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Peer reviewed

## FUNGAL INFECTIONS

# A humanized antibody against mucormycosis targets angioinvasion and augments the host immune response

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Mucormycosis is a fungal infection caused by Mucorales fungi that cause severe disease and fatality, especially in immunocompromised individuals. Although vaccines and immunotherapeutics have been successful in combating viral and bacterial infections, approved antifungal immunotherapies are yet to be realized. To address this gap, monoclonal antibodies targeting invasive fungal infections have emerged as a promising approach, particularly for immunocompromised patients who are unlikely to maximally benefit from vaccines. The Mucorales spore coat (CotH) proteins have been identified as crucial fungal invasins that bind to glucose-regulated protein 78 (GRP78) and integrins of host barrier cells. Previously, we described a murine monoclonal antibody, anti-CotH C2, which protected diabetic ketoacidosis (DKA) and neutropenic mice from mucormycosis. Here, we advanced the development of the C2 immunoglobulin G1 (IgG1) by humanizing it, establishing a stable Chinese hamster ovary cell line producing the antibody at commercial yields, and carried out optimization of the upstream and downstream manufacturing processes. The resultant humanized IgG1 (VX-01) exhibited a 10-fold increase in binding affinity to CotH proteins and conferred comparable *in vitro* and *in vivo* efficacy when compared to C2 antibody. The mechanism of protection was reliant on prevention of angioinvasion and enhancing opsonophagocytic killing. VX-01 demonstrated acceptable safety profiles with no detectable damage to host cells *in vitro* and weak or moderate binding to only cytoplasmic proteins in *ex vivo* good laboratory practice–human tissue cross-reactivity studies. Our studies warrant continued development of VX-01 as a promising adjunctive immunotherapy.

## INTRODUCTION

Mucormycosis, caused by filamentous fungi belonging to the Mucorales order, was first identified in 1885 (1). These pathogenic fungi are ubiquitous, found in various environmental sources like soil and vegetation; they are also a major cause of food spoilage, thereby making exposure to these pathogens common. Several risk factors contribute to the susceptibility to mucormycosis, including leukemia, immunosuppression in organ transplant recipients, blood dyscrasias (e.g., myelodysplastic syndrome), and diabetes mellitus (2). The incidence of diabetes-related cases has been on the rise in recent decades, fueled by global obesity rates, with an estimated 382 million people affected in 2013 and projected to reach 592 million by 2035 (3). Additionally, malnourishment, acidosis, and prematurity are also considered as potential risk factors for mucormycosis (4). Notably, mucormycosis has become the second most common invasive mold infection in major US transplant centers, trailing only aspergillosis (1). The infection is highly invasive, characterized by a mortality rate exceeding 40% despite the use of antifungal therapy and aggressive surgical intervention (2). The mortality rate escalates to nearly 100% in cases of disseminated diseases and prolonged neutropenia (2, 5). Consequently, the urgent need for innovative treatment modalities to combat mucormycosis is apparent.

Monoclonal antibodies (mAbs) have emerged as a crucial immunotherapy against infectious diseases, successfully targeting viral and bacterial pathogens (6). Because mucormycosis usually afflicts patients with severely compromised immunity, these patients are more likely to benefit from mAb treatment instead of a vaccine-based immunotherapy (7). Some of the best antigens to target for mAb therapy are those expressed on the fungal cell surface or secreted and are known to contribute to the pathogenesis of the disease (8). In this context, CotH3, a fungal cell surface protein, has been identified as the key ligand that binds to glucose-regulated protein 78 (GRP78) on human cells [e.g., nasal epithelial cells and human umbilical vein endothelial cells (HUVECs)], playing a vital role in fungal-mediated cell invasion and hematogenous dissemination (9, 10). We have shown that murine mAbs raised against CotH3 protein can protect mice from mucormycosis (11).

We humanized one of the most protective murine mAbs (named VX-01), expressed it in Chinese hamster ovary (CHO) cells, and optimized and completed manufacturing processes. VX-01 enhanced the ability of antifungal drugs to protect mice from mucormycosis through prevention of invasion and enhancing opsonophagocytosis. Last, in a good laboratory practice (GLP)–human tissue cross-reactivity study, VX-01 was shown to be safe. Thus, our study introduces a therapeutic approach against mucormycosis with the potential to treat patients with this severe infection.

## RESULTS

### Comparative analysis of binding affinity between grafted and chimeric mAbs targeting CotH3

To enhance the potential therapeutic application of the anti-CotH3 C2 antibody in treating mucormycosis, we pursued a humanization

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strategy to maintain or improve its binding affinity to its fungal CoH3 antigen. Initially, we obtained the sequences of the heavy-chain (HC) and light-chain (LC) variable regions of the C2 antibody through hybridoma cell line mRNA sequencing. N-terminal protein sequencing of the murine mAb was also conducted, revealing identical sequences of the first 10 amino acids of both the kappa LC variable region ( $V_{\kappa}$ ) and the HC variable region ( $V_H$ ) to the encoding regions of the cloned mRNA sequence of the hybridoma cells (12).

To achieve humanization, we performed alignments of the murine HC and LC variable regions with human germline antibody variable regions to identify the most suitable frameworks for the mouse C2 mAb  $V_H$  and  $V_{\kappa}$ . On the basis of the alignment analysis, we selected the human VH4-59\*01 framework for the HC humanization and the IGKV2-29\*02 (A18  $V_{\kappa}$ ) framework for the LC humanization. Initially, we generated a chimeric mAb containing the mouse  $V_H$  and  $V_{\kappa}$  fused to the human constant regions (fig. S1). We also generated a grafted antibody in which the six complementarity-determining regions (CDRs) of the C2 mouse  $V_H$  and  $V_{\kappa}$  were grafted in the selected human framework to generate a humanized clone without mutations in the framework. This step was intended to minimize the antigenicity of the antibody when given to humans.

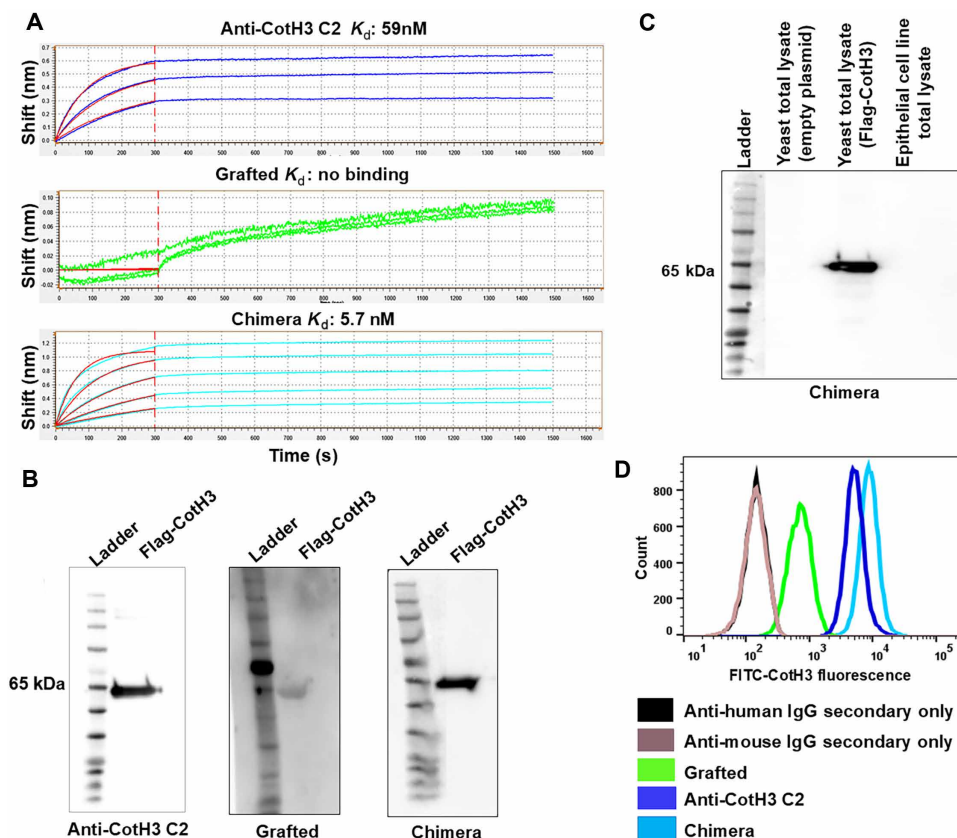
The DNA encoding the  $V_H$  variable region was cloned into a pcDNA3.4-immunoglobulin G1 (IgG1) vector, and sequencing confirmed the HC sequences of the mouse-human chimera and grafted variants. Similarly, the  $V_{\kappa}$  region for both constructs was cloned into a pcDNA3.4-kappa vector, with sequencing confirming the LC sequences.

The expression of the LCs and HCs was achieved in ExpiCHO cells, and both the chimera and grafted antibodies were purified to >95% on the basis of on SDS-polyacrylamide gel electrophoresis (PAGE) (fig. S2). We then performed biolayer interferometry (BLI) (13) to assess the binding kinetics of each mAb for the CoH3 antigen (table S1). Whereas the chimeric mAb exhibited an ~10-fold improvement in affinity over the original murine mAb [e.g., a dissociation constant ( $K_d$ ) of 5.7 nM binding affinity for the chimera versus 59 nM for the C2 parental mAb], the grafted humanized variant showed no detectable binding to CoH3 antigen (Fig. 1A).

