The Journal of Clinical Investigation
http://www.jci.org

The endothelial cell receptor GRP78 is required for mucormycosis pathogenesis in diabetic mice

Mingfu Liu,1 Brad Spellberg,2,3 Quynh T. Phan,1 Yue Fu,1,3 Yong Fu,4 Amy S. Lee,2 John E. Edwards Jr.,1,3 Scott G. Filler,1,3 and Ashraf S. Ibrahim1,3

1Division of Infectious Diseases, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, and 2Division of General Internal Medicine, Harbor-UCLA Medical Center, Torrance, California, USA. 3David Geffen School of Medicine at UCLA, Los Angeles, California, USA. 4Norris Comprehensive Cancer Center, University of Southern California Keck School of Medicine, Los Angeles, California, USA.

Mucormycosis is a fungal infection of the sinuses, brain, or lungs that causes a mortality rate of at least 50% despite first-line therapy. Because angioinvasion is a hallmark of mucormycosis infections, we sought to define the endothelial cell receptor(s) for fungi of the order Mucorales (the fungi that cause mucormycosis). Furthermore, since patients with elevated available serum iron, including those with diabetic ketoacidosis (DKA), are uniquely susceptible to mucormycosis, we sought to define the role of iron and glucose in regulating the expression of such a receptor. Here, we have identified glucose-regulated protein 78 (GRP78) as what we believe to be a novel host receptor that mediates invasion and damage of human endothelial cells by Rhizopus oryzae, the most common etiologic species of Mucorales, but not Candida albicans or Aspergillus fumigatus. Elevated concentrations of glucose and iron, consistent with those seen during DKA, enhanced GRP78 expression and the resulting R. oryzae invasion and damage of endothelial cells in a receptor-dependent manner. Mice with DKA, which have enhanced susceptibility to mucormycosis, exhibited increased expression of GRP78 in sinus, lungs, and brain compared with normal mice. Finally, GRP78-specific immune serum protected mice with DKA from mucormycosis. These results suggest a unique susceptibility of patients with DKA to mucormycosis and provide a foundation for the development of new therapeutic interventions for these deadly infections.

Introduction
Mucormycosis is a life-threatening infection caused by fungi of the order Mucorales, the most common etiologic species of which is Rhizopus oryzae. The most common predisposing risk factor for mucormycosis is diabetes mellitus, and it has been long established that patients with diabetic ketoacidosis (DKA) have a unique predisposition to this infection (1, 2). Unfortunately, despite surgical debridement and first-line antifungal therapy, the overall mortality of mucormycosis remains unacceptably high, and survivors are typically left with considerable disfigurement from the infection and surgery (2, 3). Clearly, new therapeutic strategies are needed for this deadly disease.

A hallmark of mucormycosis is the presence of extensive angioinvasion with resultant vessel thrombosis and tissue necrosis (1, 2). Ischemic necrosis of infected tissues can prevent delivery of leukocytes and antifungal agents to the foci of infection. Thus, angioinvasion is a key factor in the pathogenesis of mucormycosis. During angioinvasion, the organism invades and damages vascular endothelial cells. Therefore, understanding the mechanisms by which these processes occur may lead to new approaches to prevent and/or treat mucormycosis.

We have found that R. oryzae strains adhere to human umbilical vein endothelial cells in vitro and invade these cells by induced endocytosis (4). Endocytosed R. oryzae damages endothelial cells, and prevention of endocytosis abrogates the ability of the organisms to cause endothelial cell damage (4). In the current study, we sought to define a receptor responsible for R. oryzae adherence to and invasion through endothelial cells and to determine whether glucose and iron levels consistent with those seen during DKA regulate the expression of such a receptor.

Results
GRP78 binds to Mucorales germlings but not spores. Because R. oryzae is likely to interact with endothelial cells in the germling form during angioinvasion (4), we used the affinity purification process developed by Isberg and Leong (5) to identify an endothelial cell receptor for R. oryzae germlings. When incubated with extracts of endothelial cell membrane proteins, R. oryzae bound to a major band at 78 kDa. Lesser and inconsistent binding was found to a band at approximately 70 kDa and bands between 100–150 kDa (Figure 1A).

The major band at 78 kDa that bound to germlings was excised for protein identification by matrix-assisted laser desorption/ionization–time-of-flight tandem mass spectrometry (MALDI-TOF MS/ MS) analysis. Several potential matches were identified, including human glucose-regulated protein 78 (GRP78). Because of our interest on focusing on receptors whose expression is likely to be regulated in DKA, we selected GRP78 for further investigation. To verify that endothelial cell GRP78 bound R. oryzae germlings, we probed immunoblots containing endothelial cell membrane proteins with an anti-GRP78 polyclonal Ab raised against a synthetic peptide corresponding to amino acids 24–43 of human GRP78. This polyclonal Ab recognized the germling-bound 78-kDa band (Figure 1B). Time course studies revealed that GRP78 was bound by germlings after 1–3 hours of germination but not by R. oryzae spores (Figure 1C). Finally, endothelial cell GRP78 bound to germlings of other Mucorales family members that are known to cause mucormycosis, including another

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 2010;120(6):1914–1924. doi:10.1172/JCI42164.
strain of *R. oryzae*, and strains of *Rhizopus microsporus*, *Mucor* species, and *Cunninghamella* species.

