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 α_v 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 α_v 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 α_v 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.

Despite the widespread use of antifungal agents, disseminated candidiasis continues to be a serious problem in hospitalized patients. Previously, *Candida albicans* was the most common cause of candidemia (1). However, the epidemiology of this disease has changed, and *C. albicans* now causes less than half of these infections. In fact, the combined incidence of infections caused by *Candida glabrata*, *Candida parapsilosis*, and *Candida tropicalis* now exceeds the incidence of infections caused by *C. albicans* (2, 3). Even though the causative agents of candidemia have changed, this infection remains highly lethal. Approximately 40% of patients with candidemia die, even with currently available therapy (2, 3). A deeper understanding of the pathogenesis of this disease is essential for developing new strategies to prevent and treat invasive infections caused by multiple species of *Candida*.

During hematogenously disseminated candidiasis, blood-borne organisms must invade the endothelial cell lining of the vasculature to reach the target organs (4). A number of hyphal-associated factors have been found to participate in the pathogenic interactions of *C. albicans* with endothelial cells. *C. albicans* hyphae express invasins such as Als3 and Ssa1 that interact with specific host cell receptors and stimulate fungal endocytosis by endothelial cells in vitro (5-9). Organisms that do not form true hyphae on endothelial cells, such as *C. albicans* mutants with defects in hyphal formation, and *C. glabrata* and *C. tropicalis*, have greatly impaired capacity to invade these cells in standard assays (10, 11) and have highly attenuated virulence in immunocompetent mice (12-14). In patients, organisms that grow only as yeast in vivo are still able to cross the endothelial cell lining of the vasculature and infect target organs during disseminated infections (2, 3). Indeed, patients with candidemia caused by *C. glabrata*, which grows only in the yeast form in vivo, have at least as high mortality as those with candidemia due to *C. albicans* (2, 15). Thus, in humans, a filamentous *C. glabrata* is as virulent as hypha-forming *C. albicans*.

These data suggest that yeast-phase *Candida* spp. must be able to penetrate endothelial cells in vivo by a mechanism that is not evident in standard in vitro invasion assays.

Most assays of *Candida* invasion are performed using media that contain either heat-inactivated serum or no serum at all. Here we demonstrate that when yeast-phase *Candida* spp. are incubated with either fresh human serum or plasma, two proteins, high molecular weight kininogen and vitronectin bind to the fungal surface. Acting as bridging molecules, these serum proteins interact with the globular C1q receptor (gC1qR; also known as p33/HABP) and α v integrins on the surface of human endothelial cells and induce the adherence and endocytosis of the organism. When *C. glabrata* is coated with either human or mouse serum, there is minimal endocytosis by mouse endothelial cells, suggesting a key limitation of the mouse model to study vascular invasion by yeast phase *Candida* spp. This defect in endocytosis can be rescued in vitro by expressing either human gC1qR or human α v integrin in mouse endothelial cells. Thus, we delineate a previously unexplored mechanism by which fungi can invade human endothelial cells.

Results

Serum and plasma enhance the endocytosis of *Candida* spp. Previously, we found that yeast-phase *C. albicans*, such as live *efg1Δ/Δ cph1Δ/Δ* mutant cells or killed wild-type yeast, are very poorly endocytosed by human endothelial cells in vitro (11). Although it has been determined that yeast-phase *C. parapsilosis* cells are endocytosed by endothelial cells in vitro, this process is much slower and less efficient than the endocytosis of hyphal-phase *C. albicans* (16). A limitation of these previous experiments is that they were performed in serum-free media. It is known that serum proteins can act as bridging molecules and mediate the adherence of bacteria to endothelial cells (17, 18). Therefore, we investigated whether serum components could act as bridging molecules between *Candida* spp. and endothelial cells. Live *C. glabrata* yeast and methanol killed, yeast phase *C. albicans* cells were incubated in 20% pooled human serum that was either fresh or heat-inactivated. Killed *C. albicans* cells were 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 *efg1Δ/Δ*
60 *cph1Δ/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

heat-labile serum proteins function as bridging molecules that induce endothelial cells to endocytose yeast-phase *Candida* spp.

The globular C1q receptor (gC1qR) and α v integrins are endothelial cell receptors for serum-coated yeast-phase *C. glabrata*. To identify potential endothelial cell receptors for serum-coated organisms, we employed an affinity-purification approach (6-8) using intact *C. glabrata* cells that had been coated with either fresh or heat-inactivated serum. Among the endothelial cell membrane proteins that were found to bind to serum-coated *C. glabrata* (SI Appendix, Table S1), gC1qR was selected for in-depth analysis because it is known to bind to several different serum proteins (20). Of note, we did not detect binding of serum-coated *C. glabrata* to N-cadherin, which we have found previously to be an endothelial cell receptor for *C. albicans* Als3 and Ssa1, invasins that are expressed by hyphae (5, 6, 8). Thus, serum-coated *C. glabrata* cells interact with different endothelial cell receptors than do *C. albicans* hyphae.