To further verify the binding affinity of the antibodies, we conducted an immunoblot using recombinant CoH3 protein [expressed in *Saccharomyces cerevisiae* (11)], which showed intense recognition of the CoH3 antigen at 65 kDa by both the mouse C2 mAb and the chimeric mAb but less recognition by the grafted clone (Fig. 1B). The binding of the chimeric mAb to CoH3 antigen is specific because the mAb only recognized the CoH3 protein band from total lysate of *S. cerevisiae* expressing the Flag-tagged CoH3. The generated chimeric mAb did not recognize any proteins extracted from a human alveolar epithelial cell line (A549 cells) (Fig. 1C). Next, we compared the ability of the chimera and grafted mAbs with the C2 parent mouse clone in recognizing CoH antigens on *Rhizopus delemar*. Consistent with the binding affinity and immunoblot data, the chimeric mAb showed strong binding to *R. delemar* spores that exceeded the binding activity of the parent C2 mouse clone, whereas the grafted clone showed less binding to *R. delemar* spores (Fig. 1D). Collectively, these studies show that the chimeric mAb retains recognition of the CoH3 on *R. delemar*, whereas the grafted mAb lost

**Fig. 1. A chimeric mAb, but not grafted ones, recognized *R. delemar* CoH3.**

(A) Binding kinetics of the mouse C2, chimera, and grafted antibodies targeting CoH3 antigen. The on rates were measured between 0 and 300 s, and the off rates were measured after washing the probe at 300 s. Dissociation constants ( $K_d$ ) are shown. The variation of the response is shown as the experimental (red) and fitting curves (blue, green, or cyan); the dotted red line divides the association and dissociation steps. (B) Immunoblot analysis depicting the binding of mouse C2 antibody, grafted antibody, and the chimeric mAb to denatured CoH3 antigen. The bands indicate the specific binding of each antibody to the CoH3 antigen. (C) Immunoblot analysis evaluating binding of the chimeric mAb to CoH3 antigen or a human lung epithelial cell line total lysate. (D) Flow cytometry analysis showing binding of mouse C2 antibody, the chimeric mAb, and the grafted antibody to *R. delemar*.



most of its ability to recognize the antigen. Because our goal is to optimize the therapeutic benefit of these antibodies for treating mucormycosis by minimizing the mouse sequences in the generated mAb, we decided to introduce key mouse framework residues back to the grafted variant to restore its binding activity.

### Back mutations to LC and HC restored the binding affinity of the humanized mAb to recombinant Coth3 protein

As the first step toward restoring binding activity of the grafted and humanized mAb, we conducted a computer modeling step that used the human antibody frameworks and mouse antibody CDRs. For this purpose, Protein Data Bank (PDB) human structures 1XGP (HC) and 2W60 (LC) were chosen as homology templates for the murine variable regions, and 4LKX was used for the human framework with grafted murine CDRs. An in-depth structural assessment of the homology models was then conducted, where visual analysis and residue sequence and structure comparisons between the mouse and humanized models guided us in identifying regions where back mutations might be warranted (14). The model of the LC  $V_K$  region and HC  $V_H$  variable region is depicted in Fig. 2A.

In the case of the LC, we identified three groups of residues within the human A18 framework that could potentially affect the folding of the CDRs. The first group consisted of residues S53, R54, and F55, which were located at the beginning of framework 3 of the human chain, adjacent to CDR2. These residues were predicted to force a different conformation of LC CDR2 (CDRL2) compared with the lysine, leucine, and aspartic acid residues at these positions in the murine  $V_K$ . Consequently, the first LC variant, named LC1, was designed with the S53K (changing S to K), R54L, and F55D back

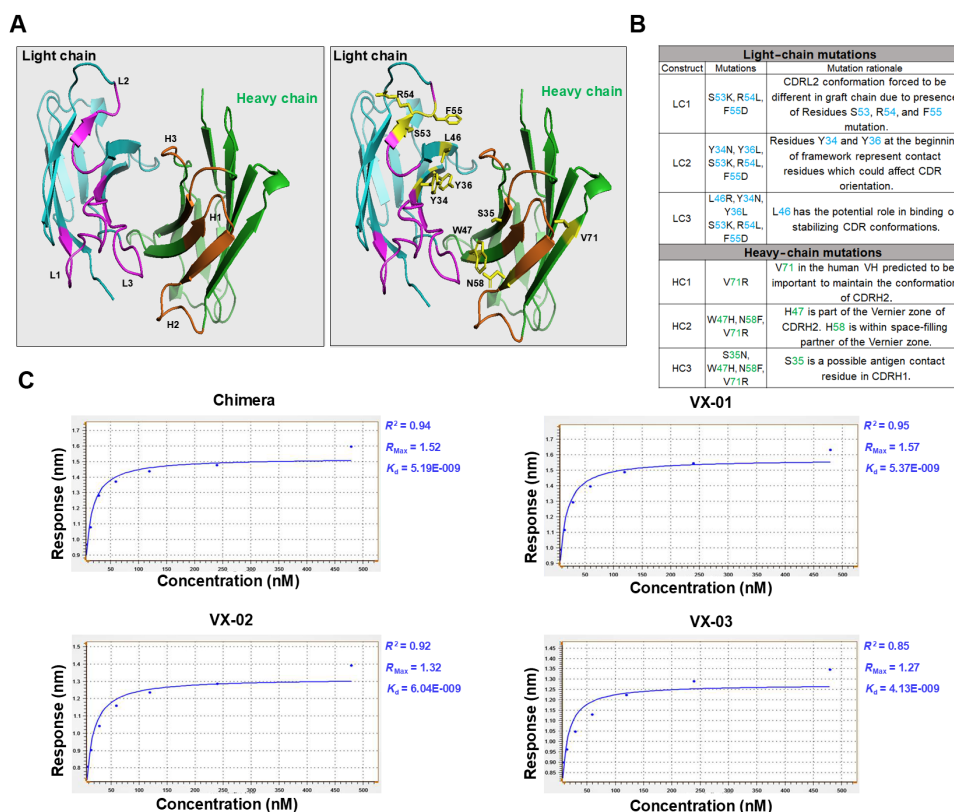
mutations at these positions. The second construct (LC2) included additional mutations on residues Y34 and Y36, situated at the beginning of framework 2 of the human acceptor framework, representing contact residues that could affect CDR orientation. Thus, the LC2 had mutations of LC1 (S53K, R54L, and F55D back mutations) and additional back mutations Y34N and Y36L. Last, the third construct LC3 included an additional mutation at residue L46R in the human framework, and this residue was predicted to potentially play a role in binding or stabilizing CDR conformations. Thus, LC3 had the following six back mutations: S53K, R54L, F55D, Y34N, Y36L, and L46R back mutations (Fig. 2B).

We also identified a total of four key residues that could affect the conformation of the CDR HC and the antigen-binding pocket. Thus, we generated three HC constructs. In HC1, we introduced a back mutation of V71R to maintain the conformation of CDRH2. In the HC2 construct, we identified W47 and N58 human amino acids that are likely located within the Vernier zone of CDRH2 and its space-filling partner at position 58, respectively, and were considered to potentially affect CDR orientation and the antigen binding pocket. Thus, these two residues were back-mutated to their mouse corresponding amino acids of W47H and N58F, and HC2 contained these two back mutations in addition to V71R. Last, in the construct HC3, the human residue S35 was also considered to potentially affect the CDR conformation and the antigen binding pocket. Thus, it was back-mutated to its mouse corresponding amino acid of N (S35N), and the HC3 construct contained V71R, W47H, N58F and S35N back mutations (Fig. 2B).

The designed humanized LCs and HCs were synthesized and cloned into antibody expression vectors. A total of 16 different

**Fig. 2. Back mutations enhanced binding of humanized mAb to Coth3 protein.**

(A) Model representation of the LC variable region (teal blue framework) and HC variable region (green framework) of the 4LKX PDB structure. The grafted CDRs from the parental C2 mAb are shown. In the left-hand panel, the CDRLs are highlighted in magenta, and the CDRHs are shown in brown. The right-hand panel highlights potential regions of conflict (yellow). (B) Summary of the three sets of framework residues (top set, LC; bottom set, HC) that could affect the binding pocket formed by the three CDRs. These residues are potential candidates for back mutation from human to murine residues. (C) Steady-state analysis was performed by BLI, whereby a 1:2 serial dilution of Coth3 from 480 to 7.5 nM (specifically, 480, 240, 120, 60, 30, 15, and 6.5 nM) was compared with buffer alone; shown are dissociation constant ( $K_d$  as an assessment for binding affinity) values for the humanized variants (VX-01, VX-02, and VX-03) compared with the chimeric mAb.



HC/LC antibody clones were generated by pairing each LC and HC variant, including the HC and LC of the grafted antibody without any back mutations in the framework. Transfection of ExpiCHO cells with small-scale plasmid preps was conducted, and the yield of each antibody clone was estimated using protein G probe capture. The chimeric mAb was also produced to serve as the reference control. This comprehensive rational design approach led to the development of engineered humanized variants that were assessed for their binding activity to Coth3 recombinant protein using BLI (13). Each variant was loaded onto protein G sensors in the binding buffer, and, then, 30 nM of Coth3 antigen was introduced, allowing us to monitor the on rates for 300 s, followed by the dissociation step for another 300 s. Only three humanized variants, namely, LC3:HC3 (VX-01), LC3:HC2 (VX-02), and LC2:HC3 (VX-03), exhibited distinct binding profiles to Coth3, similar to the reference chimera (table S2). No detectable binding was observed for any of the variants with the grafted LC or with LC-1 or with the variants containing the grafted HC or HC-1. Furthermore, all three variants, along with the chimera, demonstrated negligible dissociation.

To further characterize the binding kinetics, we performed steady-state analysis using a serial dilution of Coth3 from 480 to 7.5 nM for all three humanized variants and the chimera (15). The  $K_d$  values for the variants were similar, ranging from 4 to 6 nM. Both the chimera and VX-01 exhibited  $K_d$  values of 5.2 and 5.4 nM, respectively, with high coefficient of determination ( $R^2$ ) values of 0.94 and 0.95, indicating robust and reliable binding (Fig. 2C). Collectively, these data show that the grafted antibody's binding affinity was successfully restored through back mutations, and the objective of achieving an improvement in binding affinity above the parental murine mAb was successfully accomplished with the humanized variants.

### The back-mutated mAbs specifically bind to Coth3 protein expressed on *Mucorales* spores

We investigated the specificity of the three generated mAb variants (VX-01, VX-02, and VX-03) in binding to Coth3 protein. Flag-tagged Coth3 protein was expressed in *S. cerevisiae*, the entire cell lysate was separated on an SDS-PAGE, and immunoblot analysis was performed using each of the generated mutated mAbs (VX-01, VX-02, and VX-03). Whereas VX-01 and VX-02 recognized only the Coth3 recombinant protein at 65 kDa, VX-03 recognized two additional bands at ~48 and 35 kDa. These two additional bands recognized by VX-03 likely represent degradation of Coth3 protein because VX-03 did not recognize any bands from *S. cerevisiae* transformed with empty plasmid (Fig. 3A). None of the antibody variants recognized proteins extracted from the A549 cell line (Fig. 3A).