We next used indirect immunofluorescence to verify that GRP78 on intact endothelial cells was bound by *R. oryzae*. Endothelial cells expressed GRP78 on the cell surface (Figure 2, B and F), in accordance with previous reports (6, 7). When endothelial cells were infected with *R. oryzae* germlings, GRP78 colocalized with *R. oryzae* (Figure 2, D and H). These fungal cells were being endocytosed because they were surrounded by endothelial cell microfilaments (Figure 2, C and G). These findings confirm that during endocytosis, *R. oryzae* germlings bind to GRP78 on intact endothelial cells. GRP78 is a receptor for *R. oryzae* germlings. Because we previously showed that endocytosis of the fungus is a prerequisite for *R. oryzae* to cause endothelial cell damage (4), we sought to determine whether blocking the function or expression of GRP78 would protect endothelial cells from *R. oryzae*-induced endocytosis and subsequent damage. Endocytosis, but not adherence, of *R. oryzae* germlings was abrogated by addition of an anti-GRP78 but not control polyclonal Ab, the latter of which targeted p53, which is not expressed by endothelial cells (Figure 3A). The anti-GRP78 Ab reduced by more than 40% damage to endothelial cells caused by *R. oryzae* germlings (Figure 3B) but not *Candida albicans* (Figure 3C) or *Aspergillus fumigatus* (Figure 3D).

To complement the Ab blocking studies, we sought to suppress GRP78 expression to determine its impact on adherence, endocytosis, and endothelial cell damage. Because GRP78 is essential (8), we employed shRNA to downregulate its expression. Transduction of endothelial cells with a lentivirus containing GRP78 shRNA mediated an 80% reduction in GRP78 transcript levels compared with endothelial cells transduced by non-target shRNA lentivirus (Figure 4A). This suppression of GRP78 expression resulted in a significant reduction in endothelial cell endocytosis of *R. oryzae* germlings and subsequent endothelial cell damage (Figure 4, B and C). Collectively, these results indicate that GRP78 is essential for maximal endocytosis of *R. oryzae* germlings by endothelial cells.

As an additional confirmatory method, we used the cell line C.1, which was derived from parental dihydrofolate reductase-deficient (DHFR-deficient) CHO cells engineered to overexpress hamster GRP78 (9, 10). C.1 cells overexpressed GRP78 transcript by 26-fold compared with their parent cells (Figure 5A). The C.1 cells had a 40% increase in endocytosis of *R. oryzae* germlings, which resulted in a more than 50% increase in damage (Figure 5, B and C) compared with the parent CHO cells, which do not overexpress GRP78. These results were specific to *R. oryzae* germlings, because CHO cells overexpressing GRP78 had no effect on endocytosis of *R. oryzae* spores, which do not bind GRP78 (data not shown). Thus, the enhanced endocytosis of germlings induced by GRP78 overexpression is not the result of a generalized increase in endocytosis. These results further support the concept that GRP78 functions as an endothelial cell receptor for *R. oryzae* germlings.

Iron regulates endothelial cell damage by *R. oryzae*. Patients with elevated available serum iron, such as DKA patients (11) or those treated with deferoxamine (an iron siderophore that provides *Rhizopus* with exogenous iron) (12), are uniquely predisposed to developing mucormycosis. Furthermore, the iron chelator deferasirox, which reduces available serum iron, is effective in treating experimental hematogenously disseminated mucormycosis (13). Given the role of iron in the pathogenesis of mucormycosis infections, we sought to define the impact of iron levels on endothelial cell endocytosis of *R. oryzae*. Endothelial cell adherence, endocytosis, and damage caused by *R. oryzae* were compared following exposure to phenanthroline (an iron chelator) with or without exogenous iron (12), are uniquely predisposed to developing mucormycosis. Furthermore, the iron chelator deferasirox, which reduces available serum iron, is effective in treating experimental hematogenously disseminated mucormycosis (13). Given the role of iron in the pathogenesis of mucormycosis infections, we sought to define the impact of iron levels on endothelial cell endocytosis of *R. oryzae*. Endothelial cell adherence, endocytosis, and damage caused by *R. oryzae* were compared following exposure to phenanthroline (an iron chelator) with or without exogenous iron. As a positive control, *R. oryzae* germlings were incubated on endothelial cells that were exposed to the microfilament disruptant cytocothalasin D, which prevents endocytosis (14). None of the treatments altered fungal adherence to endothelial cells (Figure 6A). However, similar to cytocothalasin D, phenanthroline reduced endothelial cell endocytosis of *R. oryzae* by approximately 70% (Figure 6A). Further, the addition of exogenous iron completely reversed the inhibition of endocytosis caused by the iron chelator. Finally, phenanthroline prevented *R. oryzae*-induced endothelial cell damage in a concen-
Patients with DKA have expression by RT-PCR in mice with.. To determine the potential role for GRP78 in mediating susceptibility to mucormycosis in vivo, we quantified Grp78 expression by RT-PCR in mice with DKA (which, like humans, are hypersusceptible to mucormycosis) (16) and in normal mice. Mice were rendered diabetic with streptozotocin (16, 17). Diabetes was confirmed by measurement of increased urinary glucose levels. Concordant with the establishment of DKA, diabetic mice had a decrease in blood pH from 7.4 to 7.2. Blood pH rapidly decreased to 6.8 following administration of FeCl3, a result consistent with experimental iron overload (11). In contrast, administration of a similar concentration of FeCl3 in non-diabetic mice did not result in a decrease in blood pH. Similarly, incubating endothelial cells with 4–8 mg/ml glucose resulted in approximately 20% and 40% increases in endocytosis of R. oryzae compared with cells incubated in a normal physiological concentration of glucose (1 mg/ml, P = 0.005) (Figure 7C). To discern whether GRP78 overexpression in response to glucose was due to hyperglycemia itself, or rather to hyperosmolarity caused by the increased glucose, we measured the expression of GRP78 after incubating endothelial cells with similar concentrations of mannitol (i.e., 1, 4, and 8 mg/ml) for 20 hours. No change in GRP78 expression was noticed (data not shown), indicating that hyperglycemia and not hyperosmolarity is responsible for the enhanced GRP78 expression. Based on a previous hypothesis that HMG-CoA reductase inhibitors (i.e., statins) might affect mucormycosis incidence in diabetic patients (15), we also tested the impact of lovastatin on GRP78 expression and found no evidence of alterations in expression in the presence of the statin (data not shown).

