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

Quynh T. Phan[1], Norma V. Solis[1], Jianfeng Lin[1], Marc Swidergall[1,2], Shakti Singh[1], Hong Liu[1], Donald C. Sheppard[3], Ashraf S. Ibrahim[1,2], Aaron P. Mitchell[4], Scott G. Filler[1,2]*

[1]Institute for Infection and Immunity, Division of Infectious Diseases. The Lundquist Institute for Biomedical Innovation at Harbor-UCLA Medical Center, Torrance, CA 90502, USA

[2]David Geffen School of Medicine at UCLA, Los Angeles, CA 90024, USA

[3]Department of Microbiology and Immunology, Faculty of Medicine, McGill University, Montreal, Quebec, Canada

[4]Department of Microbiology, University of Georgia, Athens, Georgia 30602, USA

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.

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, afilamentous *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 αv 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 αv 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 *efg1*Δ/Δ *cph1*Δ/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

fungal cells were then rinsed and incubated with human umbilical vein endothelial cells. When the organisms were incubated with heat-inactivated serum, few cells were endocytosed, similarly to control organisms that had been incubated in serum-free medium (Fig. 1A and B). When the organisms were incubated in fresh serum, the number of endocytosed cells increased by 8- to 9-fold. Incubating *C. glabrata* and *C. albicans* with fresh serum also increased the number of cell-associated organisms, a measure of adherence (Fig. 1C and D). To verify that serum could enhance the endothelial cell interactions of live *C. albicans*, we tested an *efg1*Δ/Δ *cph1*Δ/Δ mutant strain that remains in the yeast phase when exposed to serum (14). The endocytosis and adherence of this strain were increased when it was incubated in fresh serum as compared to heat-inactivated serum (SI Appendix, Fig. S1A and B). Fresh serum also significantly enhanced the endothelial cell endocytosis and adherence of live, yeast-phase *C. parapsilosis*, *Candida krusei*, and *Candida auris*, but not *C. tropicalis* (Fig. 1E and F). Fresh human plasma was at least as effective as fresh human serum at enhancing the endocytosis and adherence of *C. glabrata* (SI Appendix, Fig. S1C and D), indicating that both plasma and serum contain factors that strongly enhance the endothelial cell interactions of yeast-phase organisms.

Although fresh serum significantly enhanced the endocytosis and adherence of multiple species of *Candida,* it only increased the adherence of *S. cerevisiae* but had no effect on endocytosis (SI Appendix, Fig. S1E and F). Thus, the bridging molecules that bind to *Candida* spp. appear to be non-functional after they bind to *S. cerevisiae*.

To verify that the serum-coated organisms were being endocytosed, endothelial cells were infected with serum-coated *C. glabrata*, fixed and then stained for actin. We observed that actin microfilaments coalesced around *C. glabrata* cells, a hallmark of endocytosis (Fig. 1G). When endothelial cells were treated with cytochalasin D to depolymerize actin, the endocytosis of serum-coated organisms was significantly decreased (Fig. 1H). Cytochalasin D also reduced 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 αv 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 αvβ3 and αvβ5 inhibited endocytosis and adherence by approximately 45% (Fig. 3A-D). Although some antibodies against integrins αvβ3 and αvβ5 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 α5 (Fig. 3E and F, SI Appendix, Fig. S4). Notably, blocking gC1qR and integrins αvβ3 and αvβ5 simultaneously resulted in near maximal reduction in endocytosis, but did not further decrease adherence (Fig. 3G and H), indicating that gC1qR and integrins αvβ3 and αvβ5 make additive contributions to endocytosis.

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

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

(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 efg1*Δ/Δ *cph1*Δ/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 sightly 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 *als3*Δ/Δ *ssa1*Δ/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 *als3*Δ/Δ *ssa1*Δ/Δ mutant with kininogen and vitronectin would restore is 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 αv integrins. When *C. glabrata* cells were incubated with vitronectin alone, endocytosis was significantly inhibited by antibodies against integrins αvβ3 and αvβ5, 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-αv 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 αvβ3 and αvβ5 (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 αv integrins, leading to the strong adherence and subsequent endocytosis of the organism.

Expression of human gC1qR and αv 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 αv), or *ITGB5* (integrin β5). 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 αv 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 αv, and integrin β5 (SI Appendix, Fig. S7F). These data demonstrate that human gC1qR and integrin αv 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 αvβ3 and αvβ5 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 αvβ3 and αvβ5, 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 αMβ2 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 *Candid* 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

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.

