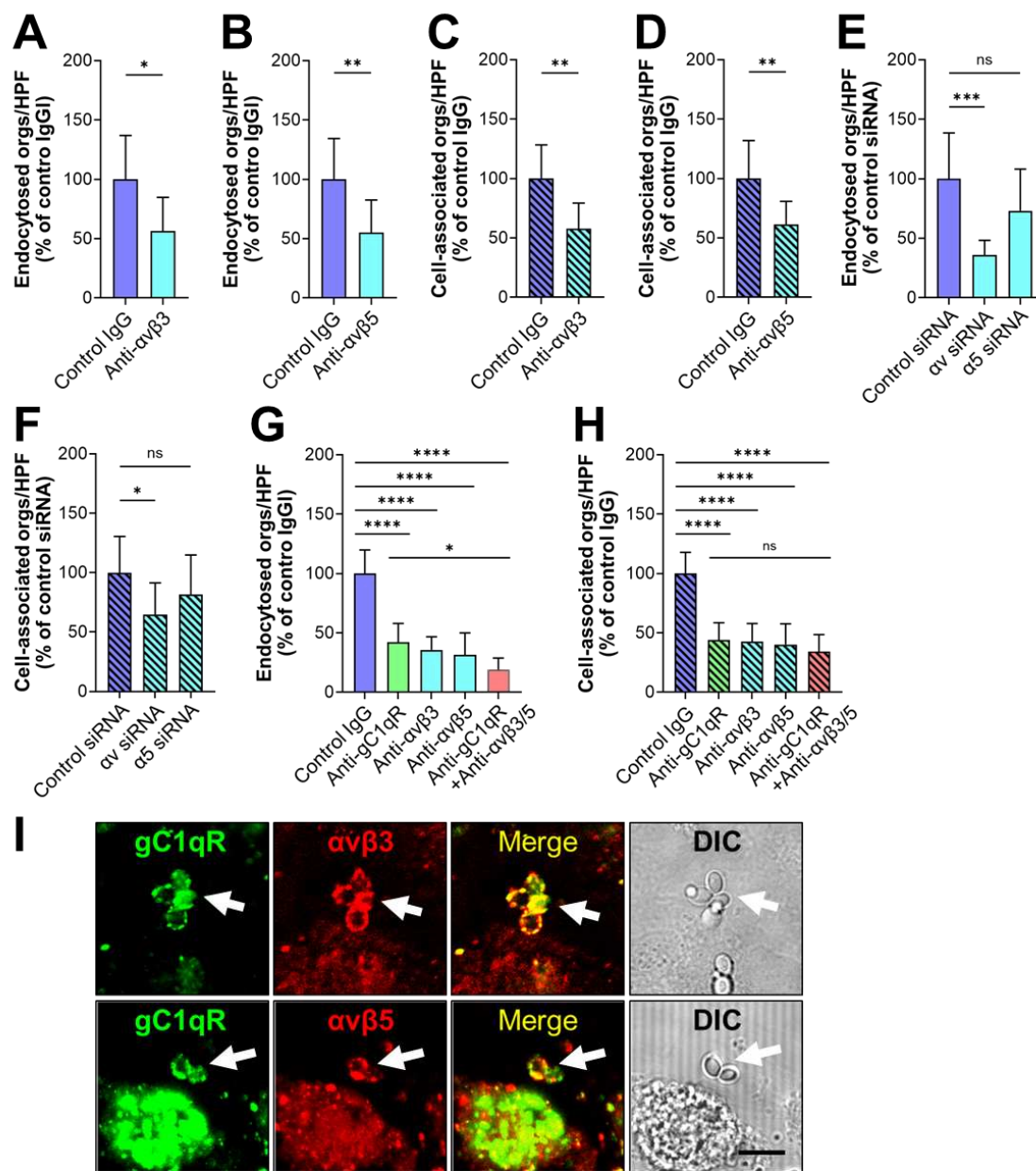
Fig. 1. Serum coating increases the endocytosis of *Candida spp.* by endothelial cells**.** (A-D), Effects of fresh and heat-inactivated human serum on the number of organisms that were endocytosed by and cell-associated (a measure of adherence) with human umbilical vein endothelial cells. (A) Endocytosis of live *C. glabrata,* (B) endocytosis of killed wild-type *C. albicans* yeast, (C) cell-association of live *C. glabrata*, and (D) cell-association of killed wild-type *C. albicans* yeast. (E-F) Endocytosis (E) and cell-association (F) of live *C. glabrata, C. parapsilosis, C. tropicalis, C. krusei,* and *C. auris*. (G) Confocal micrographs showing the

accumulation of phalloidin-stained endothelial cell actin around endocytosed *C. glabrata* cells. Results are representative of 3 independent experiments. Arrows indicate the *C. glabrata* cells and the actin that has accumulated around them. Scale bar, 10 µm. (H and I) Effects of cytochalasin D on the endocytosis (H) and cell-association (I) of live *C. glabrata* coated with fresh human serum. Results are the mean ± SD of 3 experiments each performed in triplicate. Orgs/HPF, organisms per high-power field; ns, not significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by ANOVA with the Dunnett's test for multiple comparisons.