To confirm that the increased mRNA expression of GRP78 translated into increased protein surface expression, we incubated endothelial cells with 0, 15, or 50 μM FeCl3, the latter of which mediated the strongest increase in mRNA transcription, and stained them with anti-GRP78 monoclonal Ab or isotype control. Fluorescence was quantified by flow cytometry. Surface expression of GRP78 protein increased by 150% in the presence of high iron levels (Figure 7D).

Iron- and glucose-induced GRP78 overexpression enhances susceptibility of endothelial cells to R. oryzae–induced invasion and damage. Incubation of endothelial cells with either 15 or 50 μM FeCl3, enhanced R. oryzae–induced endocytosis and subsequent damage by 80% compared with endothelial cells incubated without exogenous FeCl3 (Figure 8A). Similarly, incubation of endothelial cells with 4–8 mg/ml glucose resulted in approximately 20% and 40% increases in endocytosis of and damage caused by R. oryzae, respectively, when compared with endothelial cells incubated in 1 mg/ml glucose (Figure 8B). Importantly, the anti-GRP78 Ab blocked this enhanced endothelial cell susceptibility to R. oryzae–induced damage, confirming the specificity of the increased susceptibility to overexpression of GRP78 (Figure 9). Collectively, these results confirm that iron and glucose concentrations consistent with those seen in patients with DKA induce the overexpression of GRP78, resulting in enhanced endocytosis and damage of endothelial cells.

**Figure 2**

GRP78 on intact endothelial cells colocalizes with R. oryzae germlings that are being endocytosed. Confocal microscopic images of endothelial cells infected with R. oryzae cells that have been germinated for 1 hour (A–D) or 2 hours (E–H). Confluent endothelial cells on a 12-mm-diameter glass coverslip were infected with 10,000 R. oryzae germlings. After 60-minute incubation at 37°C, the cells were fixed with 3% paraformaldehyde, washed, blocked, and then permeabilized (31). The cells were stained with GRP78 using rabbit anti-GRP78 polyclonal Ab (Abcam), followed by a counterstain with goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes, Invitrogen) (B and F). To detect F-actin, the cells were incubated with Alexa Flour 568–labeled phalloidin (Molecular Probes) per the manufacturer’s instructions (C and G). A merged image is shown in D and H. (A and E) The same fields taken with differential interference contrast imaging. Arrows indicate GRP78 and microfilaments that have accumulated around R. oryzae. Scale bars: 30 μm (A–D) and 20 μm (E–H).
7.8 (normal for mice) to 7.3–7.2, associated with increased levels of urinary glucose (250–1,000 mg/dl) and urinary ketone bodies (≥5 mg/dl) as determined by Keto-Diastix strip testing. We also compared levels of serum-free iron (i.e., unbound by carrier proteins such as transferrin) in DKA mice with those in normal mice. In accordance with the results found in humans (11), DKA mice (n = 11) had approximately 5-fold-higher levels of serum-free iron than normal mice (median [25th quartile, 75th quartile], 7.29 [4.3, 11.8] μM vs. 1.69 [1.3, 2.3] μM; P = 0.03 by Wilcoxon rank-sum test). Finally, concordant with the regulation of GRP78 expression by iron and glucose levels, DKA mice were found to express 2- to 5-fold-higher levels of Grp78 mRNA in sinus, lungs, and brain compared with normal mice (Figure 10A).

To determine the potential for abrogation of GRP78 function as a treatment for mucormycosis, we vaccinated female BALB/c mice with recombinant hamster GRP78 (which is more than 98% identical to murine or human GRP78), and serum was collected from GRP78-immunized or control mice. The median anti-GRP78 Ab titer of serum collected from vaccinated mice was found to be 1:128,000 compared with a titer of 1:600 in the serum collected from mice vaccinated with adjuvant alone as determined by ELISA (P = 0.005). The efficacy of the anti-GRP78 serum was compared with that of the non-immune serum in protecting naive mice from *R. oryzae* infection. DKA mice were treated intraperitoneally with anti-GRP78 or non-immune serum 2 hours prior to intranasal infection with 10⁵ spores of *R. oryzae*. Another dose of serum was given on day +3 relative to infection. DKA mice that received anti-GRP78 serum had marked improvement in survival compared with mice treated with non-immune serum (Figure 10B). Lungs and brains harvested from surviving mice were found to be sterile at the end of the experiment.