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^{o}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^{o}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^{o}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^{o}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

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 30ºC 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 $5x10^7$ 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 37ºC 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 $2x10^7$ fungal cells were
388 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 $ZnCl_2$ and 3 µM $CaCl_2$. 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 $3x10^5$ *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 10^5 fungal cells in 5% CO_2 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 µM 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-αvβ3 antibody (Millipore Sigma, clone LM609, Cat. # MAB1976), αvβ5 (Millipore Sigma, clone P1F6, Cat. # MAB1961), or a combination of antibodies, each at 10 µg/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 µg/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 method[6-8]. To pull down

441 endothelial cell proteins that bound to serum-coated *C. glabrata*, 8x10^8 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^{o}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*, 1x10^8 *C. glabrata* cells were incubated
452 with 20% fresh or heat-inactivated or serum in RPMI 1640 medium for 1 hr at 37^{o}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.*

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 α5 (Millipore-Sigma, Cat. # AB1928), integrin αv (Santa Cruz Biotechnology, clone H-2, Cat # sc-376156), integrin β3 (Santa Cruz Biotechnology, clone B-7, Cat. # sc-46655), integrin β5 (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 µg/ml, for 1 hr at 37^0C. 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-αvβ3 and anti-αvβ5 antibodies to *C. glabrata* was determined by incubating *C. glabrata* cells with each antibody at a final concentration of 10 µg/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 ^{51}Cr 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 x 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 x 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 0.45um PVDF filter and stored at 4℃ (short-term) or -80℃ (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 µg/ml polybrene (Santa Cruz Biotechnology; #SC134220), centrifuged at 1000*g* for 30 min, and then incubated at 37℃ 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 µg/ml of blasticidin (Gibco; # A1113903) was added to the

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

Acknowledgments

This work was supported in part by NIH grant R01AI124566 to S.G.F., R00DE026856 to M.S., and R01AI141202 to A.S.I. The collection of umbilical cords for this project was supported in part by the National Center for Advancing Translational Sciences through UCLA CTSI Grant UL1TR001881. We thank Adam Diab for assistance with tissue culture and Nathan Wiederhold for providing the clinical isolates.

.

References

1. M. A. Pfaller *et al.*, Bloodstream infections due to *Candida* species: SENTRY antimicrobial surveillance program in North America and Latin America, 1997-1998. *Antimicrob. Agents Chemother.* **44**, 747-751. (2000).
2. M. Pfaller *et al.*, Epidemiology and outcomes of candidemia in 3648 patients: data from the Prospective Antifungal Therapy (PATH Alliance(R)) registry, 2004-2008. *Diagn Microbiol Infect Dis* **74**, 323-331 (2012).
3. A. A. Cleveland *et al.*, Changes in incidence and antifungal drug resistance in candidemia: results from population-based laboratory surveillance in Atlanta and Baltimore, 2008-2011. *Clin. Infect. Dis.* **55**, 1352-1361 (2012).
4. S. E. Grubb *et al.*, *Candida albicans*-endothelial cell interactions: a key step in the pathogenesis of systemic candidiasis. *Infect. Immun.* **76**, 4370-4377 (2008).
5. J. N. Sun *et al.*, Host cell invasion and virulence mediated by *Candida albicans* Ssa1. *PLoS Pathog.* **6**, e1001181 (2010).
6. Q. T. Phan *et al.*, Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biol* **5**, e64 (2007).
7. W. Zhu *et al.*, EGFR and HER2 receptor kinase signaling mediate epithelial cell invasion by *Candida albicans* during oropharyngeal infection. *Proc. Natl. Acad. Sci. USA* **109**, 14194-14199 (2012).
8. Q. T. Phan, R. A. Fratti, N. V. Prasadarao, J. E. Edwards, Jr., S. G. Filler, N-cadherin mediates endocytosis of *Candida albicans* by endothelial cells. *J. Biol. Chem.* **280**, 10455-10461 (2005).
9. Y. Liu, R. Mittal, N. V. Solis, N. V. Prasadarao, S. G. Filler, Mechanisms of *Candida albicans* trafficking to the brain. *PLoS Pathog.* **7**, e1002305 (2011).
10. S. G. Filler, B. O. Ibe, P. M. Luckett, J. U. Raj, J. E. Edwards, Jr., *Candida albicans* stimulates endothelial cell eicosanoid production. *J. Infect. Dis.* **164**, 928-935 (1991).
11. Q. T. Phan, P. H. Belanger, S. G. Filler, Role of hyphal formation in interactions of *Candida albicans* with endothelial cells. *Infect. Immun.* **68**, 3485-3490 (2000).
12. S. P. Saville, A. L. Lazzell, C. Monteagudo, J. L. Lopez-Ribot, Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. *Eukaryot. Cell* **2**, 1053-1060 (2003).
13. C. G. Chen *et al.*, Non-lethal *Candida albicans cph1/cph1 efg1/efg1* transcription factor mutant establishing restricted zone of infection in a mouse model of systemic infection. *Int J Immunopathol Pharmacol* **19**, 561-565 (2006).