By immunoblotting with an anti-gC1qR monoclonal antibody, we verified that gC1qR was indeed bound by serum-coated *C. glabrata* cells (Fig. 2A). To determine the functional significance of this binding, we used siRNA to knockdown gC1qR. We found the gC1qR siRNA significantly inhibited the endocytosis of serum-coated *C. glabrata* (Fig. 2B and SI Appendix, Fig. S2). The gC1qR siRNA also slightly inhibited *C. glabrata* adherence (Fig. 2C). Because gC1qR is known to be expressed both intracellularly and on the cell surface (21, 22), siRNA knockdown likely depleted both pools of this protein. To verify that surface-expressed gC1qR was required for the endocytosis of serum-coated *C. glabrata*, we tested two different anti-gC1qR monoclonal antibodies for their capacity to inhibit endothelial cell interactions of serum-coated *C. glabrata*. Antibody 74.5.2, which recognizes the high molecular weight kininogen binding site in the C-terminus of the gC1qR (23, 24), reduced endocytosis by 45% but did not significantly affect adherence (Fig. 2D and E). By contrast, antibody 60.11, which is directed against the C1q binding site in the N-terminus of the gC1qR, had no effect on either endocytosis

or adherence. Collectively, these data suggest that the gC1qR functions as an endothelial cell receptor for serum-coated *C. glabrata*.

The finding that blocking gC1qR resulted in incomplete inhibition of endocytosis prompted us to search for additional endothelial cell receptors for serum-coated *C. glabrata*. Because integrins bind to serum proteins that could potentially act as bridging molecules, we screened a panel of anti-integrin monoclonal antibodies for their capacity to block the endocytosis of serum-coated *C. glabrata*. We found that antibodies against integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ inhibited endocytosis and adherence by approximately 45% (Fig. 3A-D). Although some antibodies against integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ also bind to the surface of *C. albicans* (25, 26), flow cytometry confirmed that the monoclonal antibodies used in our experiments did not bind to *C. glabrata* (SI Appendix, Fig. S3). Endocytosis and adherence of *C. glabrata* was similarly inhibited by siRNA knockdown of integrin αv , but not by knockdown of the unrelated integrin $\alpha 5$ (Fig. 3E and F, SI Appendix, Fig. S4). Notably, blocking gC1qR and integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ simultaneously resulted in near maximal reduction in endocytosis, but did not further decrease adherence (Fig. 3G and H), indicating that gC1qR and integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ make additive contributions to endocytosis.

To further explore the relationship among the gC1qR and the integrins $\alpha v\beta 3$ and $\alpha v\beta 5$, we infected endothelial cells with serum-coated *C. glabrata*, stained them with antibodies against the three receptors, and then imaged them with confocal microscopy. We observed that all three receptors accumulated around endocytosed organisms (Fig. 3I). Collectively, these results support the model that when serum proteins bind to a *C. glabrata* cell, they interact with gC1qR and integrins $\alpha v\beta 3$ and $\alpha v\beta 5$, which causes the fungus to adhere to endothelial cells and induce its own endocytosis.

High molecular weight kininogen and vitronectin are bridging molecules that mediate the endocytosis of serum-coated organisms. Next, we sought to identify potential bridging

molecules that mediate the binding of serum-coated organisms to gC1qR and integrins $\alpha v\beta 3$ and $\alpha v\beta 5$. After incubating *C. glabrata* cells in fresh serum, we rinsed them extensively and eluted the bound serum proteins with HCl followed by Tris neutralization. The eluted proteins were separated by SDS-PAGE and analyzed by Western blotting to detect proteins that are known to bind to these receptors. Two proteins, high molecular weight kininogen and vitronectin, were identified. These proteins could be eluted from *C. glabrata* when the cells were incubated in fresh serum, but not heat-inactivated serum (Fig. 4A). To determine whether the binding of these proteins to *C. glabrata* was functionally significant, we analyzed the effects of antibodies against kininogen and vitronectin on the endocytosis of serum-coated *C. glabrata*. We found that antibodies against each protein significantly inhibited endocytosis, but had no effect on adherence (Fig. 4B, SI Appendix, Fig. S5A), suggesting that these proteins may function as bridging molecules that induce endothelial cells to endocytose *C. glabrata*.

High molecular weight kininogen is cleaved by kallikrein and other proteases, releasing bradykinin from the larger protein. The remaining protein, called HKa, consists of a 62 kDa heavy chain that is linked by a disulfide bond to a 56 kDa light chain (27). By immunoblotting with specific monoclonal antibodies and looking for bands of the appropriate molecular mass, we found that both the heavy and light chains of HKa bound to *C. glabrata* (Fig. 4A and C). Both of these chains were bound by *C. glabrata* when the cells were incubated in fresh human serum, but not with heat-inactivated serum. Collectively, these results suggest that high molecular weight kininogen is cleaved to HKa, which then binds to *C. glabrata*.

Using flow cytometry, we analyzed the relationship between the binding of kininogen and vitronectin to *C. glabrata*. We found that when the organisms were incubated with kininogen alone, very little protein bound to them (Fig. 4D and E). When the organisms were incubated with kininogen in the presence of vitronectin, kininogen binding increased significantly. By contrast, vitronectin bound to the organisms both in the presence and absence of kininogen

(Fig. 4D and F). These results suggest the model that vitronectin binds to the organism and facilitates the binding of kininogen.