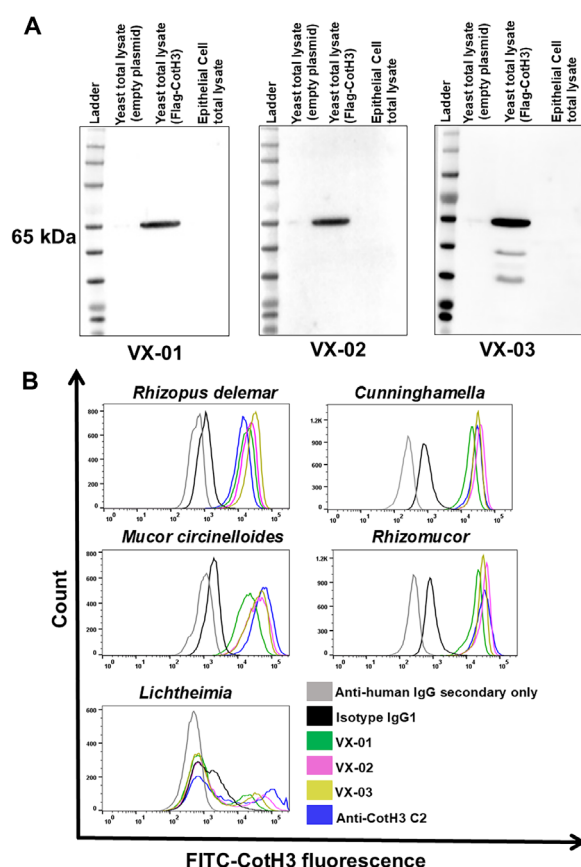
Next, we studied the binding of the humanized mAbs to *Mucorales* fungi by immunostaining and flow cytometry, with the anti-Coth3 C2 as the positive control. The results indicated that all three humanized antibodies (VX-01, VX-02, and VX-03) demonstrated recognition of various *Mucorales* fungi including *Rhizopus*, *Cunninghamella*, *Mucor circinelloides*, *Rhizomucor*, and, to a lesser extent, *Lichtheimia* (Fig. 3B and table S3). This reduced binding to *Lichtheimia* can be attributed to the reduced identity of the 16-nucleotide oligomer peptide in *Lichtheimia* (five mismatched amino acids) when compared with the other *Mucorales* fungi (fig. S3A) that the original C2 murine mAb was raised against. Alternatively, the reduced binding can be attributed to the reduced expression of CothH ortholog in *Lichtheimia* spores when compared with that in *R. delemar* (fig. S3B). Nonetheless, these data also show that the humanized variants retained the binding specificity

of the parental mouse antibody and universally recognized Coth3 on *Mucorales* fungi.

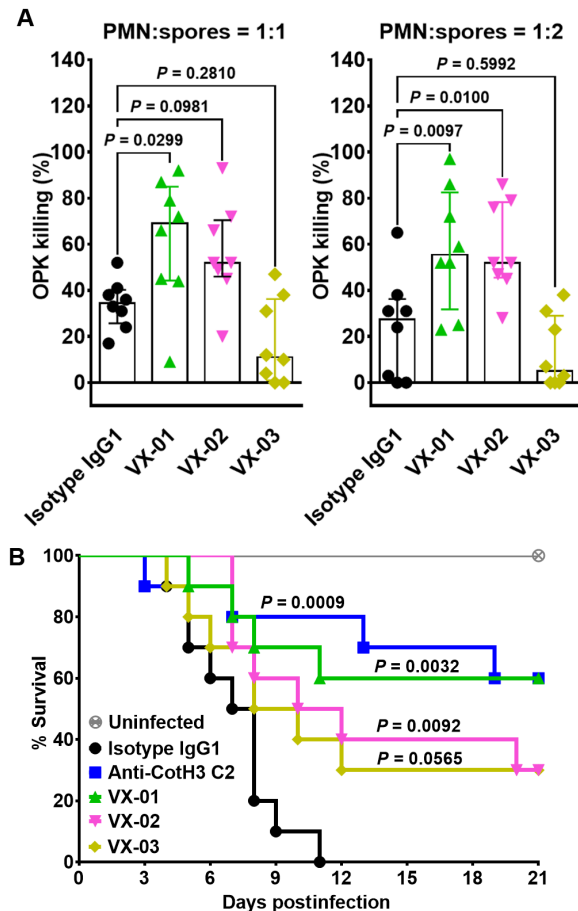
### The humanized antibodies enhance opsonophagocytic ex vivo and protect mice from mucormycosis

We previously showed that anti-Coth3 polyclonal antibodies enhance polymorphonuclear leukocyte (PMN)-mediated damage of *R. delemar* through opsonophagocytic killing (11). To further investigate the activity of the humanized antibodies in enhancing the phagocytosis of *R. delemar*, we conducted in vitro experiments using human PMNs isolated from human peripheral blood cells of healthy individuals. The extracted neutrophils were then incubated with *R. delemar* at a 1:1 or 1:2 ratio (PMN:spores) in the presence of either the control isotype IgG or the humanized antibodies at 37°C for 3 hours. Only VX-01, but not VX-02 or VX-03, more than doubled the ability of neutrophils to kill *R. delemar*, indicating that this humanized variant is opsonophagocytic and further validating its potential as a potent immunotherapeutic agent against mucormycosis (Fig. 4A).

We next evaluated the ability of the humanized antibodies to protect against murine mucormycosis. We used our established and clinically relevant neutropenic mouse model in which infection with *R. delemar* is induced through the intratracheal route. Treatment



**Fig. 3. Humanized antibody variants bind to Coth3 antigen and *Mucorales* fungi.** (A) Immunoblot analysis testing binding of VX-01, VX-02, and VX-03 to Coth3 antigen or a human lung epithelial cell line total lysate. (B) Binding of the control human isotype-matched IgG1, VX-01, VX-02, and VX-03 and the parental mouse C2 antibodies to *R. delemar*, *Cunninghamella*, *M. circinelloides*, *Rhizomucor*, and *Lichtheimia* was measured by flow cytometry.



**Fig. 4. The VX-01 humanized Ab enhanced OPK activity of *R. delemar* and protected mice from mucormycosis.** (A) The ability of PMNs harvested from healthy volunteers to phagocytize *R. delemar* was measured after 3 hours of incubation in the presence of VX-01, VX-02, and VX-03 (PMN:spore ratio 1:1 or 1:2,  $n = 8$ ). An isotype IgG1 was used as a control. Data are median  $\pm$  interquartile range. Data were analyzed by one-way ANOVA with Dunnett's multiple comparisons test. (B) Shown is the survival of neutropenic mice infected intratracheally with *R. delemar*. After 24 hours, mice were treated with 30  $\mu$ g of VX-01, VX-02, VX-03, mouse C2 antibodies, or the human isotype-matched control IgG1 ( $n = 10$  per group, except for  $n = 5$  for uninfected mice). Data were analyzed by log-rank (Mantel-Cox) test comparing each treatment with the isotype IgG1 control.

with a single dose of 30  $\mu$ g of an isotype-matched IgG1, the murine mAb (C2), or its humanized versions (VX-01, VX-02, or VX-03) was given 24 hours postinfection through the intraperitoneal route, and survival of mice was determined over a 21-day period. The dose of the antibody was selected on the basis of historical data obtained with the murine C2 antibody (11). Although VX-01 and VX-02 both significantly ( $P < 0.05$ ) prolonged overall survival of mice infected with *R. delemar* compared with that of isotype control, only VX-01 resulted in protection identical to what is afforded by the C2 clone. Specifically, C2 mouse antibody or VX-01 humanized antibody resulted in a median survival time of >21 days and 60% overall survival versus 7 days median survival time and 0% overall survival for mice treated with isotype-matched IgG1. Treatment with VX-02 resulted in a 10-day median survival time and an overall survival of 30% (Fig. 4B).

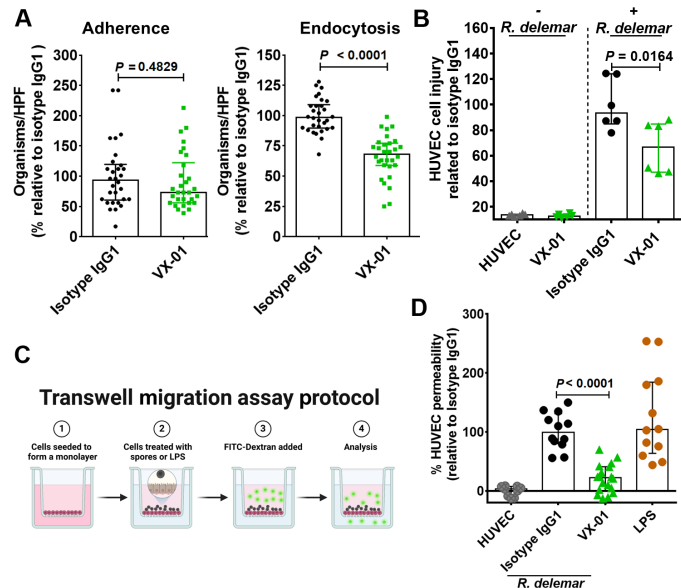
We also investigated whether the generated humanized antibodies with improved binding to CotH3 protein had any direct effect on the viability or germination of *R. delemar*. We focused on VX-01 because it gave us the most consistent results in enhancing opsonophagocytic killing activity of human neutrophils and had superior protection in mice compared with VX-02 and VX-03. Incubating *R. delemar* in the presence of varying concentrations of VX-01 (ranging from 0.1 to 100  $\mu$ g/ml) or the mouse C2 mAb for 24 hours had no effect on the metabolic activity of the fungus as determined by 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay (fig. S4). Similarly, germination of *R. delemar* spores in the presence of VX-01 (100  $\mu$ g/ml) or mouse C2 mAbs for 6 hours had no effect on the ability of the fungus to germinate or to change the length of the germ tube (fig. S5).