14. H. J. Lo *et al.*, Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90**, 939-949 (1997).
15. C. Moran, C. A. Grussemeyer, J. R. Spalding, D. K. Benjamin, Jr., S. D. Reed, Comparison of costs, length of stay, and mortality associated with *Candida glabrata* and *Candida albicans* bloodstream infections. *Am. J. Infect. Control* **38**, 78-80 (2010).
16. T. Shintaku *et al.*, Human endothelial cells internalize *Candida parapsilosis* via N-WASP-mediated endocytosis. *Infect. Immun.* **81**, 2777-2787 (2013).
17. A. L. Cheung, M. Krishnan, E. A. Jaffe, V. A. Fischetti, Fibrinogen acts as a bridging molecule in the adherence of *Staphylococcus aureus* to cultured human endothelial cells. *J. Clin. Investig.* **87**, 2236-2245 (1991).
18. B. Singh, Y. C. Su, K. Riesbeck, Vitronectin in bacterial pathogenesis: a host protein used in complement escape and cellular invasion. *Mol. Microbiol.* **78**, 545-560 (2010).
19. X. L. Xu *et al.*, Bacterial peptidoglycan triggers *Candida albicans* hyphal growth by directly activating the adenylyl cyclase Cyr1p. *Cell Host Microbe* **4**, 28-39 (2008).
20. E. I. Peerschke, B. Ghebrehiwet, The contribution of gC1qR/p33 in infection and inflammation. *Immunobiology* **212**, 333-342 (2007).
21. J. Jiang, Y. Zhang, A. R. Krainer, R. M. Xu, Crystal structure of human p32, a doughnut-shaped acidic mitochondrial matrix protein. *Proc. Natl. Acad. Sci. USA* **96**, 3572-3577 (1999).
22. F. Mahdi, Z. Shariat-Madar, R. F. Todd, 3rd, C. D. Figueroa, A. H. Schmaier, Expression and colocalization of cytokeratin 1 and urokinase plasminogen activator receptor on endothelial cells. *Blood* **97**, 2342-2350 (2001).
23. B. Ghebrehiwet, J. Jesty, E. I. Peerschke, gC1q-R/p33: structure-function predictions from the crystal structure. *Immunobiology* **205**, 421-432 (2002).
24. B. Ghebrehiwet *et al.*, Identification of functional domains on gC1Q-R, a cell surface protein that binds to the globular "heads" of C1Q, using monoclonal antibodies and synthetic peptides. *Hybridoma* **15**, 333-342 (1996).
25. S. A. Klotz, M. L. Pendrak, R. C. Hein, Antibodies to alpha5beta1 and alpha(v)beta3 integrins react with *Candida albicans* alcohol dehydrogenase. *Microbiology* **147**, 3159-3164 (2001).
26. E. Spreghini, A. Gismondi, M. Piccoli, G. Santoni, Evidence for alphavbeta3 and alphavbeta5 integrin-like vitronectin (VN) receptors in *Candida albicans* and their involvement in yeast cell adhesion to VN. *J. Infect. Dis.* **180**, 156-166 (1999).
27. G. Lalmanach, C. Naudin, F. Lecaille, H. Fritz, Kininogens: More than cysteine protease inhibitors and kinin precursors. *Biochimie* **92**, 1568-1579 (2010).
28. M. Swidergall *et al.*, Activation of EphA2-EGFR signaling in oral epithelial cells by *Candida albicans* virulence factors. *PLoS Pathog.* **17**, e1009221 (2021).
29. D. L. Moyes *et al.*, Candidalysin is a fungal peptide toxin critical for mucosal infection. *Nature* **532**, 64-68 (2016).
30. S. G. Filler, J. N. Swerdloff, C. Hobbs, P. M. Luckett, Penetration and damage of endothelial cells by *Candida albicans*. *Infect. Immun.* **63**, 976-983 (1995).
31. A. A. Sanchez *et al.*, Relationship between *Candida albicans* virulence during experimental hematogenously disseminated Infection and endothelial cell damage In vitro. *Infect. Immun.* **72**, 598-601 (2004).
32. M. Paulsson, K. Riesbeck, How bacteria hack the matrix and dodge the bullets of immunity. *Eur Respir Rev* **27** (2018).
33. M. Dehio, O. G. Gomez-Duarte, C. Dehio, T. F. Meyer, Vitronectin-dependent invasion of epithelial cells by *Neisseria gonorrhoeae* involves alpha(v) integrin receptors. *FEBS Lett* **424**, 84-88 (1998).
34. E. Buommino *et al.*, AlphaVBeta5 integrins mediates *Pseudomonas fluorescens* interaction with A549 cells. *Front Biosci (Landmark Ed)* **19**, 408-415 (2014).