To determine if kininogen and vitronectin could function as bridging molecules in the absence of other serum proteins, we incubated *C. glabrata* cells with these proteins, either alone or in combination, and then measured their endocytosis by endothelial cells. When the organisms were incubated with kininogen alone, few organisms were endocytosed, similarly to control organisms that had been incubated in BSA (Fig. 4G). When the organisms were incubated in vitronectin alone, endocytosis increased significantly, and it increased even more when the organisms were incubated in both kininogen and vitronectin. The combination of kininogen and vitronectin also significantly increased the adherence of the organisms, while kininogen and vitronectin alone had no effect (SI Appendix, Fig. S5B). Collectively, these data indicate that the human serum proteins kininogen and vitronectin function as bridging molecules that enhance the adherence and induce endocytosis of *C. glabrata* by human endothelial cells.

Next, we investigated the endothelial cell interactions of *C. albicans* cells that had been incubated in kininogen and vitronectin prior to being added to these host cells. These experiments were feasible because, unlike serum, kininogen and vitronectin did not induce significant filamentation. We found that preincubating organisms with these proteins significantly enhanced the endocytosis and adherence of the *C. albicans efg1Δ/Δ cph1Δ/Δ* mutant, which remained in the yeast phase while in contact with the endothelial cells (Fig. 5A and SI Appendix, Fig. S6A). Also, kininogen and vitronectin slightly enhanced the endocytosis of wild-type *C. albicans*, which formed hyphae on the endothelial cells, and largely rescued the endocytosis and adherence defects of the invasin-deficient *als3Δ/Δ ssa1Δ/Δ* mutant (Fig. 5B and SI Appendix, Fig. S6B).

When wild-type *C. albicans* is endocytosed by endothelial cells, it damages these cells, likely by releasing candidalysin into the invasion pocket (6, 29-31). We tested whether coating the *als3Δ/Δ ssa1Δ/Δ* mutant with kininogen and vitronectin would restore its capacity to damage

endothelial cells. While organisms coated with BSA caused minimal endothelial cell damage, cells coated with kininogen and vitronectin induced significantly greater damage (Fig. 5C). These results indicate that in the absence of invasins, bridging molecules can enhance the endocytosis of *C. albicans* hyphae, leading to subsequent endothelial cell damage.

As we had observed that fresh serum increased the endocytosis of species of *Candida*, other than *C. albicans*, we investigated whether human kininogen and vitronectin functioned as bridging molecules for these organisms. We found that these proteins significantly increased the endocytosis of *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, but not *C. auris*. (Fig. 5D). Kininogen and vitronectin also increased the endothelial cell adherence of *C. parapsilosis* and *C. krusei* (SI Appendix, Fig. S6C). Next, we tested whether the enhanced endocytosis of these organisms by kininogen and vitronectin would result in endothelial cell damage. To increase the sensitivity of the experiment, we increased the inoculum and extended the incubation period to 6 hr. None of these organisms caused detectable damage to the endothelial cells (SI Appendix, Fig. S6D), indicating that induction of endocytosis alone is not sufficient for these species of *Candida* to cause significant endothelial cell damage.

To investigate which endothelial cell receptor was responsible for interacting with each bridging molecule, we tested the inhibitory effects of specific antibodies directed against gC1qR and α v integrins. When *C. glabrata* cells were incubated with vitronectin alone, endocytosis was significantly inhibited by antibodies against integrins α v β 3 and α v β 5, but not by the anti-gC1qR antibody (Fig. 5E). None of these antibodies significantly reduced the adherence of vitronectin-coated organisms (SI Appendix, Fig. S6E). When the organisms were incubated with vitronectin and kininogen, endocytosis was inhibited by both the anti-gC1qR antibody and the anti- α v integrin antibodies (Fig. 5F). The combination of all 3 antibodies inhibited endocytosis in an additive manner and also inhibited adherence (SI Appendix, Fig. S6F). Taken together, these data support the model that vitronectin likely binds first to the fungal surface where it is recognized mainly by integrins α v β 3 and α v β 5 (Fig. 6). Binding of vitronectin enables kininogen

to bind to fungal cell surface, and the vitronectin-kininogen complex is recognized by both gC1qR and the α v integrins, leading to the strong adherence and subsequent endocytosis of the organism.

Expression of human gC1qR and α v integrins on mouse endothelial cells enhances bridging molecule mediated endocytosis. Next, we investigated whether mouse serum bridging molecules also mediated the endocytosis of *C. glabrata* by comparing the capacity of mouse and human serum to mediate endocytosis by human endothelial cells. To maximize endocytosis, we incubated the organisms in 100% serum. We observed that after 45 min, mouse serum enhanced the endocytosis of *C. glabrata* by human endothelial cells, but to a lesser extent than human serum (Fig. 7A). Mouse serum also increased adherence to human endothelial cells, but not as much as human serum (SI Appendix, Fig. S7A). These differences in endocytosis and adherence persisted even when the incubation period was increased to 3 h (Fig. 7B and SI Appendix, Fig. S7B). These results indicate that while mouse serum proteins can function as bridging molecules between *C. glabrata* and human endothelial cells, they are less effective than human serum proteins.