**Discussion**

We found that host GRP78 plays a critical role in susceptibility to mucormycosis and that increased GRP78 expression offers an explanation for the unique susceptibility to mucormycosis of hyperglycemic hosts with elevated available serum iron. While mucormycosis can occur in patients with profound neutropenia or those receiving high doses of corticosteroids (2, 18), the most common risk factor for mucormycosis is diabetes (2, 19). Although the attack rate of mucormycosis is substantially higher in patients with DKA than in diabetic patients who are not acidicotic, less than half of diabetic patients with mucormycosis are acidicotic (18, 20).

Therefore, diabetes is a risk for mucormycosis even in the absence of acidosis, but acidosis enhances the predisposition of diabetic patients to mucormycosis. The causes of this predisposition of patients with diabetes, and DKA in particular, to mucormycosis has never been adequately explained.

Our results greatly elucidate the predisposition of diabetic and DKA patients to mucormycosis. We found that GRP78 expression was enhanced in hosts with elevated available serum iron levels and high glucose concentrations, and this enhanced expression of GRP78 resulted in increased endocytosis of *R. oryzae* by human endothelial cells and subsequent enhanced damage to the cells. Furthermore, suppression of GRP78 function by Ab and its reduced expression levels by shRNA blocked *R. oryzae* uptake and resulting damage to human endothelial cells. In contrast, the anti-GRP78 Abs did not block fungal endothelial cell damage mediated by *C. albicans* or *A. fumigatus*, two other fungal pathogens that do not have an increased attack rate in patients with DKA. Most importantly, our results clearly showed that mice with DKA, which had elevated levels of glucose and available iron and overexpressed *Grp78* in relevant target tissues, were protected from mucormycosis infection when the recep-
tor was blocked by Abs. These exciting results underscore the therapeutic potential of GRP78-blocking strategies in treating or preventing mucormycosis.

Exposure to hyperglycemia of iron-sequestering proteins, such as apotransferrin and hemoglobin, has been shown to damage the proteins and cause them to release free iron in serum (21, 22). Therefore, diabetes can result in increased serum-free iron even in the absence of acidosis. Furthermore, acidosis has been shown to markedly increase dissociation of iron from sequestering proteins in serum from DKA patients, independent of glucose levels (11). Our data confirm that hyperglycemia as well as increased iron levels in the absence of hyperglycemia increase expression of GRP78 in host cells. Thus, increased GRP78 expression caused by hyperglycemia can explain the predisposition of non-acidotic diabetic patients to mucormycosis, whereas the potentiation of increased free iron levels caused by acidosis explains the marked increase in attack rate of mucormycosis in patients with DKA.

GRP78 (also known as BiP/HSPA5) was discovered as a cellular protein induced by glucose starvation (23). It is a member of the HSP70 protein family that is mainly present in the endoplasmic reticulum. It functions as a major chaperone that is involved in many cellular processes, including protein folding and assembly, marking misfolded proteins for proteosome degradation (24), regulating Ca\(^{2+}\) homeostasis, and serving as a sensor for endoplasmic reticulum stress (6).

**Figure 4**

Downregulation of endothelial cell GRP78 expression with shRNA reduces the number of endocytosed organisms and subsequent damage to endothelial cells. Endothelial cells were transduced with lentivirus containing either shRNA targeting GRP78 or a scrambled sequence (Non-target shRNA). Transduction of endothelial cells with GRP78 shRNA lentiviruses reduced GRP78 transcript levels (A), diminished the number of endocytosed *R. oryzae* germlings (data derived from >800 fungal cells interacting with approximately 250 endothelial cells each group/experiment, with an average of 76% being endocytosed in the non-target shRNA) (B), and blocked *R. oryzae*-induced endothelial cell damage (C). \*P < 0.005 compared with non-target shRNA by Wilcoxon rank-sum test for all comparisons. n = 6 slides per group from 3 independent experiments for endocytosis, and n = 6 wells per group from 2 independent experiments for damage assay. Data are expressed as median ± interquartile range.

**Figure 5**

Heterologous overexpression of GRP78 in CHO cells makes them more susceptible to *R. oryzae*-induced invasion and subsequent damage. The C.1 cell line, which was derived from parental DHFR-deficient CHO cells engineered to overexpress GRP78, was found to overexpress GRP78 (A). \*P = 0.01 compared with parent cells by nonparametric Wilcoxon rank-sum test; n = 6 per each group. C.1 cells were able to endocytose more *R. oryzae* germlings (data derived from >950 fungal cells interacting with approximately 300 CHO cells each group/experiment, with an average of 40.9% being endocytosed in the parent cells) (B) and were more susceptible to *R. oryzae*-induced damage (C). \*P < 0.005 compared with parent cells by Wilcoxon rank-sum test. n = 6 slides per group from 3 independent experiments for endocytosis, and n = 6 wells per group from 2 independent experiments for damage assay. Data are expressed as median ± interquartile range.
GRP78 has also been reported to be antiapoptotic and plays critical cytoprotective roles in early embryogenesis, oncogenesis, neurodegenerative diseases, and atherosclerosis (23). More recently, GRP78 overexpression was shown to inhibit both insulin-dependent and endoplasmic reticulum stress–induced SREBP-1 activation, resulting in reduction of hepatic steatosis in obese mice (25).