### VX-01 reduces *R. delemar* ability to invade and damage endothelial cells and abrogates *R. delemar*-mediated vascular leak

Angioinvasion and vascular leak are hallmarks of mucormycosis (2). We previously showed that anti-CotH3 antibodies protected mice infected intratracheally with *R. delemar* from angioinvasion and subsequent hematogenous dissemination to the brain (11). Thus, we investigated whether VX-01 would protect HUVECs from *R. delemar*-induced invasion and subsequent damage. Consistent with our previously reported data, VX-01 had no effect on the ability of *R. delemar* to adhere to HUVECs but significantly ( $P < 0.0001$ ) reduced *R. delemar*-mediated invasion (endocytosis) by >30% when compared with isotype-matched IgG1 (Fig. 5A). Further, incubating HUVECs with either an isotype-matched IgG1 or VX-01 (50  $\mu$ g/ml) had no damaging effect on these mammalian primary cells in the absence of *R. delemar* infection, indicating that the humanized VX-01 antibody has favorable safety profiles. Incubating *R. delemar*-infected HUVECs cells with VX-01 (50  $\mu$ g/ml) resulted in ~40% protection of these cells versus cells infected and incubated with the isotype-matched IgG1 (Fig. 5B). Furthermore, using a permeability assay in which HUVEC were grown on membrane inserts in 24-well transwell plates and using fluorescent dextran to determine migration of the fluorescence signal from the upper chamber to the lower chamber after addition of *R. delemar* (Fig. 5C), we show that VX-01 antibody almost completely abrogated the ability of *R. delemar* to cause vascular leak when compared with isotype-matched IgG1 or *Escherichia coli* lipopolysaccharide (LPS), a potent inducer of vascular permeability (Fig. 5D). Collectively, these findings underscore the potential of VX-01 as an effective agent in neutralizing angioinvasion and vascular leak, two processes that are essential to mucormycosis pathogenesis and are mediated by CotH proteins (9, 16).

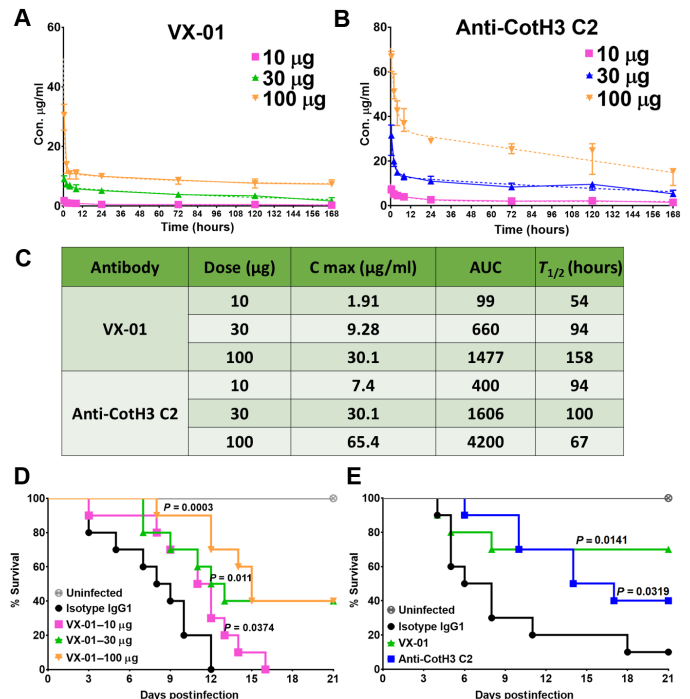
### Pharmacokinetics of VX-01 and the parental murine antibody using the neutropenic mouse model

To facilitate the clinical development of the VX-01 antibody and optimize dosing, we conducted a pharmacokinetic (PK) study to characterize its behavior in the mouse model. To mimic the clinical scenario, we compared the PK of VX-01 to the mouse C2 antibody using the neutropenic mouse model infected with *R. delemar*. VX-01 or the parental C2 antibody was administered intravenously at different doses (10, 30, or 100  $\mu$ g). Blood samples were collected at 0.5, 2, 4, 8, 24, 72, 120, and 168 hours after antibody administration. Both VX-01 and C2 displayed biexponential serum concentration-time profiles, characterized by a short distribution phase and a long



**Fig. 5. VX-01 prevents HUVECs cell damage and vascular permeabilization by *R. delemar*.** (A) Adherence and invasion (endocytosis) of *R. delemar* to HUVECs were measured in vitro in the presence of isotype IgG1 or VX-01 (50  $\mu$ g/ml). Adherence and endocytosis were determined by the differential fluorescence of the *R. delemar* under the microscope ( $n = 30$  per group) after 1.5 hours of incubation with VX-01 or isotype-matched control IgG1. HPF, high-power field. (B) The ability of *R. delemar* to injure HUVECs after 6 hours of incubation was measured in the presence of VX-01 (50  $\mu$ g/ml) by the  $^{51}\text{Cr}$  release method. The human isotype-matched IgG1 served as a control ( $n = 6$  wells from two experiments). (C) A diagram depicting the cell permeability assay. (D) The ability of *R. delemar* to induce permeability of HUVECs after 4 hours of incubation was measured in the presence of VX-01 (50  $\mu$ g/ml) by measuring the amount of fluorescent dextran migrating from the upper chamber to the lower chamber of the transwell ( $n = 9$  to 18 per group from two individual experiments). Human isotype-matched IgG1 served as a negative control, and LPS was used as an inducer of vascular permeability. Data are presented as median  $\pm$  interquartile range. Data were analyzed by unpaired, two-sided Student's *t* test.

elimination phase (Fig. 6, A to C). When examining the average concentration during the elimination phase (after 8 hours of intravenous injection), we observed that the average concentrations for 10, 30, and 100  $\mu$ g of VX-01 injections were 0.57, 4.08, and 8.94  $\mu$ g/ml, respectively. In contrast, the average concentrations for 10, 30, and 100  $\mu$ g of murine C2 antibody injections were 2.51, 9.68, and 25.6  $\mu$ g/ml, respectively. These results indicate that the murine antibody displayed greater stability in mouse serum compared with the humanized antibody. Despite lower concentrations of VX-01 in the elimination phase, it had a serum half-life comparable to C2 antibody that ranged from  $\sim 3$  to 7 days (Fig. 6C). We also compared the efficacy of the three VX-01 doses given intravenously in protecting mice from *R. delemar* intratracheal infection. Although all doses resulted in a significant ( $P < 0.05$ ) improvement in survival of mice, the 30- and 100- $\mu$ g doses resulted in a comparable enhanced survival of 40% when compared with that in mice infected with isotype-matched IgG1 (Fig. 6D). Because the intravenous and the intraperitoneal routes showed similar protection in neutropenic mice infected with *R. delemar*, we investigated the breadth of protection of VX-01 in neutropenic mice infected with second most common cause of mucormycosis, *M. circinelloides* (2). VX-01 and C2 resulted in prolonged survival of 70 and 40%, respectively, versus 10% for isotype-matched

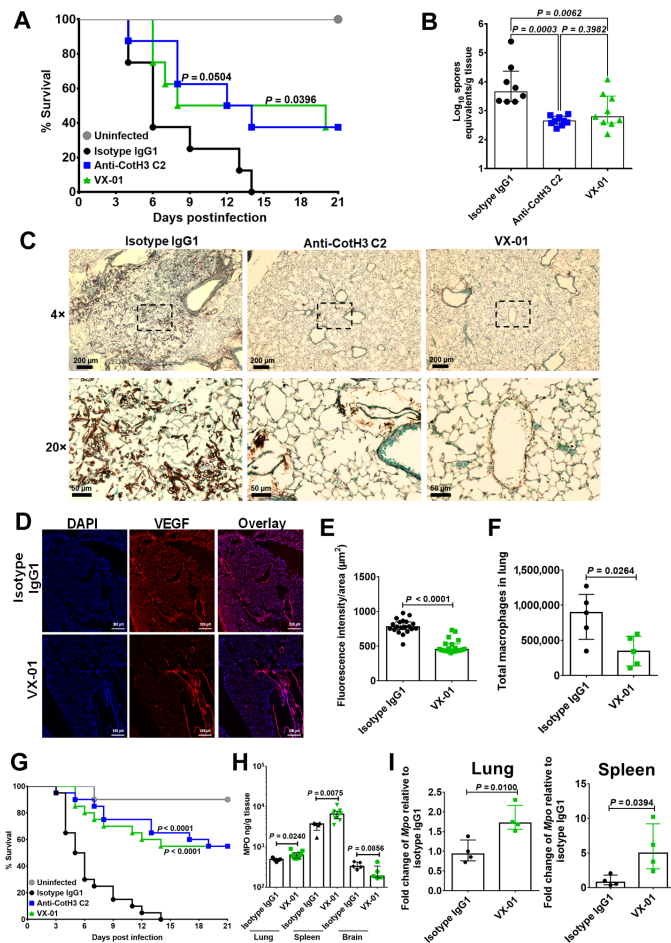


**Fig. 6. VX-01 has comparable pharmacokinetics and in vivo efficacy to C2 mouse mAb.** (A and B) VX-01 (A) or C2 mouse IgG1 (B) were given intravenously at a single dose of 10  $\mu$ g (pink), 30  $\mu$ g (green), or 100  $\mu$ g per each neutropenic mouse (orange). The solid line represents the simulated pharmacokinetic (PK) profile using in vitro parameters, and the dots represent the observed concentrations ( $n = 4$  per time point). Data are presented as median  $\pm$  interquartile range. (C) PK data for VX-01 and mouse C2 antibody, including  $C_{\text{max}}$  (peak concentration),  $T_{1/2}$  (terminal half-life), and AUC (area under the curve). Results are presented as mean values. (D and E) Shown is the survival of neutropenic mice ( $n = 10$  per group, except for  $n = 5$  for uninfected mice) infected intratracheally with *R. delemar* (D) or *M. circinelloides* (E) and 24 hours later treated with 10, 30, and 100  $\mu$ g of VX-01 or human isotype-matched IgG1 as the control group. *P* values are versus isotype-matched IgG1 treated mice. Survival data were analyzed by log-rank (Mantel-Cox) test. DAPI, 4',6'-diamidino-2-phenylindole.

placebo mice (Fig. 6E). Together, these results show that VX-01 has comparable PK parameters to C2 and produces similar protection to mice from mucormycosis due to *R. delemar* or *M. circinelloides* regardless of the antibody route of administration.