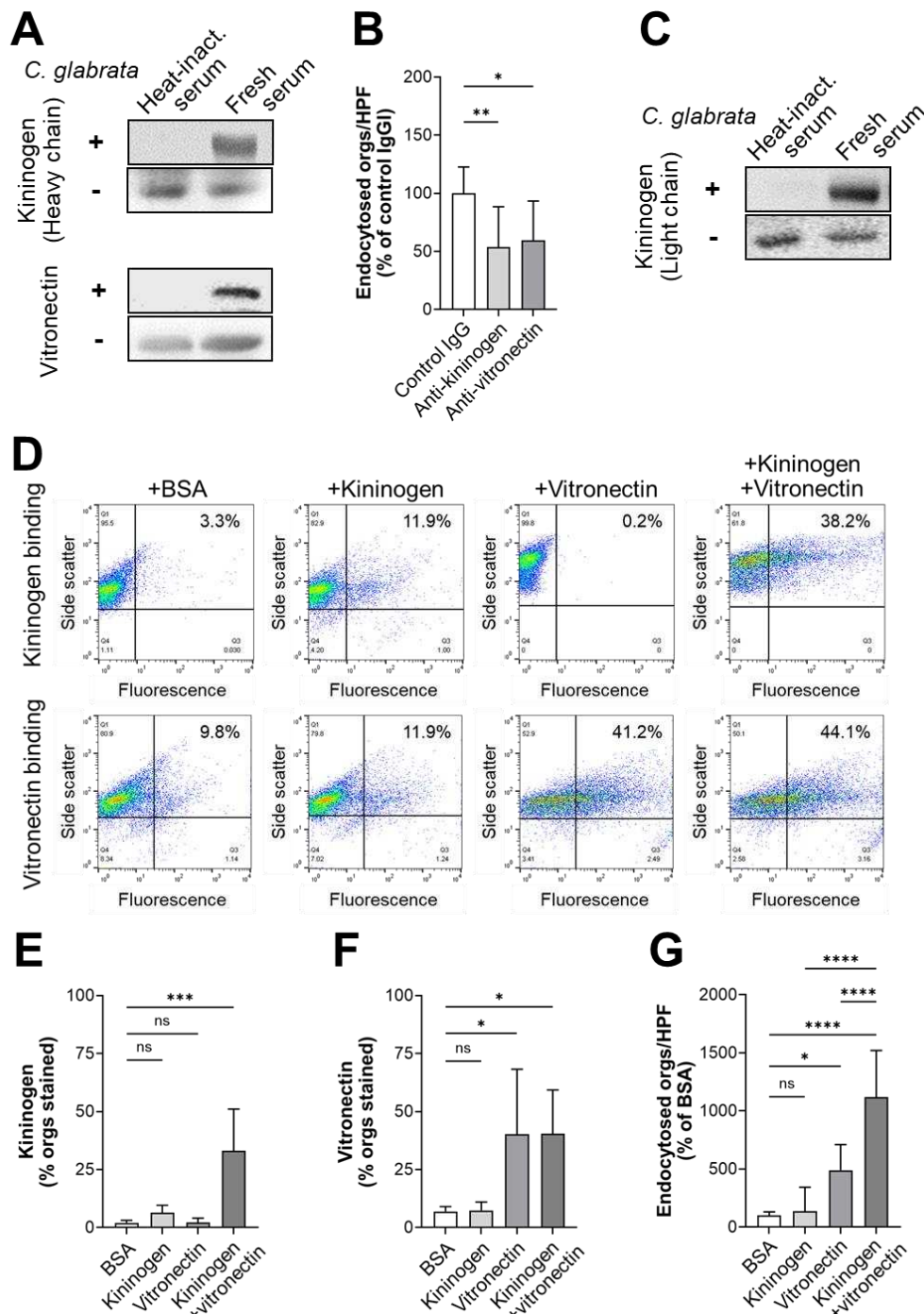
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Fig. 2 The globular C1q receptor (gC1qR) is an endothelial cell receptor for serum-coated *C. glabrata.* (A) Western blot showing that endothelial cell gC1qR binds to *C. glabrata* coated with fresh serum. Results are representative of 3 independent experiments. (B-E) Effects of inhibiting gC1qR function with siRNA knockdown (B and C) and specific monoclonal antibodies (D and E) on the endocytosis (B and D) and cell-association (C and E) of *C. glabrata* coated with fresh serum. Results shown in (B-E) are the mean ± SD of 3 experiments each performed in triplicate. Heat inact., heat inactivated serum; orgs/HPF, organisms per high-power field; ns, not significant, *$P < 0.05$, **$P < 0.01$.