To investigate whether serum bridging molecules could mediate the endocytosis of *C. glabrata* by mouse endothelial cells, we obtained primary mouse kidney and liver endothelial cells and tested their capacity to endocytose *C. glabrata* cells that had been coated with either human or mouse serum. We found that there was minimal endocytosis and adherence of organisms coated with human or mouse serum by mouse endothelial cells after both 45 min and 180 min (Fig. 7A and B, SI Appendix, Fig. S7A and B). To verify that human endothelial cells other than those obtained from umbilical cord veins were able to endocytose serum coated organisms, we tested a Tert-immortalized human microvascular endothelial (TIME) cell line. *C. glabrata* cells coated with human serum were endocytosed by and adhered to the TIME cells more than human umbilical vein endothelial cells (SI Appendix, Fig. S7C and D). Collectively,

these data indicate that while both mouse and human serum proteins can function as bridging molecules between *C. glabrata* and human endothelial cells, mouse endothelial cells have very limited capacity to endocytose organisms coated with serum from either mice or humans.

We considered the possibility that the inability of mouse endothelial cells to endocytose serum coated *C. glabrata* was due to difference between the receptors on mouse vs. human endothelial cells. To evaluate the possibility, we used lentivirus to transduce primary mouse liver endothelial cells with human *C1QBP* (gC1qR), *ITGAV* (integrin α v), or *ITGB5* (integrin β 5). Control cells were transduced with lentivirus encoding GFP. The expression of the human proteins by the transduced endothelial cells was verified by Western blotting (SI Appendix, Fig. 7E). Endothelial cells that expressed human gC1qR and integrin α v endocytosed significantly more serum-coated organisms than did the control endothelial cells (Fig. 7C). *C. albicans* also had enhanced adherence to the cells that expressed human gC1qR, integrin α v, and integrin β 5 (SI Appendix, Fig. S7F). These data demonstrate that human gC1qR and integrin α v mediate the endocytosis and adherence of serum-coated *C. glabrata*. They also suggest that these human receptors are functionally different from their mouse counterparts.

Discussion

In this study, we sought to elucidate how species of *Candida* that do not form true hyphae are able to invade vascular endothelial cells. Using *C. glabrata* as a representative fungus that grows only as yeast within the human host, we determined that proteins present in human serum act as bridging molecules between the fungus and human endothelial cells and induce the adherence and subsequent endocytosis of the organism. The data presented here indicate that binding of vitronectin to the fungal surface facilitates the subsequent binding of kininogen. Vitronectin interacts mainly with the integrins α v β 3 and α v β 5 endothelial cells, and the kininogen-vitronectin complex also interacts with gC1qR. The binding of these serum proteins to

their respective receptors causes the organism to adhere to endothelial cells and stimulates its subsequent endocytosis (Fig. 6). Not only did kininogen and vitronectin act as bridging molecules for *C. glabrata*, but they also mediated endothelial cell endocytosis of yeast-locked and invasin-deficient *C. albicans* mutants and other medically important species of *Candida*, including *C. tropicalis*, *C. parapsilosis*, and *C. krusei*. Although serum enhanced the endocytosis of *C. auris*, kininogen and vitronectin did not, suggesting that other serum proteins must function as bridging molecules for this organism. Also, serum bridging molecules did not induce the endocytosis of *S. cerevisiae*, indicating that bridging molecule-mediated endocytosis is not a general property of yeast. Taken together, these results indicate that invasion of vascular endothelial cells via bridging molecule-mediated endocytosis is a pathogenic strategy shared by many medically important *Candida* spp.

Vitronectin, which is bound by integrins $\alpha\beta3$ and $\alpha\beta5$, is known to function as a bridging molecule that mediates adherence to respiratory epithelial cells of a variety of bacteria, including nontypeable *Haemophilus influenzae*, *Moraxella catarrhalis*, group A streptococci, and *Pseudomonas aeruginosa* (reviewed in (32)). In addition to mediating adherence, vitronectin induces the internalization of *Neisseria gonorrhoeae* and *Pseudomonas fluorescens* by epithelial cells (33, 34). *C. albicans*, *C. parapsilosis* and *C. tropicalis* have been shown to bind to fluid phase vitronectin (35), and the binding of *C. albicans* to vitronectin mediates adherence to keratinocytes (36). Our findings demonstrate that *C. glabrata* and *C. krusei* are additional *Candida* spp. that bind to vitronectin. More importantly, we show that vitronectin acts as a bridging molecule that, in conjunction with kininogen, mediates the endocytosis of these organisms by human endothelial cells.