35. C. M. Lopez, R. Wallich, K. Riesbeck, C. Skerka, P. F. Zipfel, *Candida albicans* uses the surface protein Gpm1 to attach to human endothelial cells and to keratinocytes via the adhesive protein vitronectin. *PLoS ONE* **9** (2014).
36. A. Kozik *et al.*, Fibronectin-, vitronectin- and laminin-binding proteins at the cell walls of *Candida parapsilosis* and *Candida tropicalis* pathogenic yeasts. *BMC Microbiol* **15**, 197 (2015).
37. I. M. Frick *et al.*, The contact system--a novel branch of innate immunity generating antibacterial peptides. *Embo J.* **25**, 5569-5578 (2006).
38. E. C. Murphy, M. Morgelin, J. C. Cooney, I. M. Frick, Interaction of *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* with the kallikrein-kinin system. *Microbiology* **157**, 2094-2105 (2011).
39. M. Rapala-Kozik *et al.*, Kininogen adsorption to the cell surface of *Candida* spp. *Int Immunopharmacol* **8**, 237-241 (2008).
40. J. Karkowska-Kuleta *et al.*, Molecular determinants of the interaction between human high molecular weight kininogen and *Candida albicans* cell wall: Identification of kininogen-binding proteins on fungal cell wall and mapping the cell wall-binding regions on kininogen molecule. *Peptides* **32**, 2488-2496 (2011).
41. J. Karkowska-Kuleta, A. Kozik, M. Rapala-Kozik, Binding and activation of the human plasma kinin-forming system on the cell walls of *Candida albicans* and *Candida tropicalis*. *Biol Chem* **391**, 97-103 (2010).
42. T. Chavakis *et al.*, High molecular weight kininogen regulates platelet-leukocyte interactions by bridging Mac-1 and glycoprotein Ib. *J. Biol. Chem.* **278**, 45375-45381 (2003).
43. K. Joseph, B. Ghebrehiwet, E. I. Peerschke, K. B. Reid, A. P. Kaplan, Identification of the zinc-dependent endothelial cell binding protein for high molecular weight kininogen and factor XII: identity with the receptor that binds to the globular "heads" of C1q (gC1q-R). *Proc. Natl. Acad. Sci. USA* **93**, 8552-8557 (1996).
44. L. Braun, B. Ghebrehiwet, P. Cossart, gC1q-R/p32, a C1q-binding protein, is a receptor for the InlB invasion protein of *Listeria monocytogenes*. *Embo J.* **19**, 1458-1466 (2000).
45. A. Cortegiani *et al.*, Epidemiology, clinical characteristics, resistance, and treatment of infections by *Candida auris*. *J Intensive Care* **6**, 69 (2018).
46. M. Arendrup, T. Horn, N. Frimodt-Moller, In vivo pathogenicity of eight medically relevant *Candida* species in an animal model. *Infection* **30**, 286-291 (2002).
47. S. Singh *et al.*, The NDV-3A vaccine protects mice from multidrug resistant *Candida auris* infection. *PLoS Pathog.* **15**, e1007460 (2019).
48. E. A. Jaffe, R. L. Nachman, C. G. Becker, C. R. Minick, Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Investig.* **52**, 2745-2756 (1973).
49. H. Park *et al.*, Role of the fungal Ras-protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. *Cell. Microbiol.* **7**, 499-510 (2005).
50. Y. Fu *et al.*, Investigation of the function of *Candida albicans* Als3 by heterologous expression in *Candida glabrata*. *Infect. Immun.* **81**, 2528-2535 (2013).
51. S. Bergmann *et al.*, Integrin-linked kinase is required for vitronectin-mediated internalization of *Streptococcus pneumoniae* by host cells. *J. Cell Sci.* **122**, 256-267 (2009).