Despite its main function as a cellular chaperone protein, recent studies reported the translocation of a fraction of GRP78 to the cell surface in a variety of cells (26). In fact, GRP78 has been reported to function as a receptor for a variety of ligands, including (a) the angiogenesis inhibitor Kringle 5 (7), (b) the activated proteinase inhibitor α2-macroglobulin (27), (c) a synthetic 12-aa peptide (28), (d) dengue virus serotype 2 (29), and (e) a coreceptor for Coxsackievirus A9 (30). To our knowledge, this is the first report that GRP78 acts as a receptor for fungal pathogens. We found that GRP78 acts as a receptor for invasion but not adherence of Mucorales to endothelial cells. Similarly, N-cadherin was shown to mediate invasion but not adherence of C. albicans to endothelial cells (31), demonstrating that adherence and invasion are two independent processes mediated by different receptors. The fungal ligand for GRP78 that mediates invasion of endothelial cells is under active investigation.

With regard to the effect of iron on GRP78, previous work demonstrated paradoxical effects of iron on GRP78 expression in animal models. For example, mRNA and protein levels of GRP78 were decreased in iron-fed C57BL/6 mice, while they were unchanged in iron-fed 129/Sv mice (32). In contrast, rats with chronic or acute iron overload had increased GRP78 expression in hearts and livers compared with control rats (33). We found iron to have a drastic effect on increasing the cell surface expression of GRP78. Higher glucose concentrations also increased expression of GRP78, but to a lesser extent. These results are in agreement with the findings of Mote et al., who reported that Chinese hamster lung fibroblasts expressed 30% more GRP78 when cultured in medium with a glucose concentration of 4.5 mg/ml compared with medium with a glucose concentration of 1.5 mg/ml (34).

Our findings that DKA mice have elevated available serum iron and increased serum glucose, and expressed more GRP78 in their organs, underscore the physiological relevance of our in vitro findings. More importantly, we found that anti-GRP78 Ab protected DKA mice from R. oryzae infection. The mechanism of immunological protection is currently under investigation.

In summary, multiple independent lines of investigation demonstrate that GRP78 functions as a receptor for Mucorales that facilitates fungus-induced penetration and subsequent damage of endothelial cells. Additionally, expression of the receptor and subsequent invasion of and damage to endothelial cells in a receptor-dependent manner were increased in the presence of elevated concentrations of iron and glucose, consistent with those seen in patients with DKA. Most important, anti-GRP78 Ab protected DKA mice from infection with mucormycosis. These results provide insight into why patients with DKA are uniquely susceptible to mucormycosis infections and provide a foundation for therapeutic interventions against extremely lethal mucormycosis.

**Methods**

**R. oryzae and culture conditions.** Several clinical Mucorales isolates were used in this study. R. oryzae 99-880 and Mucor sp 99-932 are brain isolates, while R. oryzae 99-892 and Rhizopus sp 99-1150 were isolated from lungs of infected patients and obtained from the Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA. Cunninghamella bertholletiae 182 is also a clinical isolate and was a gift from Thomas Walsh (NIH, Bethesda, Maryland, USA). Rhizopus microsporus ATCC 20577 is an environmental isolate obtained from ATCC. A. fumigatus AF293
and C. albicans SC5314 are clinical isolates that were used to determine whether anti-GRP78 Ab blocks endothelial cell damage caused by these two organisms. Mucorales were grown on potato dextrose agar (PDA; BD Biosciences —Diagnostic Systems) plates for 3–5 days at 37°C, while A. fumigatus and C. albicans were grown on Sabouraud dextrose agar (SDA) plates for 2 weeks and 48 hours, respectively, at 37°C. The sporangiospores were collected in endotoxin-free Dulbecco’s PBS containing 0.01% Tween 80 (for Mucorales) and 0.2% Tween 80 (for A. fumigatus), washed with PBS, and counted with a hemocytometer to prepare the final inocula. For C. albicans, blastospores were collected in PBS after the organisms were grown in YPD medium (1% yeast extract [Difco Laboratories], 2% Bacto Peptone [Difco Laboratories], and 2% glucose [Sigma-Aldrich]) at 30°C overnight. To form germ tubes, spores were incubated in liquid YPD medium at 37°C with shaking for 1–3 hours based on the assay under study. Germ tubes were washed twice with RPMI 1640 without glucose (Irvine Scientific) for all assays used, except in experiments involving isolation of the endothelial cell receptor, for which the germ tubes were washed twice with PBS (plus Ca²⁺ and Mg²⁺).

**Endothelial cells and CHO cells.** Endothelial cells were collected from umbilical vein endothelial cells by the method of Jaffe et al. (35). The cells were harvested by using collagenase and were grown in M-199 (Gibco BRL) enriched with 10% fetal bovine serum, 10% defined bovine calf serum, l-glutamine, penicillin, and streptomycin (all from Gemini Bio-Products). Second-passage cells were grown to confluency in 96-well tissue culture plates (Costar) on the assay under study. The reagents were tested for endotoxin using a chromogenic limulus amebocyte lysate assay (BioWhittaker Inc.), and the endotoxin concentrations were less than 0.01 IU/ml. Endothelial cell collection was approved by the Institutional Review Board of Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center. CHO cell line C.1, which was derived from parental DHFR-deficient CHO cells engineered to overexpress GRP78β, was a gift of Randall Kaufman, University of Michigan, Ann Arbor, Michigan, USA (9, 10).