Studies of the interaction of kininogen with microbial pathogens have focused mainly on its proteolytic cleavage to release bradykinin and other fragments with antimicrobial activity. Kininogen has been found to bind to *S. aureus*, *Salmonella typhimurium*, and *Bacteroides* spp. (37, 38). Rapala-Kozik et al., have determined that virtually all medically important *Candida* spp.

bind kininogen (39-41). In contrast to the results shown here, they found that kininogen could bind to the fungus in the absence of additional serum proteins, whereas we found that there was minimal binding of kininogen to *C. glabrata* unless vitronectin was present. The likely explanation for these divergent results is that the other investigators used a more sensitive assay that was able to detect the binding of even low amounts of kininogen to the fungal surface. Nevertheless, our results indicate that vitronectin dramatically increases the amount of kininogen that binds to *C. glabrata* and enables kininogen to function as a bridging molecule that enhances fungal endocytosis.

Although the function of kininogen as a bridging molecule between microbial pathogens and host cells has not been appreciated previously, it is known that kininogen can bind to glycoprotein 1b on platelets and integrin $\alpha M\beta 2$ on neutrophils to enhance the co-adherence of these two cells (42). We determined that unlike platelets and neutrophils, endothelial cells bind kininogen via gC1qR, a result that has been reported by others (43). gC1qR has also been found to be a receptor for *Listeria monocytogenes* that mediates the internalization of this organism. However, this bacterium binds directly to gC1qR, and the interaction can be blocked by both C1q and monoclonal antibody 60.11, which is directed against the C1q binding site of gC1qR (44). By contrast, we found that while monoclonal antibody 60.11 did not inhibit bridging molecule-mediated endocytosis of *C. glabrata*, monoclonal antibody 74.5.2, which is directed against the kininogen binding site of gC1qR, was highly inhibitory. These results support the model that when kininogen is bound to the surface of *Candida* spp., it interacts with gC1qR on endothelial cells and stimulates the endocytosis of the organisms.

Patients with hematogenously disseminated candidiasis due to *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. auris* have at least as high mortality as those who are infected with *C. albicans* (2, 15, 45). These data suggest that in humans, these different species of *Candida* have similar virulence. In immunocompetent mice, *C. albicans* is highly virulent, and most wild-type strains are capable of causing a lethal infection. By contrast, intravenous infection of

immunocompetent mice with *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. auris* induces minimal mortality even when high inocula are used (46, 47). Thus in mice, these species of *Candida* have greatly attenuated virulence. A possible explanation for this discrepancy is that *C. albicans* is able to form hyphae that express invasins such as Als3 and Ssa1 that interact directly with endothelial cell receptors and induce endothelial cell endocytosis. By contrast, other species of *Candida*, such as *C. glabrata* invade endothelial cells by bridging molecule-mediated endocytosis, a process that occurs inefficiently in mice.

The results of the in vitro studies supported this concept. *C. albicans* was endocytosed avidly by human endothelial cells in the absence of serum and coating the organism with serum only increased endocytosis slightly. *C. glabrata* was avidly endocytosed by human endothelial cells only when it was coated with serum proteins. Both mouse and human proteins increased endocytosis by human cells, indicating that mouse serum proteins can function as bridging molecules, albeit not as well as human proteins. Importantly, *C. glabrata* was poorly endocytosed by mouse liver and kidney endothelial cells when it was coated with either mouse or human serum. When the mouse endothelial cells were engineered to express human gC1qR or integrin α_v , they gained the capacity to endocytose serum-coated *C. glabrata*. These data indicate that a key difference between mice and humans is that mouse gC1qR and integrin α_v do not support bridging molecule-mediated endocytosis of *C. glabrata*.

Although the mouse model of disseminated candidiasis is an excellent model for investigating antifungal efficacy and many aspects of fungal pathogenicity, our results suggest that this model is not optimal for investigating how *C. glabrata* and possibly other *Candida* spp. other than *C. albicans* disseminate hematogenously because mouse endothelial cells do not support bridging molecule-mediated vascular invasion. Even though mice inoculated intravenously with these organisms still contain some fungal cells in their tissues, we speculate that the organisms must egress from the vasculature by another mechanism(s) that has less pathogenic impact. This possibility is currently being investigated.

The results presented here indicate that many medically important species of *Candida* can utilize serum proteins as bridging molecules to induce their own endocytosis by human vascular endothelial cells. Because this mechanism is shared by multiple *Candida spp.*, it represents a promising therapeutic target for preventing or ameliorating hematogenously disseminated candidiasis.

Methods

Ethics statement. All animal work was approved by the Institutional Animal Care and Use Committee (IACUC) of the Lundquist Institute for Biomedical Innovation at Harbor-UCLA Medical Center. The collection of blood from normal human volunteers was performed under protocol 30636-01R, which was approved by the IRB of the Lundquist Institute. Informed consent was obtained prior to phlebotomy.

Serum and plasma. After obtaining informed consent, blood was collected by venipuncture from healthy volunteers. Blood was also collected from anesthetized Balb/C mice by cardiac puncture. To obtain serum, the blood was allowed to clot at room temperature for 30 min and then centrifuged at 2000 rpm for 10 min at 4°C. After collecting the serum, samples from individual donors were pooled and stored in aliquots in liquid nitrogen. To make heat-inactivated serum, the fresh serum was incubated at 56°C for 1 hr and stored in aliquots in liquid nitrogen.