### VX-01 protects neutropenic and DKA mice from pulmonary mucormycosis primarily by prevention of angioinvasion and enhancement of phagocyte killing

Having optimized the dose and route of administration of VX-01, we embarked on a detailed study to characterize the therapeutic benefits of the humanized antibody. We tested VX-01 in the neutropenic mouse model of *R. delemar* infection and used survival, tissue fungal burden, and histopathological examination as end points. As expected, both VX-01 and the mouse C2 antibodies resulted in similar protection characterized by a median survival time of 14 and 13 days, respectively, and an overall survival of 38% when compared with isotype-matched IgG1-treated placebo mice (Fig. 7A). Additionally, both antibodies resulted in similar reduction of lung fungal burden of  $\sim 1$ -log versus isotype-matched placebo mice when the organ was harvested on day 4 postinfection (Fig. 7B). Last, histopathological examination of lung tissues sampled on the day 4



**Fig. 7. VX-01 protects neutropenic and DKA mice from *R. delemar* infection and enhances neutrophil influx.** (A and B) Neutropenic mice were treated with a 30- $\mu$ g single dose (intraperitoneal route) of VX-01 or mouse C2 antibodies at 24 hours after intratracheal infection. Survival (A) and lung fungal burden (B) were measured ( $n = 8$  or 9 per group, except for  $n = 5$  for uninfected mice). (C to F) Neutropenic mice were treated as in (A), and tissues were collected 96 hours after intratracheal infection. (C) Grocott's methenamine silver staining of the lung is shown to evaluate hyphae invasion. Scale bars, 200  $\mu$ m (low magnification) and 50  $\mu$ m (high magnification, inset). (D) Immunohistochemistry staining showing VEGF expression (red) in lungs of neutropenic mice infected with *R. delemar* and treated with isotype IgG1 control or VX-01. Scale bars, 500  $\mu$ m. (E) Quantification of fluorescence intensity of the VEGF staining in micrographs of (D) ( $n = 20$  random fields). (F) The number of macrophages in the lungs of infected mice was quantified by flow cytometry after staining with cell surface marker of macrophages ( $n = 5$  per group). (G) DKA mice ( $n = 20$  per group, except for 10 for uninfected mice) were infected with *R. delemar* and treated as in (A), and survival was measured. (H) MPO abundance was measured in the lungs and spleens of DKA mice ( $n = 5$  or 10 mice per group) treated with VX-01 or isotype control. Lungs were harvested 96 hours after intratracheal infection with *R. delemar*. A single dose of 30  $\mu$ g of VX-01 or human isotype-matched IgG1 was given 24 hours after infection. MPO values are presented as raw values in nanograms per gram of the organs. (I) RT-PCR of *Mpo* gene expression in lungs and spleen of DKA mice ( $n = 4$  mice per group) infected with *R. delemar* and treated with either VX-01 or isotype-matched IgG1 as in (H). Data in (B) are presented as median  $\pm$  interquartile range and were analyzed by one-way ANOVA with Tukey's multiple comparisons test. Data in (E), (F), (H), and (I) are presented as median  $\pm$  interquartile range and were analyzed by the unpaired, two-sided Student's *t* test. Data in (A) and (G) were analyzed by log-rank (Mantel-Cox) test comparing with isotype IgG1 control.

postinfection showed that mice treated with the isotype IgG displayed wide fungal infiltration and abscesses with tissue edema. In contrast, the lungs of mice treated with either VX-01 or C2 showed no visible fungal presence and normal lung architecture (Fig. 7C).

We investigated the mechanism by which VX-01 protected neutropenic mice from mucormycosis. To investigate whether VX-01 prevented fungal invasion, we conducted immunohistochemistry staining of lung sections of mice infected with *R. delemar* and treated with either VX-01 or isotype-matched IgG1 for vascular endothelial growth factor (VEGF), a potent angiogenesis and microvascular permeability enhancing cytokine (17). VX-01 treatment significantly ( $P < 0.0001$ ) reduced expression of VEGF in the lungs of mice infected with *R. delemar* compared with that in lung sections from infected mice treated with isotype-matched IgG1 (Fig. 7, D and E). Consistent with the VX-01 effect on reducing lung fungal burden and decreasing fungal angiogenesis (Fig. 7, B to E), we detected fewer lung macrophages in lungs of infected mice treated with VX-01 versus those treated with isotype-matched IgG1 (Fig. 7F). These results indicate that the VX-01 mechanism of protection in neutropenic mice is reliant on prevention of angiogenesis.

We also validated the therapeutic benefits of VX-01 compared with that of C2 using the DKA mouse model of mucormycosis (18). A single dose of 30  $\mu$ g of VX-01 or C2 significantly ( $P < 0.0001$ ) improved overall survival of DKA mice compared with mice treated with the isotype-matched IgG1 (Fig. 7G). Because DKA mice have intact neutrophil count and because we previously showed that anti-CoH3 antibodies enhance mouse neutrophil function (11), we evaluated the role of VX-01 on neutrophil chemotaxis. We focused on myeloperoxidase (MPO), which is recognized for its pivotal role in neutrophil function and antimicrobial defense (19). As expected, the DKA mice treated with the VX-01 exhibited substantially higher concentrations of MPO in the lungs and spleen, but not in the brain (Fig. 7H), likely because VX-01 prevents angiogenesis and dissemination to secondary target organs (11). We further investigated the mechanism by which VX-01 augments MPO production in target organs. Lungs and spleens collected from infected mice and treated with VX-01 or isotype-matched IgG1 were gently disrupted to produce single cells followed by flow cytometry analysis to identify live neutrophil populations. There was no difference in the number of live neutrophils in lungs or spleens harvested from mice treated with VX-01 or isotype-matched IgG1 (fig. S6, A and B). Furthermore, there was no difference in intracellular MPO staining among neutrophils identified in lungs or spleens of mice treated with VX-01 or isotype-matched IgG1 (fig. S6, C and D). To investigate whether enhanced mRNA expression of the gene encoding MPO in infected organs treated with VX-01 or isotype-matched IgG1 accounted for the increased detection of MPO in the lungs and spleens of infected mice that were treated with VX-01, we harvested lungs or spleens from mice infected with *R. delemar*, treated them with a single dose of VX-01 on day 4 postinfection, and conducted reverse transcription polymerase chain reaction (RT-PCR) targeting the *MPO* gene. The lungs or spleens harvested from VX-01-treated mice showed 1.5- to 5-fold increase in *MPO* gene expression when compared with corresponding organs harvested from mice treated with isotype-matched IgG1 (Fig. 7I). These results show that VX-01 augments expression of MPO in live neutrophils and underscores the role of VX-01 in opsonophagocytic killing activity in the DKA mouse model.

### VX-01 can be combined with antifungal drugs to protect mice from mucormycosis

Because any immunotherapy-based drug is likely to be used as an adjunctive therapy with currently used antifungal drugs, we tested the effect of administering VX-01 with either liposomal amphotericin B (LAMB; Fig. 8, A to C) or posaconazole (POSA; Fig. 8, D to F) using the neutropenic mouse model. LAMB is considered a first-line therapy for mucormycosis, whereas POSA is reserved for salvage or step-down therapies (20). We used a severe model of infection, initiating fungal intratracheal inoculation and starting treatment 48 hours postinfection to mimic delayed diagnosis of the disease and to enhance the chance of detecting improved efficacy of combined therapy. Treatment with VX-01 alone demonstrated reduced but significant ( $P < 0.05$ ) protection (20% survival in Fig. 8, A and D). Conversely, treatment with LAMB or POSA alone did not improve survival, with 20 and 0% survival, respectively (Fig. 8, A and D). However, mice treated with a combination of VX-01 and either LAMB or POSA achieved 70% survival (Fig. 8, A and D) and remained healthy throughout the experiment. In an independent experiment, the tissue fungal burden of lung and brain (18) corroborated the survival studies, with combination therapy with LAMB (Fig. 8, B and C) or POSA (Fig. 8, E and F) outperforming monotherapy, reducing lung and brain fungal burden by ~2-log. Last, we confirmed these results against murine mucormycosis due to *M. circinelloides* by using the same delayed and severe model. Again, combination therapy resulted

in enhanced survival of neutropenic mice over mice treated with isotype-matched IgG1 or monotherapy with ~40% overall survival for either LAMB or POSA combinations (fig. S7, A and B). These findings collectively demonstrate that VX-01 immunotherapy enhances the efficacy of antifungals for treating mucormycosis.

### Prolonged exposure of *R. delemar* to VX-01 does not induce resistance

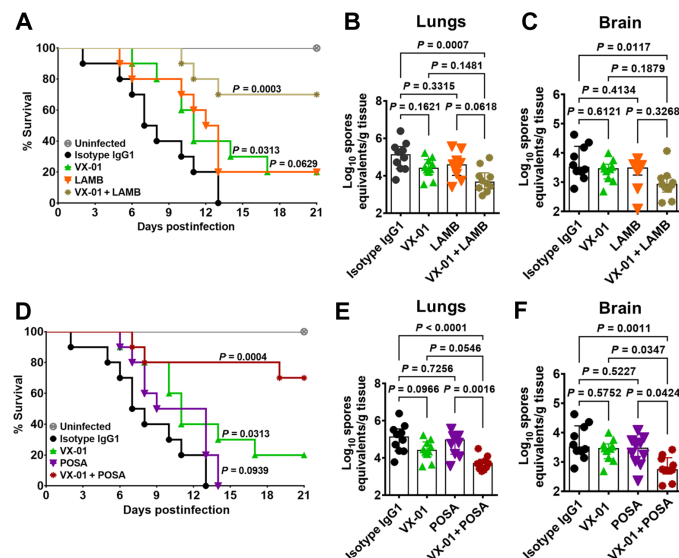
Antibody resistance, characterized by the ability of pathogens to evade the effects of therapeutic antibodies, is a concern that could limit the effectiveness of antibody-based treatments especially with repeated dosing that is likely to be needed for treating patients with mucormycosis (21). To investigate the possibility of Mucorales developing resistance to VX-01, *R. delemar* was serially passaged in the presence of VX-01 or human isotype-matched IgG1 for 20 generations, followed by flow cytometry analysis to confirm sustained binding of VX-01 to the fungal spores. VX-01 maintained recognition of *R. delemar* that has been passaged for 20 generations in the presence of VX-01, thereby indicating the lack of resistance to VX-01 in this fungal isolate (fig. S8A). Next, we compared the protective activity of VX-01 against an *R. delemar* strain that was passaged for 20 generations with that of wild-type *R. delemar* using the neutropenic mouse model of intratracheal infection. VX-01 had a similar protective activity of 40% overall survival against the wild-type *R. delemar* and the strain that was passaged for 20 generations in the presence of VX-01 (fig. S8B) ( $P < 0.01$ ). Therefore, these data suggest that treatment with VX-01 may not induce resistance.

### VX-01 does not bind to cell surface proteins but has weak to moderate binding affinity to cytoplasmic proteins of human tissues

To further investigate the toxicity of VX-01, we conducted GLP-tissue cross-reactivity studies using human samples from at least three donors per tissue (Charles River Laboratories). VX-01 was applied at two concentrations (2.5 and 0.5  $\mu\text{g}/\text{ml}$ ) to cryosections of normal tissues. VX-01 showed cross-reactive binding in epithelial cells of multiple tissues, including neurons and glial cells in the central nervous system; mononuclear cells in lymphoid tissues, including mucosal associated lymphoid tissue and peripheral blood; and in male and female reproductive tissues (stromal and germ cells). All of the tissue elements with test article binding had granular cytoplasmic staining only with no membrane labeling. In general, the character and distribution of cellular staining were similar at both concentrations but reduced in intensity and frequency or absent at 0.5  $\mu\text{g}/\text{ml}$  (fig. S9).