**Extraction of endothelial cell membrane proteins.** Endothelial cell membrane proteins were extracted according to the method of Isberg and Leong (5). Briefly, confluent endothelial cells in 100-mm-diameter tissue culture dishes were rinsed twice with warm Dulbecco’s PBS containing Ca²⁺ and Mg²⁺ (PBS-CM) and then incubated with EZ-Link Sulfo-NHS-LS-Biotin (0.5 mg/ml; Pierce) in PBS-CM for 12 minutes at 37°C in 5% CO₂. The cells were then rinsed extensively with cold PBS-CM and scrapped from the tissue culture dishes. The endothelial cells were collected by centrifugation at 500 g for 5 minutes at 4°C and then lysed by incubation for 20 minutes on ice in PBS-CM containing 5.8% n-octyl-β-d-glucopyranoside (w/v) (Calbiochem) and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) (Sigma-Aldrich). The cell debris was removed by centrifugation at 5,000 g for 5 minutes at 4°C. The supernatant was collected and centrifuged at 100,000 g for 1 hour at 4°C. The concentration of the endothelial cell proteins in the resulting supernatant was determined using the Bradford method (Bio-Rad).

**Isolation of endothelial cell receptors that bind to Mucorales.** Live Mucorales spores (8 × 10⁶) or an equivalent volume of 1–3 hour germlings (approximately 2 × 10⁶ cells) were incubated for 1 hour on ice with 250 μg of biotin-labeled endothelial cell surface proteins in PBS-CM plus 1.5% n-octyl-β-d-glucopyranoside and protease inhibitors. The unbound endothelial cell proteins were washed away by 3 rinses with this buffer. The endothelial cell proteins that remained bound to the fungal cells were eluted twice with 6 M urea (Fluka), and the supernatant was concentrated to an appropriate volume with a Microcon centrifugal filter (10,000 MWCO; Millipore). The proteins were then treated with Western Blocking Reagent (Roche) and probed with a mouse anti-biotin monoclonal Ab (Sigma-Aldrich). The membrane was then washed and incubated with secondary Ab, HRP-conjugated sheep anti-mouse IgG (Sigma-Aldrich) and probed with anti-mouse Alexa Fluor 488-labeled Ab (D). Data are presented as percent of median fluorescent cells ± interquartile range. *P < 0.01 versus pH 7.4 or the same pH with phenanthroline; **P < 0.05 versus 1 mg/ml glucose or 0 FeCl₃ by Wilcoxon rank-sum test.
stained with SYPRO Ruby (Molecular Probes, Invitrogen). The major band at approximately 75 kDa was excised and microsequenced using MALDITOF MS/MS (Emory University Microchemical Facility).

To confirm the identity of GRP78, endothelial cell membrane proteins that bound to R. oryzae were separated on an SDS-polyacrylamide gel and transferred to PVDF-plus membranes. Membranes were probed with a rabbit anti-GRP78 Ab (Abcam), followed by HRP-conjugated goat anti-rabbit IgG (Pierce) as a secondary Ab. After incubation with SuperSignal West Dura Extended Duration Substrate (Pierce), the signals were detected using a CCD camera.

**Colocalization of GRP78 with phagocytosed R. oryzae germlings by indirect immunofluorescence.** We used a modification of our previously described method (31). Confluent endothelial cells on a 12-mm-diameter glass coverslip were infected with 10^5/ml R. oryzae cells in RPMI 1640 medium that had been pregerminated for 1 or 3 hours. After 60 minutes incubation at 37°C, the cells were gently washed twice with HBSS to remove unbound organisms and then fixed with 3% paraformaldehyde. After washing with 1% BSA (Fisher) prepared in PBS-CM, the cells were incubated for 1 hour with rabbit anti-GRP78 Ab (Abcam), then counterstained with Alexa Fluor 488-labeled goat anti-rabbit IgG (Molecular Probes, Invitrogen). Cells were then permeabilized for 5 minutes in 0.5% Triton X-100 and incubated with Alexa Fluor 586-labeled phalloidin (Molecular Probes) for 1 hour to detect F-actin. After washing, the coverslip was mounted on a glass slide with a drop of ProLong Gold antifade reagent (Molecular Probes, Invitrogen) and viewed by confocal microscopy. The final confocal images were produced by combining optical sections taken through the z axis.

**Interactions of fungi with endothelial or CHO cells.** The number of organisms endocytosed by endothelial cells or CHO cells was determined using a modification of our previously described differential fluorescence assay (14). Briefly, 12-mm glass coverslips in a 24-well cell culture plate were coated with fibronectin for at least 4 hours and seeded with endothelial or CHO cells until confluency. After washing twice with prewarmed HBSS, the cells were then infected with 10^5 cells of R. oryzae in RPMI 1640 medium that had been germinated for 1 hour. Following incubation for 3 hours, the cells were fixed in 3% paraformaldehyde and were stained for 1 hour with 1% Uvitex (a gift from Jay Isharani, Ciba-Geigy, Greensboro, North Carolina, USA), which specifically binds to the chitin of the fungal cell wall. After washing 3 times with PBS, the coverslips were mounted on a glass slide with a drop of ProLong Gold antifade reagent and sealed with nail polish. The total number of cell-associated organisms (i.e., germlings adhering to monolayer) was determined by phase-contrast microscopy. The same field was examined by epifluorescence microscopy, and the number of uninternalized germlings (which were brightly fluorescent) was determined. The number of endocytosed organisms was calculated by subtracting the number of fluorescent organisms from the total number of visible organisms. At least 400 organisms were counted in 20–40 different fields on each slide. Two slides per arm were used for each experiment, and the experiment was performed in triplicate on different days.