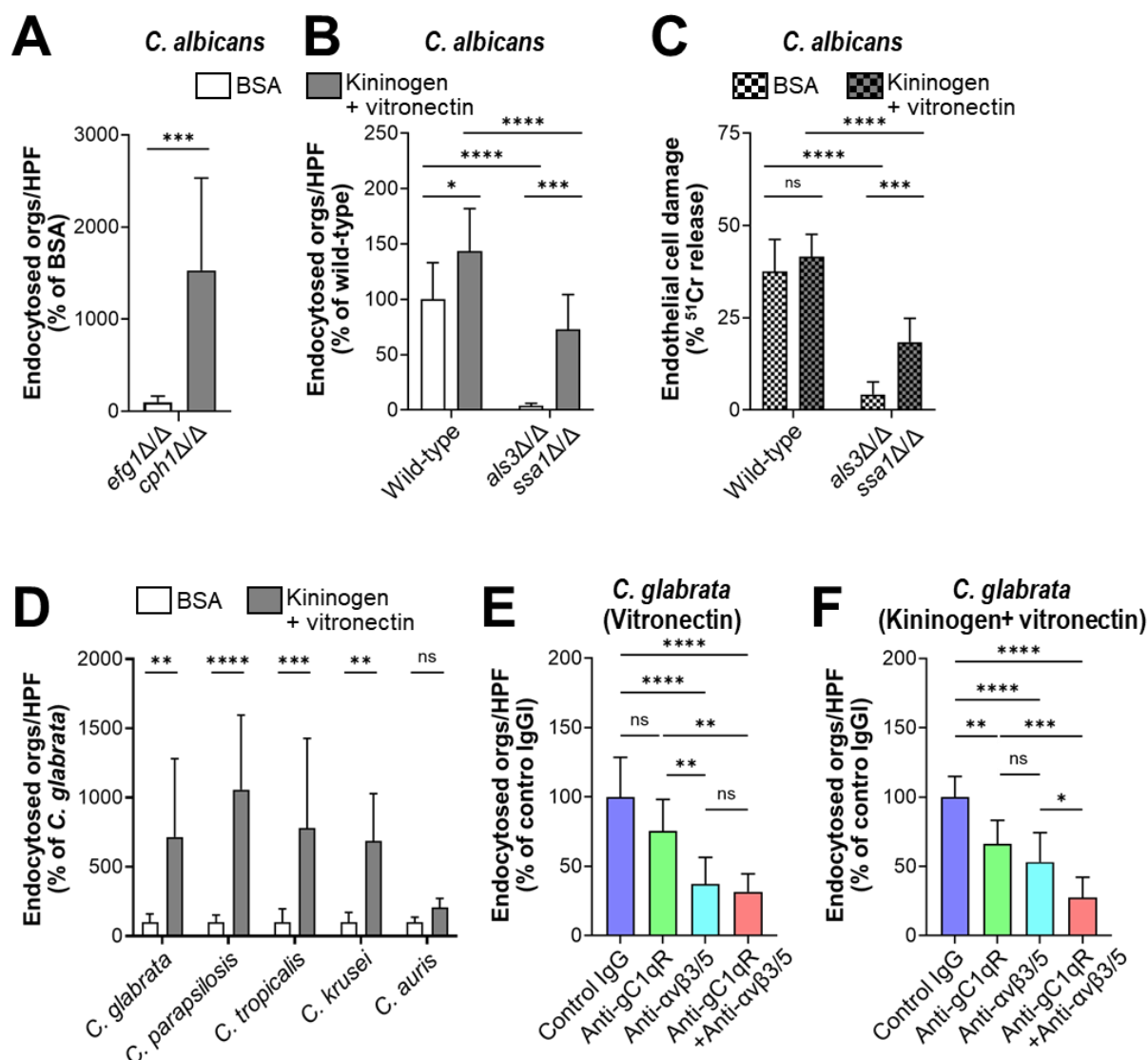**Fig. 3.** Integrins αvβ3 and αvβ5 are endothelial cell receptors for serum-coated *C. glabrata*. (A-F) Effects of inhibiting αv integrin function with specific monoclonal antibodies (A-D) and siRNA knockdown (E-F) on the endocytosis (A, B, E) and cell-association (C, D, F) of serum-coated *C. glabrata*. (G and H) Inhibition of gC1qR (with monoclonal antibody 74.5.2) and αv integrins has an additive effect on decreasing the endocytosis (G) but not cell-association of serum-coated *C. glabrata* (H). (I) Confocal micrographs showing the accumulation of gC1qR and integrins αvβ3 and αvβ5 around serum-coated *C. glabrata* cells. Representative results of 3 independent experiments. Arrows indicate the *C. glabrata* cells and the endothelial cell receptors that have accumulated around them. Scale bar, 7 μm. Results shown in (A-H) are the mean ± SD of 3 experiments each performed in triplicate. Orgs/HPF, organisms per high power field; ns, not significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

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Fig. 4. High molecular weight kininogen and vitronectin function as bridging molecules. (A) Western blots showing that the heavy chain of high molecular weight kininogen and vitronectin bind to *C. glabrata* cells that have been incubated in fresh human serum. In each pair of blots, the upper panel shows the proteins that were eluted from *C. glabrata* and lower panel shows the proteins present in serum in the absence of *C. glabrata*. (B) Effects of anti-kininogen and anti-vitronectin antibodies on the endocytosis of serum-coated *C. glabrata* by endothelial cells. (C) Western blot showing that the kininogen light chain binds to *C. glabrata* cells that have been