### Transfected and clonally derived CHO cells can produce commercial yields of VX-01

Because of these encouraging results, we initiated the manufacturing process development of VX-01. Stable clonal cell lines expressing VX-01 were generated using JOINN-CHO host cells (CHO-K1 derivative). Top clones showed a productivity range of 2.2 to 3.2 g/liter with the peak viable cell density (VCD) ranging from  $3.4 \times 10^6$  to  $7.1 \times 10^6$  cells/ml in a shake flask fed-batch evaluation. The research cell banks of three top clones were prepared, were free from mycoplasma, and were sterile. The cell count, viability, and recovery rate matched with those upon freezing and were within the acceptable ranges (cell viability of >90% before and after thawing and total number of cells of  $\sim 10 \times 10^6$  per vial). Additionally, using the



**Fig. 8. VX-01 treatment shows improved efficacy when administered with clinically used antifungal drugs.** (A to C) Neutropenic mice ( $n = 10$  per group, except for  $n = 5$  for infected mice) were infected with *R. delemar* and, 48 hours later, were treated with a single dose of 30  $\mu\text{g}$  of VX-01, LAMB [10 mg/kg, once a day (qd)] for 4 days, or a combination of both. End points were survival (A) or tissue fungal burden in the lung (B) or brain (C) as determined by qPCR. (D to F) The same model was used with similar treatment except that LAMB treatment was substituted with posaconazole [30 mg/kg, twice a day (bid) for 7 days] ( $n = 10$  per group, except for  $n = 5$  for uninfected mice). End points were survival (D) or tissue fungal burden in lung (E) or brain (F) as determined by qPCR. Survival data in (A) and (D) were analyzed by log-rank (Mantel-Cox) test. P values in the survival curve are versus isotype-matched IgG1 treatment. Data in (B), (C), (E), and (F) are presented as median  $\pm$  interquartile range. Statistical comparisons in (B), (C), (E), and (F) were made by one-way ANOVA with Tukey's multiple comparisons test.

National Institute of Allergy and Infectious Diseases preclinical service, a master cell bank was established and used for conducting upstream and downstream manufacturing processes; the production process successfully scaled up to a 200-liter bioreactor, and production was followed by conventional downstream purification for generation of bulk-drug substances. These studies showed that the top clone of CHO producing VX-01 could reach a VCD of  $3.0 \times 10^6$  to  $5.5 \times 10^6$  cells/ml with viability of >85% after 14 days of culturing in different bioreactor scales from Ambr250, at 2, 10, and 50 liters (fig. S10, A and B). All bioreactors in different scales produced acceptable titers of 2.5 to 3.5 g/liter during the production stage (fig. S10C).

## DISCUSSION

Mucormycosis, the third most common invasive fungal infection in hematopoietic centers, poses a major global health challenge. Despite antifungal drug treatment and aggressive surgical debridement, mortality rates remain unacceptably high. The COVID-19 pandemic exacerbated the situation; thousands of COVID-associated mucormycosis (CAM) cases were reported around the globe (22). Most of the CAM cases were reported in India among patients treated with high doses of corticosteroids with diabetes as an underlying predisposing factor (22). The use of corticosteroids exacerbates hyperglycemia and elevates available serum iron, likely resulting in increased expression of GRP78 (10, 16), which, besides acting as a co-receptor for severe acute respiratory syndrome coronavirus 2 (23, 24), binds to Coth3 invasin on inhaled *Mucorales* spores favoring nasal epithelial cell invasion and subsequent hematogenous dissemination (9, 16, 22, 25). In addition, corticosteroids are known to impair the ability of macrophages to phagocytose microbes and suppress their inflammatory response (26). Therefore, interrupting interactions between Coth fungal ligands and their host receptors has the potential to introduce a potent adjunctive therapy that will minimize host cell invasion during mucormycosis. In this context, the use of antibodies carries the promise of blocking invasion and potentially enhance immune recognition and pathogen elimination by mediating opsonophagocytic killing activity in a host that usually suffers from immunosuppression.

We previously reported the isolation of a murine anti-Coth mAb (C2) that was protective against murine mucormycosis especially when combined with currently used antifungal drugs to treat the disease (11). Although the mechanism of C2 protection was not fully characterized in that study, polyclonal antibodies targeting Coth3 were found to prevent host cell invasion and subsequent damage and enhance opsonophagocytic killing activity of phagocytes (11). To fully realize the therapeutic potential of C2, a humanization step is needed to (i) minimize immunogenicity, (ii) enable human effector functions (e.g., opsonophagocytic killing) through the human C region, and (iii) increase the antibody half-life in serum (27). Antibody humanization, first described by Jones and colleagues in 1986, involves implanting a mouse CDRs into a full human antibody framework to achieve a grafted humanized antibody with the same antigen-binding specificity (28). In certain cases, loss of recognition of the antibody binding target occurs because of the introduction of the human sequences. In this case and to fully restore the binding affinity, additional contact residues in the human frame must be mutated back to the mouse residues (29). Our humanization process with back mutations to 10 amino acid residues resulted in isolating a humanized clone that avidly binds to Coth3 by ~10-fold

higher than the murine C2 and has a binding affinity similar to a chimeric mAb. There are several factors that can contribute to the enhanced binding affinity of the humanized VX-01 and chimera antibodies, including that (i) the human Fc region used in VX-01 and the chimera antibodies can influence overall binding by providing better stability and a more optimal conformation for the Fab region, potentially enhancing binding affinity, and that (ii) the glycosylation patterns on the Fc region can affect the overall structure and function of the antibody. Human cells such as CHO cells often produce antibodies with different glycosylation patterns compared with mouse cells (30). Although the enhanced binding of VX-01 was only achieved by mutating 10 amino acids, it is prudent to mention that US Food and Drug Administration–approved mAbs have up to 16 framework mutations (31).

VX-01 was able to block *R. delemar*–induced HUVEC injury and vascular permeability in vitro, processes that are critical in mucormycosis pathogenesis (20). In the neutropenic mouse model, VX-01 appears to protect mice from mucormycosis by reducing the ability of *R. delemar* to angio-invade as shown by the reduced expression of VEGF, a marker of angiogenesis and microvascular permeability (17), in the lungs of mice treated with VX-01 compared with that in lungs treated with isotype-matched control IgG1. These results were corroborated with reduced lung fungal burden in VX-01–treated mice, which reduced the numbers of lung macrophages (Fig. 7, C to E). In the DKA mouse model, it appears that the mechanism of VX-01–mediated protection against mucormycosis might constitute both prevention of angiogenesis and augmenting of opsonophagocytic killing activity, as demonstrated by enhanced detection of total MPO in lungs and spleens of DKA mice coupled with enhanced expression of the MPO gene in live neutrophils isolated from both organs (Fig. 7, H and I). It is prudent to mention that the number of neutrophils detected in organs harvested from VX-01–treated or isotype IgG1–treated mice was similar (fig. S6), hence suggesting that the augmented expression of *Mpo* gene in live neutrophils from VX-01–treated mice is followed by enhanced secretion of MPO to the infection milieu in a mechanism that is primarily not reliant on NETosis (32, 33).

Although the immunomodulatory function of VX-01 is expected to be maximized in individuals who have some immune effector functions (e.g., DKA hosts or immunocompetent patients with trauma), neutropenic hosts maintain tissue macrophages that are derived from embryonic and not hematopoietic origin (34). Thus, it is possible that opsonophagocytic killing activity of tissue resident phagocytes might be modulated by VX-01 in neutropenic hosts. The ability of VX-01 to prevent invasion and damage to host cells and to enhance opsonophagocytic killing activity is a feature maintained from the murine antibodies and is likely critical for demonstrating improved efficacy in combination with antifungal activity. It is thought that the reduction of tissue infarction by diminishing the ability of *Mucorales* to invade host tissues and the reduction of the tissue fungal load through opsonophagocytic killing activity are essential for adequate delivery of antifungals to the site of infection.

The field of therapeutic antibodies has experienced rapid growth and widespread application in treating cancer, autoimmune diseases, and infectious diseases (35). Invasive fungal infections pose a persistent threat, necessitating new therapeutic options. mAbs have emerged as promising candidates for eliminating microbial pathogens, as evidenced by our findings demonstrating enhanced neutrophil and peritoneal macrophage activity against *R. delemar* in vitro

and in vivo. Unlike bacterial and viral infections, fungal infections lack efficient vaccines because of their occurrence predominantly in patients who are severely immunocompromised, making it challenging to develop autologous protection through vaccination. Hence, passive immunization using mAbs remains a promising approach, particularly for mucormycosis, which exhibits resistance to most antifungal drugs and high mortality rates. We found that the combination of humanized mAbs with antifungal drug treatments improved survival of mice and eliminated fungal presence in the lungs and brain, where antifungal drugs alone proved ineffective.

Before advancing to human clinical trials, it is imperative to assess the functionality and potential therapeutic effects of the humanized antibody within a relevant in vivo model (36). Mice are commonly used as a preclinical model because of their physiological similarities to humans and the availability of mouse strains with different genetic backgrounds (37). Previous research has shown that human IgG subclasses exhibit comparable relative affinities for mouse Fc $\gamma$  receptors (Fc $\gamma$ Rs) as they do for their human counterparts, suggesting that preclinical assessment of human IgG1 in mouse models may mimic Fc $\gamma$ R-mediated effector functions (38). Consequently, evaluating the humanized antibody's functionality in mice has become a standard practice in preclinical research and drug development (39). The PK studies showed that the 30- $\mu$ g therapeutic dose of either murine C2 or humanized VX-01 has a serum half-life of ~4 days, which is consistent with the reported half-life of IgG in mice (40). Further, despite the murine C2 antibody having more than two times higher concentration in the elimination phase compared with VX-01, both treatments resulted in similar survival rates (Fig. 4B). This may be attributed to the 10-fold increase in the binding affinity of VX-01 to CoH3 antigen on Mucorales when compared with the murine C2 antibody (compare Fig. 1A for C2 and Fig. 2C for VX-01). It is anticipated that VX-01 (an IgG1) is likely to have a serum half-life of ~3 weeks in humans (41, 42). Thus, with the higher binding capacity of VX-01, it is anticipated that this antibody could be dosed in humans once every 3 to 4 weeks when given with antifungal therapy at a low dose of about 1.25 mg/kg. Given that our manufacturing processes indicated that the transfected CHO cells can produce VX-01 in titers close to 2 to 3 g/liter, a 200-liter fermentation run can produce at least 5000 doses of the antibody, assuming an average patient of 60 kg.

