The monoclonal antibody Ca37, developed against *Candida albicans* alcohol dehydrogenase, inhibits the yeast *in vitro* and *in vivo*

Aitziber Antoran1, Leire Aparicio-Fernandez2, Aize Pellon1,4, Idoia Buldain1, Leire Martin-Souto1, Aitor Rementeria1, Mahmoud A. Ghannoum2, Beth Burgwyn Fuchs3, Eleftherios Mylonakis3, Fernando L. Hernando1✉ & Andoni Ramirez-Garcia1

*Candida albicans* is a commensal yeast able to cause life threatening invasive infections particularly in immunocompromised patients. Despite the availability of antifungal treatments, mortality rates are still unacceptably high and drug resistance is increasing. We, therefore, generated the Ca37 monoclonal antibody against the *C. albicans* alcohol dehydrogenase (Adh)1. Our data showed that Ca37 was able to detect *C. albicans* cells, and it bound to Adh1 in yeast and Adh2 in hyphae among the cell wall-associated proteins. Moreover, Ca37 was able to inhibit candidal growth following 18 h incubation time and reduced the minimal inhibitory concentration of amphotericin B or fluconazole when used in combination with those antifungals. In addition, the antibody prolonged the survival of *C. albicans* infected-*Galleria mellonella* larvae, when *C. albicans* was exposed to antibody prior to inoculating *G. mellonella* or by direct application as a therapeutic agent on infected larvae. In conclusion, the Ca37 monoclonal antibody proved to be effective against *C. albicans*, both *in vitro* and *in vivo*, and to act together with antifungal drugs, suggesting Adh proteins could be interesting therapeutic targets against this pathogen.

*Candida albicans* is a commensal microorganism typically present in the mucosal and gastrointestinal tracts of humans. However, in some cases, it can become pathogenic, causing diseases that range from superficial mucosal infections to invasive candidiasis, which comprises bloodstream infection (candidemia) and/or deep-seated candidiasis1. *Candida* spp. are among the most isolated pathogens in hospital settings and in fact, *C. albicans* is the fungus most associated with nosocomial infections, with a mortality rate that can reach up to 50%2. Due to the increasing number of immunocompromised patients and the aging of the population, it is tempting to speculate that in the following years infections due to this fungus may increase.

Currently, only four antifungal classes are available against invasive candidiasis, and drug resistance has been described to all of them3. In fact, since the appearance of the echinocandins more than 10 years ago, no new class of antifungal drugs has been introduced. There is, therefore, an urgent need for new treatments to combat fungal infections4, and inhibitory antibodies are a promising avenue. Monoclonal antibodies (mAb) are being studied as a possible therapy for candidiasis and other fungal infections, either alone or in combination with antifungals5-8.

Our research group focused on the interaction between the host and *C. albicans*, and found several proteins related to yeast pathogenicity9-11. One of these, alcohol dehydrogenase (Adh), despite its metabolic role in the cytoplasm, has been identified as an allergen and antigen12 that is also located on the cell wall13-15, where it may play a role as an adhesin, binding the host's fibronectin16 and serum plasminogen17. *C. albicans* Adh1

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1Fungal and Bacterial Biomics Research Group. Department of Immunology, Microbiology and Parasitology. Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Leioa, Spain. 2Department of Dermatology and Center for Medical Mycology, Case Western Reserve University, and University Hospitals Cleveland Medical Center, Cleveland, Ohio, USA. 3Division of Infectious Diseases, Rhode Island Hospital, Warren Alpert Medical School of Brown University, Providence, Rhode Island, USA. 4Present address: Aize Pellon, Centre for Host-Microbiome Interactions, Mucosal and Salivary Biology Division, King’s College London Dental Institute, London, United Kingdom. ✉E-mail: fl.hernando@ehu.eus
protein has also been associated with the promotion of cancer because it catalyzes the production of carcinogenic acetaldehyde\textsuperscript{18–21}.