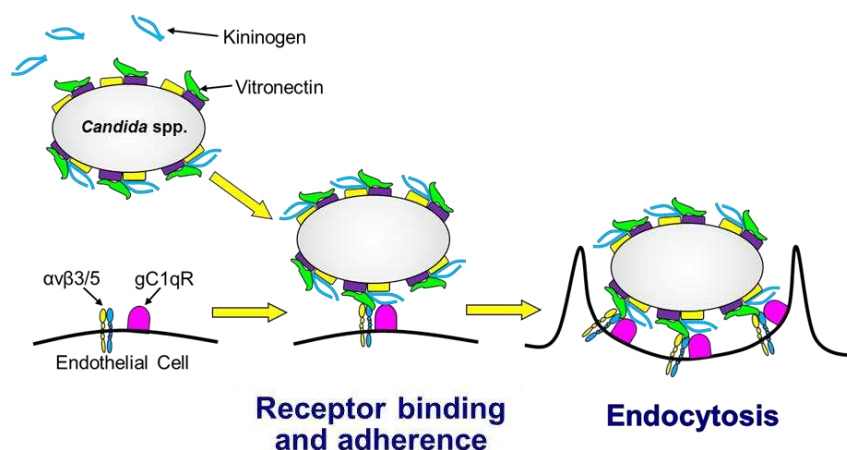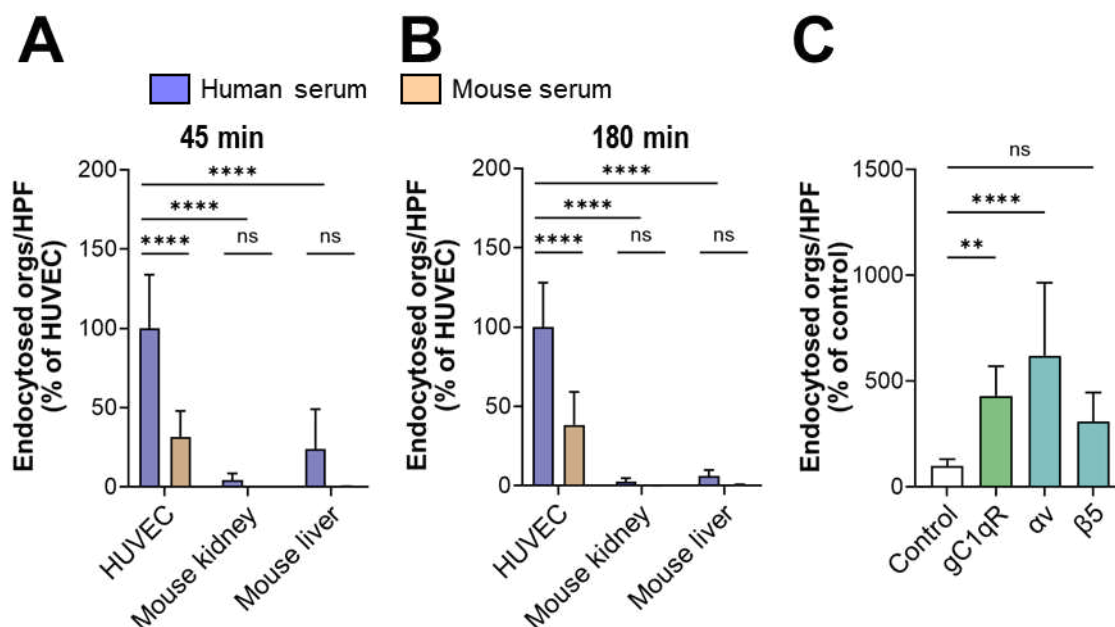
incubated in fresh human serum. (D) Flow cytometric detection of the binding of kininogen (top row) and vitronectin (bottom row) to *C. glabrata* cells that had been incubated for 1 h with BSA without kininogen or vitronectin, kininogen alone, vitronectin alone, or kininogen and vitronectin. Numbers in the upper right hand corner indicate the percentage of positive cells. Results are representative of 5 (kininogen) or 4 (vitronectin) separate experiments, each of which analyzed 10,000 cells. (E-F) Summary of combined flow cytometry results showing the binding of kininogen (E) and vitronectin (F) to *C. glabrata* cells. (G) Endocytosis of *C. glabrata* cells that had been coated with the indicated proteins. Data in (B) and (G) are the mean ± SD of 3 experiments each performed in triplicate. Orgs/HPF, organisms per high power field; ns, not significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Fig. 5. Kininogen and vitronectin interact with gC1qR and αv integrins to induce endocytosis. (A and B) Effects of BSA or kininogen and vitronectin on the endocytosis the indicated strains of *C. albicans*. (C) Effects of BSA or kininogen on endothelial cell damage caused by the indicated strains of *C. albicans.* (D) Kininogen and vitronectin increase endothelial cell endocytosis of the indicated *Candida spp.