**R. oryzae–induced endothelial or CHO cell damage was quantified by using a chromium (\(^{51}\text{Cr}\)) release assay (36).** Briefly, endothelial cells or CHO cells grown in 96-well tissue culture plates containing detachable wells were incubated with 1 μCi per well of Na\(^{51}\text{CrO}_4\) (ICN) in M-199 medium (for endothelial cells) or Alpha minimum Eagle’s medium (for CHO cells) for 16 hours.
On the day of the experiment, the unincorporated $^{51}$Cr was aspirated, and the wells were washed twice with warmed HBSS (Irvine Scientific). Cells were infected with fungal germlings (1.5 × 10^5 germinated for 1 hour) suspended in 150 μl RPMI 1640 medium (Irvine Scientific) supplemented with glutamine. Spontaneous $^{51}$Cr release was determined by incubating endothelial or CHO cells in RPMI 1640 medium supplemented with glutamine without R. oryzae. After 3 hours of incubation at 37°C in a 5% CO₂ incubator, 50% of the medium was aspirated from each well and transferred to glass tubes, and the cells were manually detached and placed into another set of tubes. The amount of $^{51}$Cr in the aspirate and the detached well was determined by gamma counting. The total amount of $^{51}$Cr incorporated by endothelial cells in each well equaled the sum of radioactive counts per minute of the aspirated medium plus the radioactive counts of the corresponding detached wells. After the data were corrected for variations in the amount of tracer incorporated in each well, the percentage of specific endothelial cell release of $^{51}$Cr was calculated by the following formula: [(experimental release × 2) – (spontaneous release × 2)]/[(total incorporation – (spontaneous release × 2)]. Each experimental condition was tested at least in triplicate and the experiment repeated at least once.

To determine the effects of chelating endothelial cell iron on interactions with R. oryzae, endothelial cells were incubated with different concentrations of phenanthroline for 16 hours. To prevent chelation of the radioisotope, $^{51}$Cr was added to endothelial cells 24 hours prior to the addition of phenanthroline (37). To confirm that the effects of phenanthroline on R. oryzae–induced endocytosis by and damage of endothelial cells were due to chelation of endothelial cell iron, exogenous iron in the form of hemin was added to endothelial cells at a final concentration of 20 μM, 2 hours before phenanthroline (37). As a positive control for prevention of endocytosis, the microfilament disrupting cytochalasin D (Sigma-Aldrich) was added at a concentration of 200 nM simultaneously with R. oryzae germlings, and endocytosis was determined as above (14).

Transduction of endothelial cells with shRNA lentiviral particles. The shRNA lentiviral particles, including TurboGFP control (SHC000V), non-target control (SHC002V), and GRP78 target (TRCN01024) were purchased from Sigma-Aldrich. The non-target control contains a scrambled sequence (CAACAAGATGAAGAGCACC) not targeting any known human gene, while lentiviruses targeting the GRP78 gene contain sequence CTTGTGGTTGCTGACTGAG. The transductions were performed according to the manufacturer’s protocol. Briefly, 1.6 × 10^6 endothelial cells were seeded into 96-well plate and incubated for about 20 hours at 37°C in a 5% CO₂ incubator. Cells were infected with lentiviral particles at an MOI of 20 in the presence of 8 μg/ml polybrene (Sigma-Aldrich) overnight. The transduced cells were incubated in fresh M199 medium for 4 more days. Puromycin at 0.2 μg/ml was added to select for puromycin-resistant cell pools, which usually took approximately 10 days of incubating at 37°C in 5% CO₂. The puromycin-resistant cells were passaged until an appropriate amount of cells was obtained for endocytosis or damage assays as above. Reduction of GRP78 expression was confirmed by using real time RT-PCR (see below).

Quantification of GRP78 expression. For quantification of GRP78 expression in endothelial or CHO cells, real time RT-PCR was carried out using a Power SYBR Green Cells-to-CT kit (Applied Biosystems) to extract RNA from 2 × 10^4 cells. Primers to amplify GRP78 from endothelial cells and CHO cells were 5′-GGAAAGAAGGTTACCCTGC-3′ and 5′-AGAGAGAGCACATCGAAGGT-3′. Primers 5′-ACCATCTTCCAGAGCCGAG-3′ and 5′-TAACGAGTGGTGCTGCGAG-3′ were used to amplify the housekeeping gene GAPDH, which was used as a control.