Mechanisms of antibody resistance include altered antigen expression or binding, impaired complement-mediated cytotoxicity or antibody-dependent cellular cytotoxicity, altered intracellular signaling effects, and inhibition of direct induction of cell death (43). Despite these challenges, mAbs offer promise as targeted therapies because of their high specificity and reduced toxicity. Our studies of passaging *R. delemar* in the presence of VX-01 for 20 generations and then evaluating the binding ability and therapeutic efficacy of VX-01 against the 20th generation indicate that resistance to VX-01 is a low probability. This could be attributed to the fact that VX-01 has no static or cidal activity against Mucorales fungi and, therefore, is less likely to exert any pressure on the fungus.

Furthermore, as the number of mAbs in the market grows, the safety profile of these antibodies, such as host cross-activity and immunogenicity, becomes increasingly important (44). VX-01 had no damaging effect on HUVEC cells even at the high concentration of 50  $\mu$ g/ml, which still effectively prevented *R. delemar* invasion and HUVEC damage. Further, human tissue cross-reactivity studies showed a favorable safety profile of weak or moderate binding of

VX-01 to only cytoplasmic proteins without any indication of binding to cell surface proteins. Although this result was not expected because CoH antigens are present only in Mucorales fungi and absent from human cells, cytoplasmic protein binding is considered of little toxicologic importance because of the limited ability of antibody therapeutics to access the cytoplasmic compartment in vivo (45, 46). GLP toxicity and rat tissue cross-reactivity studies are now underway to fully evaluate the safety of VX-01 before advancing it to clinical trials.

Some limitations to the study exist. Although we have shown that the mouse C2 mAb protects against murine mucormycosis due to *Rhizopus*, *Mucor*, and *Lichtheimia* (albeit to, a lesser extent, probably due to the lower expression of CoH3 in *Lichtheimia*) (11), here, we only showed efficacy of VX-01 in treating murine mucormycosis due to *R. delemar* or *M. circinelloides*. Additionally, the productivity of VX-01 in CHO cells can be improved to higher titers of  $\geq 5$  g/liter. This would be beneficial for clinical use. Last, although our studies of passaging *R. delemar* in VX-01 for 20 generations studies showed that fungal resistance to VX-01 did not develop, clinical resistance of *R. delemar* or other Mucorales fungi to VX-01 cannot be entirely ruled out.

In conclusion, our study highlights the efficacy and reliability of the humanized antibody targeting CoH3 proteins as a therapy, particularly when combined with antifungal drugs, for combating mucormycosis. This research is especially timely, given the surge in mucormycosis cases in India during the COVID-19 pandemic. Future research efforts will focus on optimizing gene-encoded mAb technology in small and large animal models, using fed-batch or perfusion modes to achieve multi-gram production, and developing delivery systems for clinical use based on feedback from large animal data. These endeavors aim to pave the way for improved treatment options and better patient outcomes in the battle against invasive fungal infections.

## MATERIALS AND METHODS

### Study design

This study aimed to evaluate the efficacy and safety of humanized mAbs targeting Mucorales fungi. The humanization process involved cloning and humanizing the variable regions of the C2 mAb, which was originally derived from a mouse hybridoma. The resulting humanized antibodies were subjected to a series of in vitro and in vivo tests to assess their binding affinity, functional activity, and therapeutic potential. The sample sizes for in vitro and in vivo experiments were determined on the basis of prior studies and power analyses to ensure reliable effect measurements, with at least two experiments conducted in vitro and 6 to 10 mice per group in vivo. Data collection was guided by predefined end points such as survival times and fungal burden measurements, with stopping rules in place for early termination upon achieving primary end points or detecting adverse effects. The primary and secondary end points were prespecified, and statistical corrections for multiple comparisons were applied. Experimental design included controlled laboratory experiments with randomization of treatment groups and blinding during outcome assessment and data analysis to minimize bias. Randomization was used for group assignments, and blinding was used to ensure objective measurement and analysis of results.

### Study approval

All procedures involving mice were approved by the Institutional Animal Care and Use Committee of the Lundquist Institute for

Biomedical Innovations at Harbor–University of California at Los Angeles (UCLA) Medical Center, according to the National Institutes of Health guidelines for animal housing and care (protocol nos. 11671 and 056151). Human endothelial cell and neutrophil collection was approved by the Institutional Review Board (IRB) of the Lundquist Institute for Biomedical Innovations at Harbor–UCLA Medical Center. Because umbilical cords are collected without donor identifiers, the IRB considers them medical waste not subject to informed consent. Blood collection for neutrophil harvest was obtained after the volunteer signing an IRB-approved consent.

### Organisms and culture conditions

*R. delemar* 99-880 (formerly classified as *Rhizopus oryzae*) and *M. circinelloides* f. *jenssenii* UTHSCSA DI15-131 are clinical isolates obtained from the Fungus Testing Laboratory at the University of Texas Health Sciences Center at San Antonio (UTHSCSA). *Cunninghamella bertholletiae* 182, also a clinical isolate, was a gift from T. Walsh (Center for Innovation Therapeutics and Diagnostics, Richmond, Virginia, USA). *Lichtheimia corymbifera* is another clinical isolate obtained from the Deferasirox–AmBisome Therapy for Mucormycosis (DEFEAT Mucor) clinical study (47). Last, *Rhizomucor* was obtained from a patient seen at the Harbor–UCLA Medical Center. Growth conditions and enumeration of cells and germination were previously described (11, 48).

### Cloning the V<sub>H</sub> and V<sub>K</sub> regions, humanization of the C2 mAb, and antibody production

The C2 mAb was isotyped to IgG1 kappa LC. Thus, a reverse primer based on the mouse kappa LC constant region was used for 5′ rapid amplification cDNA ends (5′ RACE) PCR to amplify the LC variable region. A reverse primer based on the mouse HC constant region was used for 5′ RACE PCR to amplify the V<sub>H</sub>. The amplified PCR products were cloned into a TOPO vector for sequencing analysis. To confirm the hybridoma sequencing result, the N-terminal protein sequencing was performed by Creative Proteomics using Edman degradation on the HCs and LCs of the murine mAb, which had been separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Alignments of the murine V<sub>H</sub> and V<sub>K</sub> regions with human germline antibody variable regions were performed to identify the best frameworks as acceptors for the murine C2 V<sub>H</sub> and V<sub>K</sub>. On the basis of the alignment analysis, the human VH4-59\*01 was selected for the HC humanization, and the A18 V<sub>K</sub> was selected for the LC humanization. The gene encoding the designed grafted humanized variant was custom synthesized by Bio Basic.

A chimera and a grafted humanized IgG were then constructed and generated by AvantGen. The cloned V<sub>H</sub> was fused with human IgG1 coupled with a V<sub>K</sub> fused to the human kappa constant region to generate the chimera. The grafted humanized version was fused with the selected human antibody frameworks and mouse antibody CDRs. For this, the DNA encoding the V<sub>H</sub> variable region was cloned into Bam HI and Apa I sites of AvantGen's pcDNA3.4-IgG1 vector, and the sequencing was confirmed. V<sub>K</sub> regions for these constructs were cloned into the Afl II and Acc65 I sites of AvantGen's pcDNA3.4-kappa vector, and the sequencing was confirmed. Large-scale plasmid preparations were prepared and used for transfecting ExpiCHO cells. Secreted antibody was purified from the culture medium 10 days post-transfection using protein A affinity chromatography. The purified antibody was analyzed by SDS-PAGE gel to determine the purity of the antibody, which was estimated to be

greater than 95% pure, and the antibody concentration was determined using ultraviolet absorbance at 280 nm.

### Biolayer interferometry

The purified preparations of the chimera and grafted humanized antibodies were then used to assess the affinity of each clone for the Coth3 antigen by BLI using the Gator system (ProbeLife). A protein G–coated sensor probe (160006, ProbeLife) was loaded with the bivalent antibody test clone at a concentration of 5 µg/ml in ProbeLife's kinetic buffer [phosphate-buffered saline (PBS) (pH 7.4), 0.02% bovine serum albumin (BSA), 0.002% Tween 20, and 0.005% sodium azide (NaN<sub>3</sub>)]. A serial dilution of Coth3 antigen of 400, 200, 100, 50, 25, 12.5, 6.25 nM and buffer only (0 nM) were added to the sensors. All reactions were performed at 25°C. The values were chosen when three or more concentrations yield the same kinetic values. The original murine anti-Coth3 C2 was used as the reference antibody.

### Back mutations in the grafted humanized variable regions

To restore the binding activity of the humanized antibody, computer modeling and analysis were performed for the C2 murine mAb and humanized grafted antibodies with the selected human antibody frameworks and mouse antibody CDRs. PDB structures 1XGP (HC) and 2W60 (LC) were chosen as homology templates for the murine variable regions and 4LKX for the human framework with the grafted murine CDRs. A structural assessment of the homology models was then carried out; models were visually assessed using PyMOL (Schrödinger Inc.), and residue sequence/structure commonalities and differences between the two models (mouse and humanized V<sub>H</sub> and V<sub>K</sub>) were used to guide where back mutations might be warranted. DNA fragments encoding the humanized variants with back mutations were custom synthesized by Integrated DNA Technologies and cloned into AvantGen's antibody expression vectors. Transfection of ExpiCHO cells and antibody expression and purification were conducted as above.

### Immunoblotting

The Flag tag–fused Coth3 antigen construct in the yeast plasmid pXW55-URA3 was previously described in detail (11). For Western blotting, 0.1 µg of Flag-Coth3 or 10 µg of whole-cell lysate was used to separate proteins with SDS-PAGE. Separated proteins were transferred to PVDF membranes (GE Water & Process Technologies) and treated with Western blocking reagent (Roche) for overnight at 4°C. C2 murine IgG1 or the generated chimera, humanized, or back-mutated humanized antibodies (0.02 µg/ml each) were used as primary antibodies. After 1 hour, horseradish peroxidase (HRP; 0.2 µg/ml)–conjugated anti-human IgG (Invitrogen, catalog no. 31412) or HRP-conjugated anti-mouse IgG (Invitrogen, catalog no. G-21040) secondary antibody was added for another 1 hour at room temperature. Flag-Coth3 bands were visualized by adding the HRP substrate (SuperSignal West Dura Extended Duration Substrate, Thermo Fisher Scientific), and the chemiluminescent signal was detected using an In-gel Azure Imager c400 fluorescence system (Azure Biosystems).