To obtain plasma, fresh human blood was transferred to 4 ml vacutainer tubes containing 7.2 mg of K₂EDTA (BD, Inc.). The tubes were then centrifuged at 2000 rpm for 10 minutes at 4°C, after which the plasma was collected, pooled, aliquoted, and stored in liquid nitrogen. Heat-inactivated plasma was made by incubating fresh plasma at 56°C for 1 hr.

Host cells, fungal strains and growth conditions. Human umbilical vein endothelial cells were isolated from umbilical cords and grown as described (30, 48). Mouse kidney endothelial

cells (Cell Biologics), mouse liver endothelial cells (Cell Biologics), and hTert-immortalized human microvascular endothelial cells (American Type Culture Collection) were purchased and grown according to the suppliers' instructions.

The fungal strains used in this work are listed in Supplemental Table 2. For use in the experiments, the organisms were grown overnight in yeast extract peptone dextrose (YPD) broth at 30°C in a shaking incubator. They were harvested by centrifugation, washed twice with PBS and enumerated with a hemacytometer as previously described (30). To produce killed organisms, cells of *C. albicans* strain DIC185 were pelleted by centrifugation and then resuspended in 100% methanol for 2 min. The killed organisms were recovered by centrifugation and washed two times with PBS. Fungal killing was verified by plating a sample of the cells onto YPD agar.

Strain DSC10 was constructed by plating strain CAN34 (*efg1Δ/Δ cph1Δ/Δ*) on minimal medium containing 5-fluororotic acid. The resulting Ura⁻ strain was transformed with a PstI/NotI-digested fragment of pBSK-URA3 (49) to restore the *URA3-IRO1* locus. Proper integration was verified by PCR.

Coating fungal cells with bridging molecules. To coat the organisms with serum or plasma, approximately 5×10^7 fungal cells were mixed with either RPMI 1640 medium alone (Irvine Scientific) or RPMI 1640 medium containing 20% fresh or heat-inactivated human serum and then incubated for 1 hr at 37°C in a shaking incubator. In some experiments, the human serum was replaced with human plasma to which CaCl₂ was added to reverse the effects of the EDTA. In experiments comparing mouse with human serum, the organisms were incubated with either 100% mouse or human serum. After coating, the fungal cells were washed twice with PBS, diluted, and counted for use in the assays described below.

To coat the organisms with bridging molecules, approximately 2×10^7 fungal cells were incubated with human kininogen (10 µg/ml; Molecular Innovations, Inc., Cat. # HK-TC) and/or

human vitronectin (30 µg/ml; Molecular Innovations Inc., Cat. # HVN-U) in RPMI 1640 medium supplemented with 50 µM ZnCl₂ and 3 µM CaCl₂. Control cells were incubated with BSA (Sigma-Aldrich). The cells were incubated for 1 hr at 37°C in a shaking incubator and processed as described above.

Confocal microscopy. The confocal microscopy was performed as previously described (6). Briefly, endothelial cells were grown to confluency on fibronectin-coated glass coverslips and then infected with 3x10⁵ *C. glabrata* cells. After 45 min, the cells were fixed with 3% paraformaldehyde and blocked with 5% goat serum containing 0.05% Triton X-100. The cells were incubated with Alexa Fluor 568-labeled phalloidin (Thermo Fisher Scientific, Cat. #A12380), rabbit anti-gC1qR antibody (Santa Cruz Biotechnology, Cat. #sc-48795), anti-integrin αvβ3 monoclonal antibody (Millipore-Sigma, clone LM609, Cat. # MAB1976), or anti-integrin αvβ5 monoclonal antibody (Millipore-Sigma, clone P1f6, Cat. # MAB1961). After extensive rinsing, the cells were incubated with the appropriate Alexa Fluor labeled secondary antibody (Thermo Fisher Scientific, Cat. #A-11031 or A-11034), rinsed, and then imaged by confocal microscopy. The *C. glabrata* cells were viewed by differential interference contrast. Consecutive z-stacks were combined to create the final images.

Endocytosis assay. The endocytosis of the various organisms by endothelial cells was determined by our standard differential fluorescence assay as described previously (6, 50). Briefly, endothelial cells grown on fibronectin-coated glass coverslips were incubated with 10⁵ fungal cells in 5% CO₂ at 37°C for 45 or 180 min. Next, the cells were fixed in 3% paraformaldehyde, and the non-endocytosed organisms were stained with an anti-*Candida* antibody (Meridian Life Science, Cat. # B65411R) that had been conjugated with Alexa Fluor 568 (Thermo Fisher Scientific, Cat. # A-10235). After rinsing the cells extensively with PBS, the endothelial cells were permeabilized in 0.05% Triton X-100 (Sigma-Aldrich), and the cell-

associated organisms were stained with the anti-*Candida* antibody conjugated with Alexa Fluor 488 (Thermo Fisher Scientific). The coverslips were mounted inverted on microscope slides and viewed with an epifluorescent microscope. The number of endocytosed organisms was determined by scoring at least 100 organisms per slide. Each experiment was performed at least three times in triplicate.