Effect of acidosis, iron, glucose, mannitol, and statins on R. oryzae–endothelial cell interactions. To study the effect of acidosis, endothelial cells exposed to different pHs, ranging from 6.8 to 7.4, were grown in MEM buffered with HEPES for 5 hours in the presence or absence of phenanthroline. Next, endothelial cells were washed twice with cold PBS, and GRP78 total expression was quantified by real-time RT-PCR. To study the effect of glucose and iron on endothelial cell GRP78 expression levels and subsequent interactions of endothelial cells with R. oryzae germlings, we incubated endothelial cells in MEM with varying FeCl₃ or glucose concentrations for 5 hours or 20 hours, respectively (pilot studies demonstrated maximum enhancement of GRP78 expression at these time points). To study the effect of hyperosmolarity and statins on GRP78 expression, endothelial cells in MEM were incubated with 1, 4, or 8 mg/ml mannitol or 5, 20, or 40 μg/ml lovastatin for 20 hours. MEM did not have any glutamate, since this acid was found to induce expression of GRP78 (38). GRP78 expression, endocytosis, and damage assays were conducted as above.

Cell surface expression of GRP78 on endothelial cells exposed to varying concentrations of FeCl₃ was quantified using FACS analysis. Briefly, endothelial cells grown in 25-cm flasks were dissociated using 1.5 ml enzyme-free dissociation buffer (Invitrogen). Cells were blocked with 50% goat serum, then stained with monoclonal anti-GRP78 Ab (BD Biosciences) at 1:100 for 1 hour. Endothelial cells were counterstained with Alexa Fluor 488-labeled anti-mouse IgG at 1:100 for 1 hour. Endothelial cells exposed
to a similar concentration of FeCl₃ or glucose and stained with an isotype matching control IgG (BD Biosciences) were used as negative control. A FACSCaliber (BD) instrument equipped with an argon laser emitting at 488 nm was used for flow cytometric analysis. Fluorescence data were collected with logarithmic amplifiers. The population percent fluorescence of $5 \times 10^3$ events was calculated using CellQuest software (BD).

**In vivo studies.** For in vivo studies, DKA was induced in BALB/c male mice ($\geq 20$ g) (National Cancer Institute) with a single i.p. injection of 190 mg/kg streptozotocin in 0.2 ml citrate buffer 10 days prior to fungal challenge (17). Glycosuria and ketonuria were confirmed in all mice 7 days after streptozotocin treatment. To determine the available serum iron in DKA versus normal mice, serum samples were obtained from mice ($n = 11$) and the serum iron levels measured using the method of Artis et al. (11).

For quantification of GRP78 expression in mouse organs, lungs, brain, or sinus from normal or DKA mice were harvested 14 days following DKA induction (17). Organs were stored in RNAlater solution (Ambion). Approximately 25 mg of brain or lung tissues was processed for RNA extraction using the RNAqueous-4PCR Kit (Ambion). Sinus bone was homogenized in liquid nitrogen (39), and RNA was extracted using the QIAGEN RNeasy Kit. For mouse GRP78 expression, primers 5'-TCTTGGCTATTCAAGGTTGGTTG-3' and 5'-TTCTTTCACAAATACGCTCAAG-3' were used, while primers to amplify the housekeeping gene GAPDH were as above. Calculations and statistical analyses were carried out as described in ABI PRISM 7000 Sequence Detection System User Bulletin 2 (Applied Biosystems).

To generate immune serum for passive immunization, normal BALB/c mice were immunized by s.c. injection of 20 μg recombinant hamster GRP78 (which is more than 98% identical to murine or human GRP78) mixed with CFA (Sigma-Aldrich) or with CFA alone mixed with PBS to generate non-immune control serum (40, 41). Mice were boosted in incomplete Freund’s adjuvant (IFA) 3 weeks later. Twelve days after the boost, serum was collected from GRP78-immunized or control mice (i.e., mice vaccinated with CFA/IFA without GRP78). Anti-GRP78 Ab titers were determined by using ELISA plates coated with 5 μg/ml of recombinant hamster GRP78 as we previously described (40). Immune or control sera (0.25 ml) were administered i.p. to DKA recipient mice 2 hours before intranasal infection with $10^5$ R. oryzae 99-880 spores. Sera doses were repeated 3 days after infection, and survival of mice was followed for 90 days after infection. All procedures involving mice were approved by the Institutional Animal Use and Care Committee of the Los Angeles Biomedical Research Institute at Harbor–UCL Medical Center, according to the NIH guidelines for animal housing and care.

**Figure 10**

GRP78 is overexpressed in DKA mice and anti-GRP78 immune serum protects mice from mucormycosis. (A) Different organs harvested from DKA or normal mice ($n = 7$ per group) were processed for GRP78 quantification by real-time RT-PCR. *$P < 0.05$ compared with normal mice. Data are expressed as median ± interquartile range. (B) Survival of mice ($n = 18$ from 2 independent experiments with similar results) infected intranasally with $R$. oryzae ($10^6$ spores actual inoculum) and treated with anti-GRP78 immune or non-immune sera. **$P = 0.037$ by log-rank test. The experiment was terminated on day 90, with all remaining mice appearing healthy.
This research was conducted at the research facilities of the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center.

Address correspondence to: Ashraf S. Ibrahim, Division of Infectious Diseases, Harbor-UCLA Medical Center, 1124 West Carson St., St. John’s Cardiovascular Research Center, Torrance, California 90502, USA. Phone: 310.222.6424; Fax: 310.782.2016; E-mail: ibrahim@labimed.org.

This work was presented in part at the Interscience Conference on Antimicrobial Agents and Chemotherapy in San Francisco, California, USA, on September 12-15, 2009 (abstract M360).