### Binding of the produced antibodies to Coth proteins expressed on Mucorales

Mucorales spores were collected in endotoxin-free PBS containing 0.01% Tween 80. Collected spores were washed with PBS and then fixed in 4% paraformaldehyde. The fixed cells were washed three

times with PBS, and  $2 \times 10^6$  cells/100  $\mu$ l were used for each staining. Briefly, cell pellets (100  $\mu$ l) were blocked using 2% BSA in PBS per tube. Each antibody (20  $\mu$ g/ml) was used as the primary antibody in the blocking buffer. After 1 hour, the cells were washed three times with PBS and 0.05% Tween 80. Then, Alexa Fluor 488–IgG anti-human IgG secondary antibody (10  $\mu$ g/ml; Invitrogen, catalog no. A11013) in blocking buffer was added for another 1 hour at room temperature followed with washing three times with PBS and 0.05% Tween 80. One-milliliter samples of stained spores were analyzed using a LSR II (Becton Dickinson) instrument equipped with an argon laser emitting at 488 nm. Fluorescence emission was read with a 515/40 band-pass filter. Fluorescence data were collected with logarithmic amplifiers. The mean fluorescence intensities of  $2 \times 10^4$  events were calculated using FlowJo v10 software (16).

### Effect of antibodies on the growth of *R. delemar* in vitro and PMN killing assay

*R. delemar* spores ( $1 \times 10^5$  cells/ml) were cocultured in 96-well flat-bottom plates at 37°C for 6 or 24 hours with or without VX-01, mouse C2 antibody, isotype-matched IgG1, or medium alone and processed to determine the effect of the mAbs on the growth of the organism using XTT assay as we previously described (11). For the PMN killing assay, *R. delemar* spores were collected, washed twice with Hanks' balanced salt solution, and counted. Spore counts were adjusted to  $1 \times 10^5$  spores/ml using 1 $\times$  RPMI 1640 medium supplemented with 10% human serum (GeminiBio), and 1% penicillin/streptomycin. One hundred microliters of spores from the above stock were added to Eppendorf tubes. This was followed by adding 3  $\mu$ l of stock (100  $\mu$ g/ml) of either VX-01, VX-02, VX-03, or the isotype-matched IgG1 prepared in 1 $\times$  RPMI 1640 medium containing 10% human serum and 1% penicillin/streptomycin, and the tubes were stored on ice for 30 min. Next, the tubes were warmed to room temperature for 30 min before adding human neutrophils isolated from healthy volunteers after an IRB-approved consent form was obtained. Neutrophils were purified as described before (49), suspended in RPMI 1640 medium supplemented with 10% human serum and 1% penicillin/streptomycin, and added at 1:1 or 1:2 ratio of neutrophils to spores. Tubes were incubated at 37°C for 3 hours, followed by 1-s sonication to kill all remaining neutrophils. Tubes were then stored at 4°C overnight before serial dilution and plated on potato dextrose agar and 0.1% Triton plates. Surviving colonies were counted, and percentage of killing was determined by comparing colony counts with those collected from tubes treated similarly without the addition of neutrophils.

### Fungal adhesion, invasion, endothelial cell damage, and transwell permeability assays

The number of *R. delemar* organisms adhering to and invading HUVECs was determined using a modification of our previously described differential fluorescence assay (50). At least 100 organisms were counted in 20 different fields on each slide. One slide per arm was used for each experiment, and the experiment was performed in triplicates on different days. For the damage assay, HUVECs were collected by the method of Jaffe *et al.* (51). HUVECs were propagated and processed for *Rhizopus*-induced damage assay according to our previously described method (48). To study the effect of VX-01 on vascular permeability in vitro, HUVECs were seeded on 24-well Corning transwell plates with permeable polyester inserts (0.4  $\mu$ m, Thermo Fisher Scientific) coated with fibronectin and processed for

the effect of VX-01 on vascular permeability as we previously described using VX-01 or isotype-matched IgG1 (50  $\mu$ g/ml) (48).

### In vivo efficacy evaluation

All animal studies used either the DKA or neutropenic mouse models using male Institute of Cancer Research mice (20 to 23 g) obtained from Envigo (11). Neutropenic or DKA mice were treated with a single dose of 30  $\mu$ g of VX-01 humanized antibody or isotype-matched human IgG1 starting 24 or 48 hours after infection administered by intraperitoneal injection. Survival served as the primary end point. As a secondary end point and in some experiments, the fungal burden in the lungs and brains (primary and secondary target organs) was determined 96 hours after infection by quantitative PCR (qPCR) assay, as we previously described (18). Values were expressed as log<sub>10</sub> spore equivalent per gram of tissue. Histopathological examination was carried out on sections of the harvested organs after fixing in 10% zinc formalin. The fixed organs were embedded in paraffin, and 5-mm sections were stained with hematoxylin and eosin.

For determining the protective activity of the mAbs with antifungal drugs, neutropenic mice were infected with *R. delemar* or *M. circinelloides* (52) and treated with the VX-01 humanized antibody, isotype IgG, POSA (Merck and Co.), LAMB (Gilead Sciences), or a combination of the VX-01 and either antifungal drug starting 48 hours after infection. VX-01 was given once by intraperitoneal injection at 30  $\mu$ g, POSA was administered by oral gavage at 30 mg/kg twice daily for 7 days, and LAMB was given intravenously at 10 mg/kg per day for 4 days. Survival and tissue fungal burden served as end points as above. The MPO assay was conducted as we previously described (11).

### PK study

For the PK studies, infected neutropenic mice were intravenously injected with VX-01 or anti-CotH3 C2 antibodies at 10, 30, or 100  $\mu$ g 24 hours after infection. Mouse serum samples were collected at 0.5, 2, 4, 8, 24, 72, 120, and 168 hours after the antibody injection. The antibody concentration in the mouse serum samples was detected by enzyme-linked immunosorbent assay using plates coated with recombinant CotH3 and anti-human IgG-HRP or anti-mouse IgG-HRP (2  $\mu$ g/ml) as the secondary antibody. The mAb concentration was determined from a standard curve obtained from serum spiked with known concentrations of the antibody. The mean concentration-time data for each antibody was evaluated, and peak concentration ( $C_{\max}$ ), the area under the curve (AUC), and terminal half-life ( $T_{1/2}$ ) were calculated as was previously described (53).

### Flow cytometry analysis

DKA or neutropenic mice were euthanized at day 4 postinfection. After disrupting the excised lungs or spleen with a gentleMACs tissue dissociator (Miltenyi Biotec) to yield single cells, red blood cells were removed by treating with ACK lysis buffer (Thermo Fisher Scientific). The resulting single-cell suspension was counted, and  $\sim 2 \times 10^6$  cells were used for antibody staining. The antibodies used were brilliant ultraviolet 395 anti-CD45 (BDB564279, Invitrogen), fluorescein isothiocyanate (FITC)–Ly6G mAb (1A8–Ly6g) (50-112-2179, Invitrogen), phycoerythrin (PE)–cyanine 7 (Cy7) anti-Ly6C (BDB560593, Invitrogen), FITC anti-CD103 (11-1031-82, Invitrogen), Pacific Blue anti-CD11b (RM2828, Invitrogen), PE–Cy7 anti-CD11c (558079, BD Biosciences), and Alexa Fluor 700 anti-major histocompatibility complex class II (MHCII) (56-5321-82, Invitrogen). The

staining protocols were followed as previously described (54). Neutrophils were identified as CD45<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>low</sup>, and MPO expression in neutrophils was further stained with Alexa Fluor 647 mouse anti-mouse MPO (BDB570233, Thermo Fisher Scientific) after cell fixation using intracellular fixation buffer (501129058, Thermo Fisher Scientific) followed by permeabilization using permeabilization buffer (501129059, Thermo Fisher Scientific). Macrophages were identified as CD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>var</sup>, and dendritic cells were identified as CD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>var</sup>CD103<sup>+</sup>CD11b<sup>+</sup>. Data were acquired using a five-laser BD FACSymphony A5 and analyzed with FlowJo Software V10. The gating strategy is diagrammed in fig. S11.

## Immunohistochemistry

To evaluate the effect of VX-01 on the in vivo treatment of angioinvasion and vascular permeability, we conducted immunohistochemistry on lung sections of neutropenic mice infected with *R. delemar* and treated with a single dose of 30 µg of VX-01 or human isotype-matched IgG1 given 24 hours postinfection. Lungs were harvested on day 4 postinfection and snap frozen in optimal cutting temperature tissue embedding medium (4585, Thermo Fisher Scientific), after which 10-µm-thick sections were cut with a cryostat, dried for 1 hour, and fixed with ice-cold methanol (55). The cryosections were rinsed with PBS, blocked with 1% goat serum, and then stained with VEGF rabbit mAb at 10 µg/ml (MA5-32038, Thermo Fisher Scientific), followed by goat anti-rabbit IgG (H+L) Alexa Fluor 568-labeled secondary antibody (A-11011, Thermo Fisher Scientific). The stained slides were mounted by ProLong gold antifade reagent with 4',6-diamidino-2-phenylindole and imaged by all-in-one fluorescence microscopy (BZ-X series, Keyence). The same image acquisition settings were used to enable comparison of fluorescence intensity among the different samples. The quantification of VEGF was taken from 20 random individual fields from two mice per group through bz-X810 software.

## Statistical analysis

Individual-level data are presented in data file S1. The data were collected, graphed, and statistically analyzed using Microsoft Office 360 and Graph Pad 8.0 (GraphPad Software). All results presented in the graphs are the median ± interquartile range. *n* indicates the number of biological replicates. Data are normally distributed; survival data are consistent with proportional hazards in a Cox regression model. The unpaired, two-sided Student's *t* test was used to compare two datasets. The one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test was used to analyze the opsonophagocytic killing assay. The one-way ANOVA with Tukey's multiple comparisons tests was used for tissue fungal burden analyses. The nonparametric log-rank (Mantel-Cox) test was used to determine differences in mouse survival times. *P* < 0.05 was considered significant.

## Supplementary Materials

### The PDF file includes:

Figs. S1 to S11

Tables S1 to S3

### Other Supplementary Material for this manuscript includes the following:

Data file S1

MDAR Reproducibility Checklist

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