The effects of depolymerizing microfilaments on endocytosis was determined by incubating the endothelial cells with 0.4 μ M cytochalasin D (Sigma-Aldrich) for 45 min prior to infection. Control endothelial cells were incubated in the diluent (0.1% DMSO) in parallel. The cytochalasin D and DMSO remained in the medium for the duration of the infection. To determine the effects of blocking endothelial cell receptors on endocytosis, the endothelial cells were incubated with anti-gC1qR antibodies (Santa Cruz Biotechnology, clone 74.5.2 Cat. # sc-23885 and Abcam, clone 60.11, Cat. # ab24733), anti- α v β 3 antibody (Millipore Sigma, clone LM609, Cat. # MAB1976), α v β 5 (Millipore Sigma, clone P1F6, Cat. # MAB1961), or a combination of antibodies, each at 10 μ g/ml. Control cells were incubated in the same concentration of mouse IgG (R&D Systems, clone 11711; # MAB002). The endothelial cells were incubated with the antibodies for 1 hr prior to infection and the antibodies remained in the medium for the duration of infection.

To determine the effects of inhibit bridging molecules on endocytosis, *C. glabrata* cells were coated with 20% heat-inactivated or fresh serum in the presence of an anti-kininogen antibody (Santa Cruz Biotechnology, clone 2B5, Cat. # sc-23914), an anti-vitronectin antibody (Millipore Sigma, clone 8E6(LJ8), Cat. # MAB88917), or an isotype control IgG, each at 10 μ g/ml. The organisms were then washed twice with PBS, counted, and used in the endocytosis assay.

Protein purification and Western blotting. Endothelial cell membrane proteins were isolated using glucopyranoside according to our previously described method⁶⁻⁸. To pull down

endothelial cell proteins that bound to serum-coated *C. glabrata*, 8×10^8 organisms that had been coated with fresh or heat-inactivated serum were incubated with 1 mg of endothelial cell membrane proteins on ice for 1 hr. Unbound proteins were removed by rinsing with 1.5% glucopyranoside, after which the bound proteins were eluted with 6M Urea. Samples were added to SDS-PAGE sample buffer, heated to 90°C for 5 min, and then separated by SDS-PAGE. After staining the gel with Instant Blue (Expedeon, Cat. #ISB1L), selected bands were excised and the proteins in them were sequenced by MS-MS at the UCLA proteomics core facility. To verify that gC1qR bound to *C. glabrata* cells that had been coated with fresh serum, the pull-down assay was repeated and Western blotting was performed with the anti-gC1qR antibody (clone 74.5.2).

To detect serum proteins that bind to *C. glabrata*, 1×10^8 *C. glabrata* cells were incubated with 20% fresh or heat-inactivated serum in RPMI 1640 medium for 1 hr at 37°C. Unbound serum proteins were removed by rinsing the cells twice with PBS, after which bound serum proteins were eluted with 2M HCl, pH 2.0 and immediately neutralized with Tris buffer, pH 8.0. The proteins were separated by SDS-PAGE and Western blotting using an anti-kininogen heavy chain antibody (Santa Cruz Biotechnology, clone 2B5, Cat. # sc-23914), anti-light chain antibody (Santa Cruz Biotechnology, clone 14J09, Cat. # sc-80524), and anti-vitronectin antibody (clone 8E6(LJ8)) was performed to detect kininogen and vitronectin that had been eluted from *C. glabrata*.

siRNA. Knockdown of endothelial cell surface proteins was accomplished using siRNA. The endothelial cells were transfected with gC1qR siRNA (Santa Cruz Biotechnology, Cat. # sc-42880), integrin $\alpha 5$ siRNA (Santa Cruz Biotechnology, Cat. # sc-29372), integrin αv siRNA (Santa Cruz Biotechnology, Cat. # sc-29373), or scrambled control siRNA (Qiagen, Cat. # 1027281) using Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer's instructions. After 48 hr, the transfected endothelial cells were infected with serum coated *C.*

glabrata and the number of endocytosed organisms was determined. Knockdown of each protein was verified by immunoblotting with antibodies against gC1qR (clone 74.5.2), integrin $\alpha 5$ (Millipore-Sigma, Cat. # AB1928), integrin αv (Santa Cruz Biotechnology, clone H-2, Cat # sc-376156), integrin $\beta 3$ (Santa Cruz Biotechnology, clone B-7, Cat. # sc-46655), integrin $\beta 5$ (Santa Cruz Biotechnology, clone F-5, Cat. # sc-398214), or actin (Millipore-Sigma, clone C4, Cat. # A5441-100UL)

Flow cytometry. The binding of kininogen and vitronectin to *C. glabrata* was analyzed by a modification of a previously described method (51). *C. glabrata* cells were incubated with kininogen that had been labeled with Alexa Fluor 568 (Thermo Fisher Scientific, Cat. #A20184) and/or unlabeled vitronectin, both at a final concentration of 30 μ g/ml, for 1 hr at 37°C. Control cells were incubated in a similar concentration of BSA. The unbound proteins were removed by washing the cells twice with PBS. Next, the cells were incubated with the anti-vitronectin antibody followed by the Alexa Fluor 488-labeled goat anti-mouse secondary antibody. The fluorescence of the cells was then quantified using a Becton Dickinson FACScalibur flow cytometer, analyzing 10,000 cells per sample using the FlowJo software.