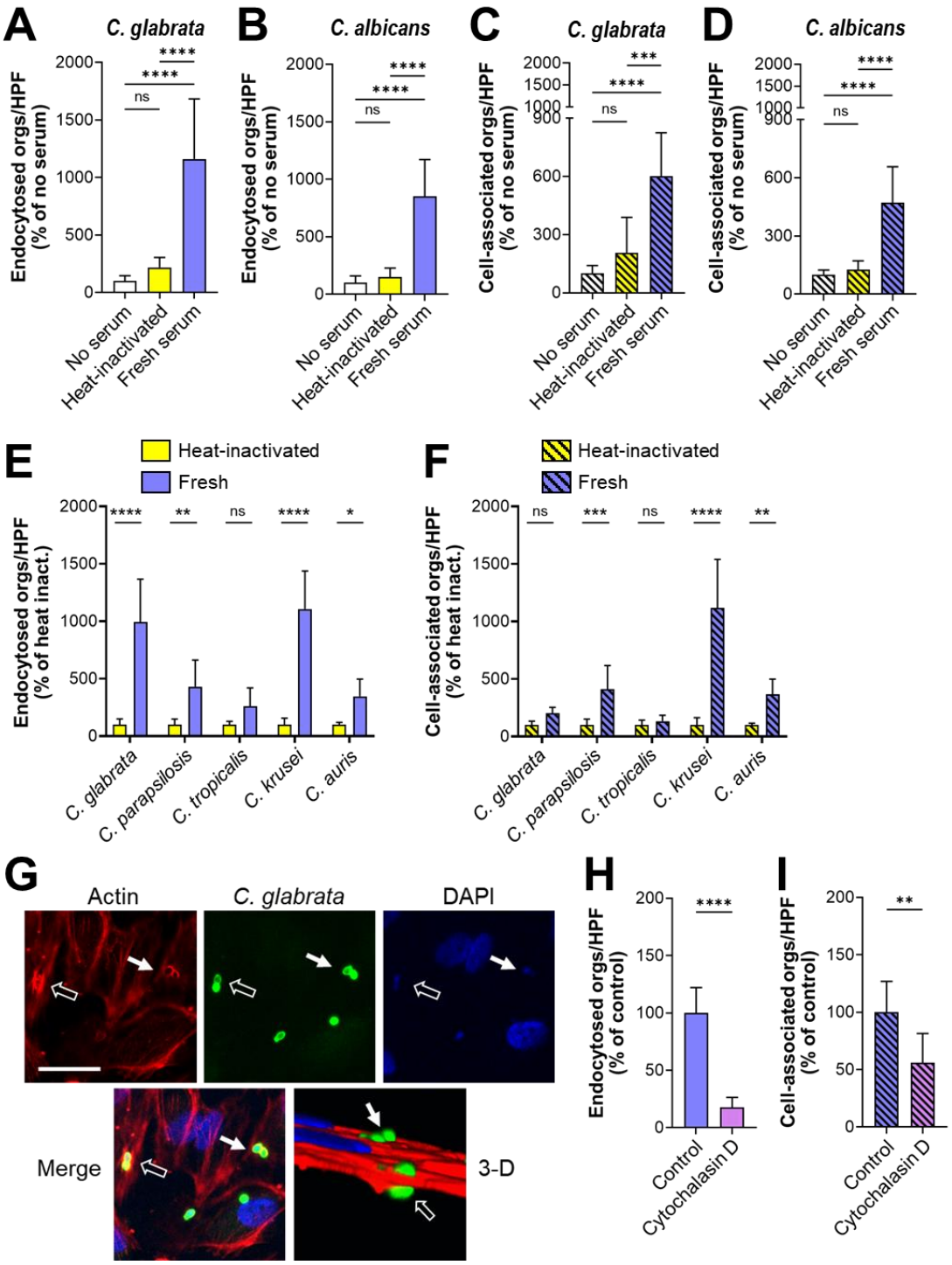

Fig. 1. Serum coating increases the endocytosis of *Candida spp.* by endothelial cells**.** (A-D), Effects of fresh and heat-inactivated human serum on the number of organisms that were endocytosed by and cell-associated (a measure of adherence) with human umbilical vein endothelial cells. (A) Endocytosis 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.

.

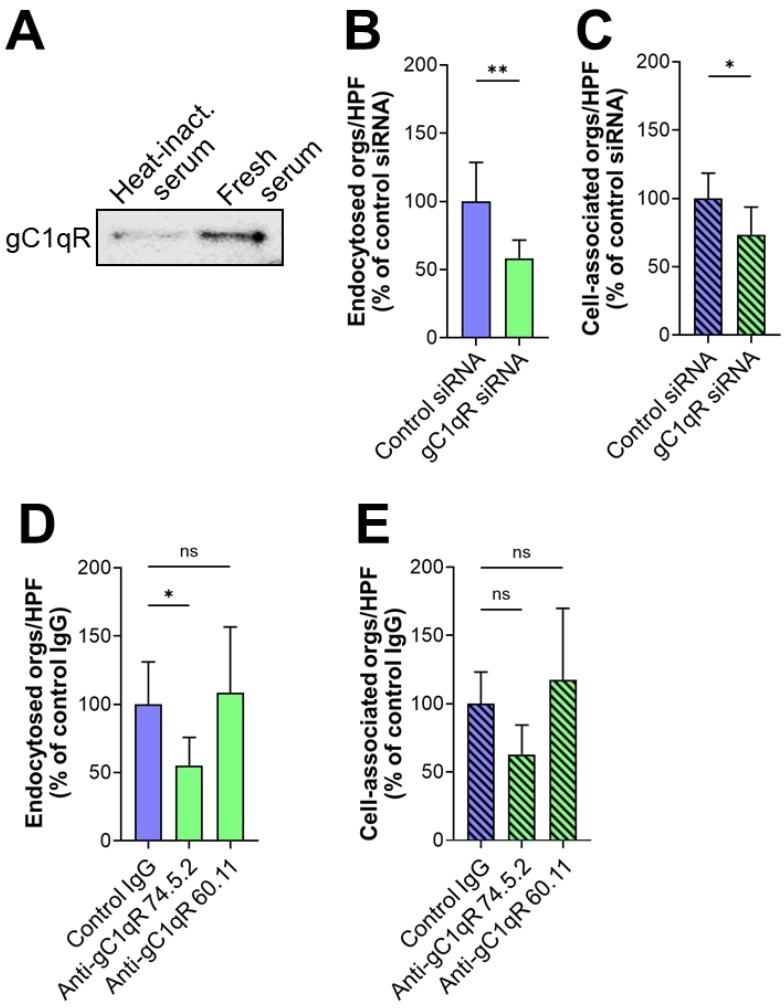

Fig. 2 The globular C1q receptor (gC1qR) is an endothelial cell receptor for serum-coated *C. glabrata.* (A) Western blot showing that endothelial cell gC1qR binds to *C. glabrata* coated with fresh serum. Results are representative of 3 independent experiments. (B-E) Effects of inhibiting gC1qR function 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.