* (E and F) Inhibition of endocytosis of *C. glabrata* coated with either vitronectin alone (E) or vitronectin and kininogen (F) by antibodies against gC1qR (clone 74.5.2) and/or integrins αvβ3 and αvβ5. Data are the mean ± SD of 3 experiments each performed in triplicate. Orgs/HPF, organisms per high power field; ns, not significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Fig. 6. Model of how kininogen and vitronectin function as bridging molecules that bind to *Candida spp.* and induce endocytosis by human endothelial cells. Vitronectin binds to the surface of the organism, which enhances the binding of kininogen. When the organism comes in contact with the endothelial cell, vitronectin interacts mainly with the integrins αvβ3 and αvβ5 and whereas the vitronectin-kininogen complex interacts with both the αv integrins and gC1qR on the endothelial cells surface. These interactions mediate the adherence of the fungus to the endothelial cell and induce the endothelial cell to endocytose the organism.

Fig. 7. Mouse endothelial cells poorly support bridging molecule-mediated endocytosis. (A and B) Endocytosis of *C. glabrata* coated with either human or mouse serum by the indicated endothelial cells after 45 min (A) and 180 min (B). (C) Endocytosis of *C. glabrata* coated with fresh human serum by mouse liver endothelial cells expressing human gC1qR, integrin αv, or integrin β5. Data are the mean ± SD of 3 experiments each performed in triplicate. HUVEC, human umbilical vein endothelial cell; orgs/HPF, organisms per high power field; ns, not significant; **$P < 0.01$, ****$P < 0.0001$. ***$P < 0.001$, ****$P < 0.0001$.