The potential binding the anti- $\alpha v\beta 3$ and anti- $\alpha v\beta 5$ antibodies to *C. glabrata* was determined by incubating *C. glabrata* cells with each antibody at a final concentration of 10 μ g/ml, followed by the Alexa Fluor 488-labeled goat anti-mouse secondary antibody. The fluorescence of the cells was then quantified by flow cytometer, analyzing 10,000 cells per sample.

Endothelial cell damage assay. The capacity of wild-type (CAI4-URA) and *als3 Δ / Δ ssa1 Δ / Δ* *C. albicans* strains to damage human umbilical vein endothelial cells was determined using our previously described ⁵¹Cr release assay (6). Endothelial cells were grown in a 96-well tissue

culture plate containing detachable wells and loaded with ^{51}Cr . The *C. albicans* were coated with either BSA or kininogen and vitronectin and rinsed, after which 4×10^4 fungal cells were added to individual wells of endothelial cells. After incubation for 3 h, the medium above the cells was aspirated and the wells were detached from each other. The amount of ^{51}Cr released into the medium and remaining in the endothelial cells was determined using a gamma counter.

When the *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C. auris* strains were tested in the damage assay, they were processed similarly to the *C. albicans* cells except that the inoculum was increased to 2×10^5 cells per well and the incubation period was increased to 6 h.

Lentivirus packaging and host cell transduction. The transfer vectors (pLenti-EF1A-EGFP-Blast, pLenti-EF1A-hITGAV-NEO, pLenti-EF1A-hITGB5-NEO or pLenti-EF1A-hC1QBP-Blast) were constructed by cloning eGFP, hC1QBP [NM_001212.4], hITGAV [NM_002210], or hITGB5 [NM_002213] into pLenti-Cas9-Blast (Addgene; # 52962) or pLenti-Cas9-NEO at the BamHI and XbaI sites. The virus was produced by transfecting HEK293T cells with plasmid psPAX2 (Addgene; # 12260), plasmid pCMV-VSVG (Addgene; # 8454), and transfer vector at 1:1:1 molar ratio using the X-tremeGENE 9 DNA transfection reagent (Sigma-Aldrich; # 6365787001) according to the manufacturer's instructions. The supernatant containing the virus was collected at 60 h post-transfection, passed through a 0.45 μm PVDF filter and stored at 4°C (short-term) or -80°C (long-term).

For transduction, mouse primary liver endothelial cells were seeded into a 6-well plate in mouse endothelial cell medium (Cell Biologics, Inc. # M1168). The cells were transduced with lentivirus in the presence of 8 $\mu\text{g}/\text{ml}$ polybrene (Santa Cruz Biotechnology; #SC134220), centrifuged at 1000g for 30 min, and then incubated at 37°C in 5% CO_2 overnight. The next morning, the cells were transferred to 10 cm diameter tissue culture dishes. For the cells transduced with hC1QBP, 10 $\mu\text{g}/\text{ml}$ of blasticidin (Gibco; # A1113903) was added to the

medium 2 d post transduction to select for transduced cells and selection was maintained for 7 d. Expression of eGFP was determined by fluorescent microscopy and expression of gC1qR, integrin α v, and integrin β 5 were verified via immunoblotting of whole cell lysates with an anti-gC1qR antibody (clone 60.11), anti- integrin α v antibody (MilliporeSigma; #AB1930), and anti- integrin β 5 antibody (My Biosource, Inc; # MBS617750). Total loading was determined by immunoblotting with an anti-GAPDH antibody (Cell Signaling; # 5174).

Statistical analysis. All data were analyzed using Prism (GraphPad). Differences among experimental groups were analyzed by one-way analysis of variance followed by pair-wise analysis with Dunnett's multiple comparison test. When a single pair of data was analyzed, the 2-way student's t-test assuming unequal variance was used. *P* values ≤ 0.05 were considered to be significant.

Data availability

The raw data that support the findings of this study are available from the corresponding author upon request.

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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 μ m. (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 $\pm$ 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 $\pm$ 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 $\alpha v\beta 3$ and $\alpha v\beta 5$ 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 $\alpha v\beta 3$ and $\alpha v\beta 5$ 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 μ m. Results shown in (A-H) are the mean $\pm$ 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. 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 $\pm$ 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 $\alpha v\beta 3$ and $\alpha v\beta 5$. Data are the mean $\pm$ 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 $\alpha v \beta 3$ and $\alpha v \beta 5$ 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 α_v , or integrin β_5 . Data are the mean $\pm$ SD of 3 experiments each performed in triplicate. HUVEC, human umbilical vein endothelial cell; orgs/HPF, organisms per high power field; ns, not significant; ** $P < 0.01$, **** $P < 0.0001$. *** $P < 0.001$, **** $P < 0.0001$.