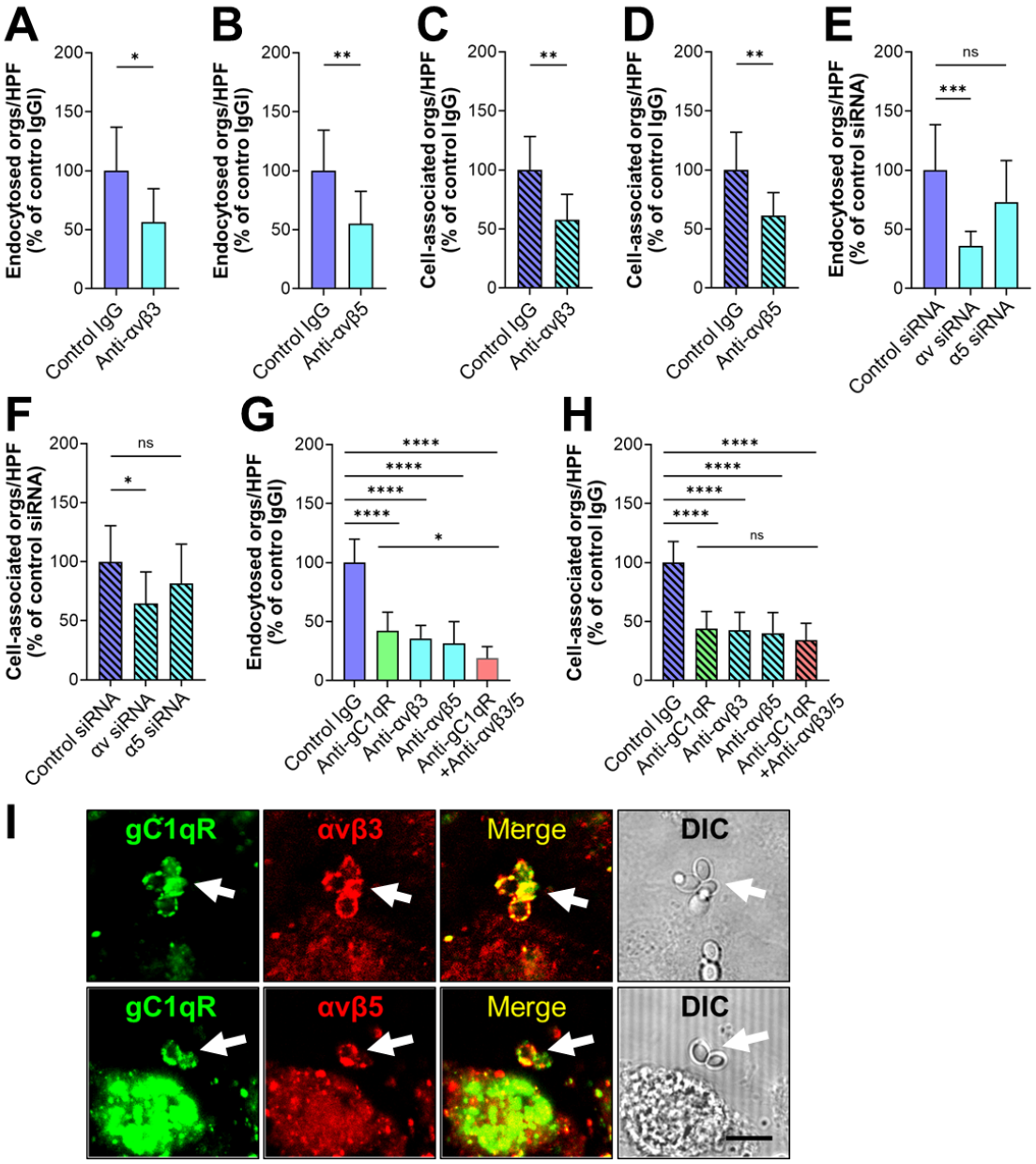

Fig. 3. Integrins αvβ3 and αvβ5 are endothelial cell receptors for serum-coated *C. glabrata.* (A-F) Effects of inhibiting αv integrin function with specific monoclonal antibodies (A-D) and siRNA knockdown (E-F) on the endocytosis (A, B, E) and cell-association (C, D, F) of serum-coated *C. glabrata.* (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.

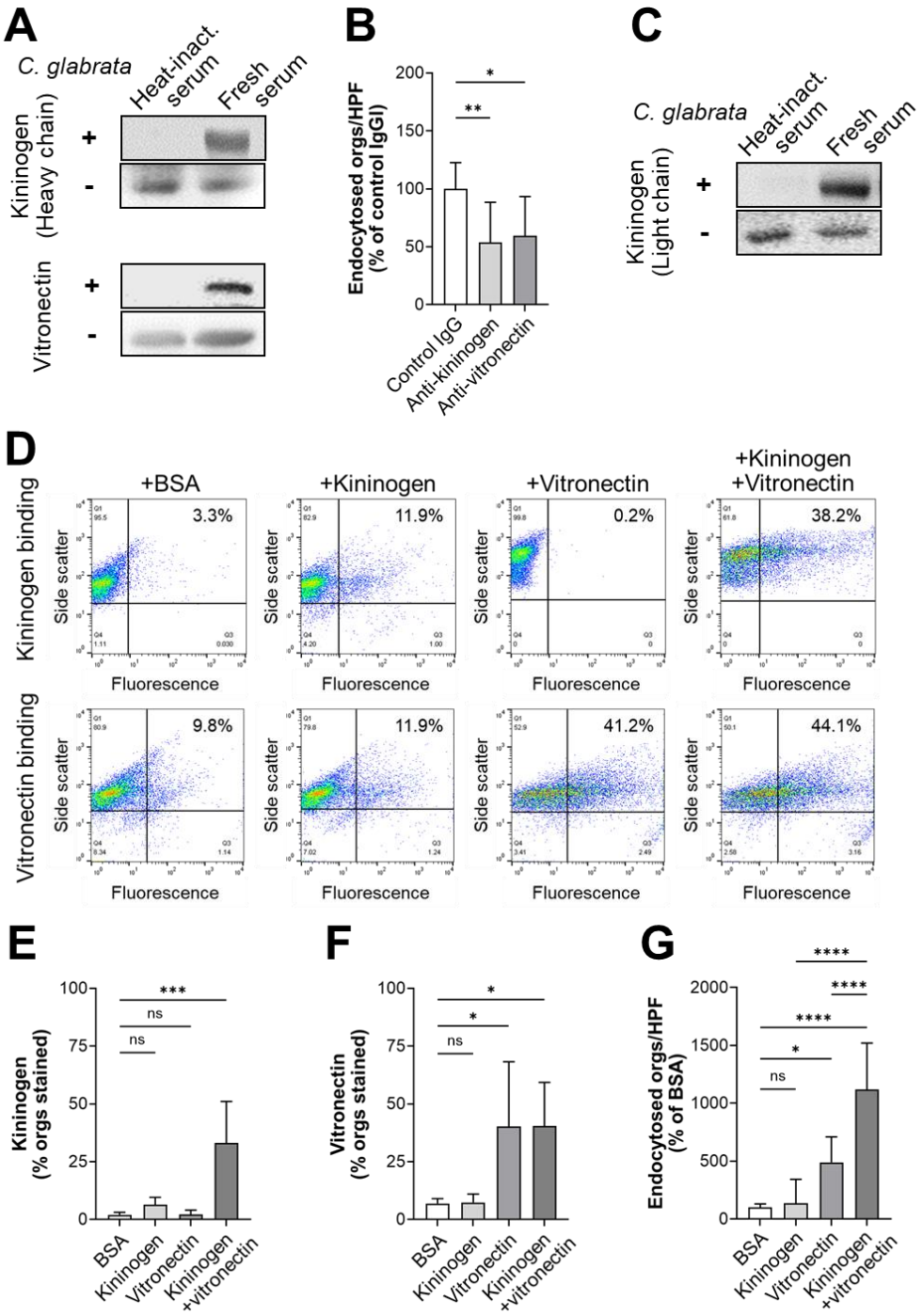

Fig. 4. High molecular weight kininogen and vitronectin function as bridging molecules. (A) Western blots showing that the heavy chain of high molecular weight kininogen and vitronectin bind to *C. glabrata* cells that have been incubated in fresh human serum. In each pair of blots, the upper panel shows 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.

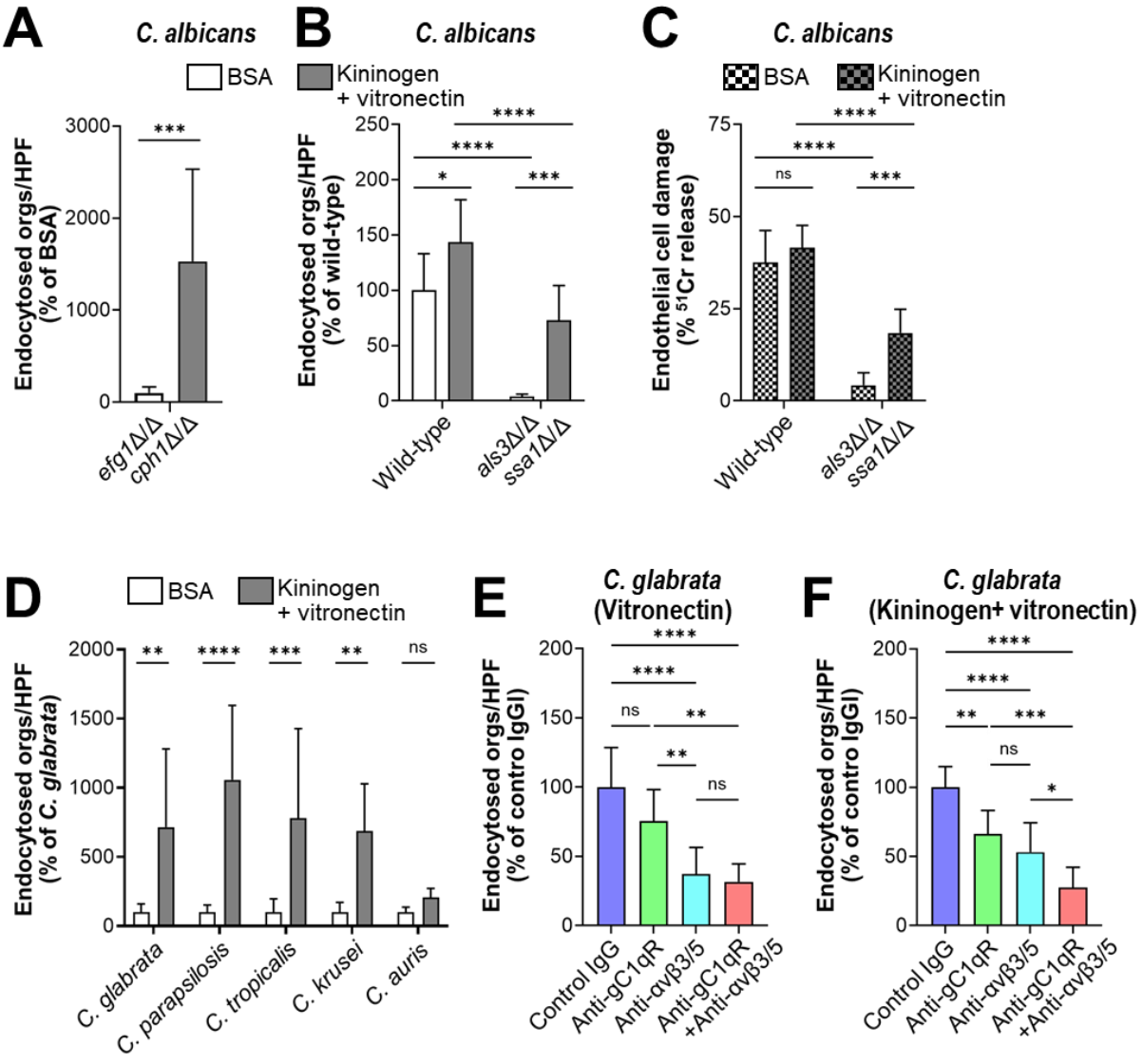

Fig. 5. Kininogen and vitronectin interact with gC1qR and αv integrins to induce endocytosis. (A and B) Effects of BSA or kininogen and vitronectin on the endocytosis the indicated strains of *C. albicans*. (C) Effects of BSA or kininogen on endothelial cell damage caused by the indicated strains of *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.

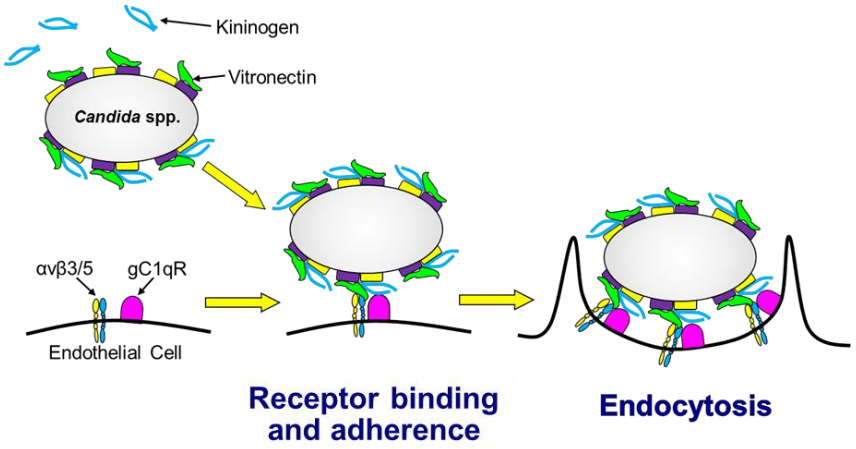

Fig. 6. Model of how kininogen and vitronectin function as bridging molecules that bind to *Candida spp.* and induce endocytosis by human endothelial cells. Vitronectin binds to the surface of the organism, which enhances the binding of kininogen. When the organism comes in contact with the endothelial 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.

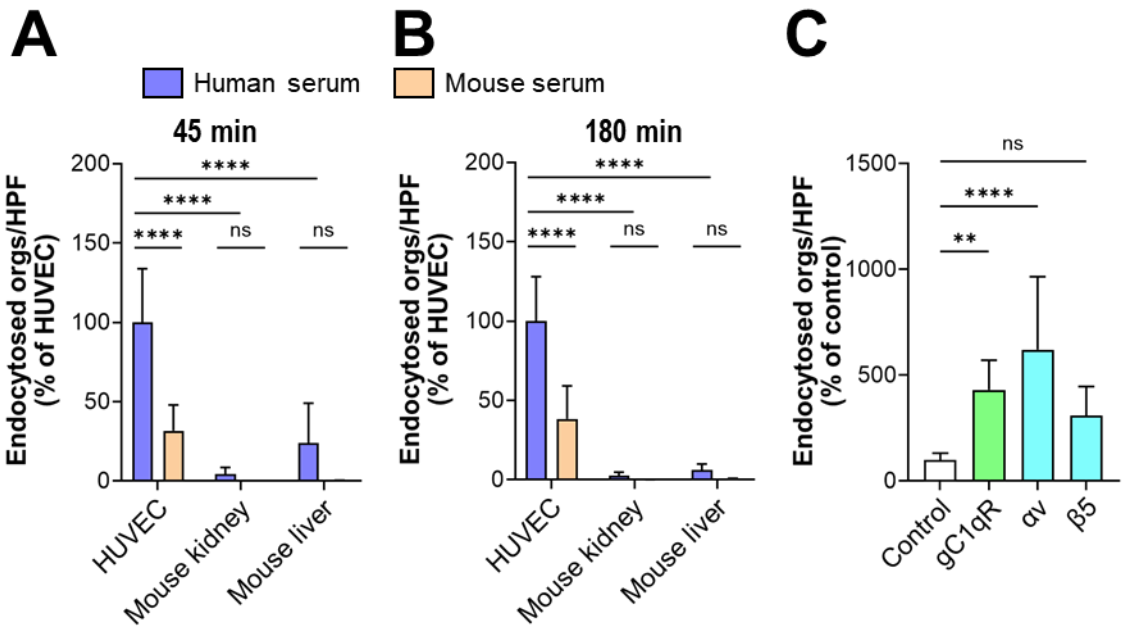

Fig. 7. Mouse endothelial cells poorly support bridging molecule-mediated endocytosis. (A and B) Endocytosis of *C. glabrata* coated with either human or mouse serum by the indicated endothelial cells after 45 min (A) and 180 min (B). (C) Endocytosis of *C. glabrata* coated with fresh human serum by 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.