Therefore, considering the importance of the Adh1 of \textit{C. albicans} and the fact that the homology between the human and fungal Adh1 is low (32% of identity, as compared by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), a tool for gene and protein sequence alignment comparison), the aim of this work was to produce and study the effect of an anti-Adh1 (Ca37) mAb on the growth and viability of \textit{C. albicans}, and to test its potential use in therapy.

**Results**

**Characterization of the specificity of the Ca37 monoclonal antibody.** \textit{C. albicans} specificity of the antibody was examined on cytosolic and cell wall-associated protein (CWP) fractions of the fungus and of THP-1 human monocyte cells. (d) Recognition of the Ca37 over the yeast cell wall-associated protein fraction of other \textit{Candida} species. 2-DE western blotting of \textit{C. albicans} (e) yeast and (f) hyphal cell wall-associated proteins. The identified spots are marked in red. (g) Growth curves of \textit{C. albicans} using four different media: minimum media (MM) with different glucose concentrations (0.5%, 1% and 2%) and Sabouraud Dextrose broth (SDB). The time of yeast cell collection is marked in red. (h) Expression of the cell wall-associated proteins recognized by Ca37 in exponential and stationary phase grown in the mentioned four media. The red arrow marks the experimental molecular weight of the Adh1 protein. Blots have been cropped in order to facilitate the comparison among them; full-length blots are presented in Supplementary Figs. 1 and 2.
yeasts and hyphae (Fig. 1b). The Ca37 antibody did not bind to any THP-1 cytosolic protein (Fig. 1c), and regarding the ability to bind other Candida species, the mAb recognized bands from Candida parapsilosis, Candida glabrata and Candida auris CWP fraction (Fig. 1e), although the pattern was different in each species. Only C. albicans yeast and hyphal CWP fractions were used for two-dimensional electrophoresis (2-DE) and WB analysis, because of the importance of this fraction owing to its closer association with the surrounding medium. In this fraction, the most immunoreactive spots were identified as alcohol dehydrogenase (Fig. 1f). However, the most immunoreactive spot from the yeast extract was identified as Adh1, whereas the one in hyphal extract was determined to be Adh2 (Table 1).

On the other hand, to test the expression of the C. albicans Adh1 in different media and growth phases, CWP from yeasts collected in exponential (8 h) and stationary phases (27 h) using 4 different media (minimal medium (MM) supplement with 0.5%, 1% and 2% glucose and Sabouraud Dextrose Broth (SDB), Fig. 1g) were analysed (Fig. 1h). Adh was present in all the textured conditions. A differential expression of some bands was observed in different conditions, being, in general, more reactive in stationary phase, but no significant differences were found. The original WB of all the blots presented in Fig. 1 can be found in Supplementary Figs. 1 and 2.

In addition, an indirect immunofluorescence (IIF) assay was carried out to study whether the fungal proteins identified on the CWP fraction were accessible to antibody recognition on the cell surface (Fig. 2). Ca37 bound to C. albicans only when cells were pre-treated with sodium metaperiodate or when the incubation time was extended to 18 h, indicating that the mAb needs the elimination of the carbohydrates achieved by oxidation with metaperiodate or extended incubation times in order to penetrate the carbohydrate layer of the cell wall and detect the Adh proteins. In the second case (18 h incubation), the antibody was able to bind both yeast and hyphal cells, although not every cell was bound by the mAb, nor was the entire cell surface covered by the mAb (Fig. 2c), indicating low epitope availability. We also performed the IIF assay over an ADH1 null mutant strain,22 but in both cases (with metaperiodate treatment and extended time of incubation) a recognition of the Ca37 mAb was observed (Fig. 2d,e, respectively) in a similar way than in its wild type (data not shown) and the control strain.

**In vitro assessment of Ca37 antibody.** After confirming antibody recognition, the effect on C. albicans NCPF 3153 and CECT 13062 strains growth was tested. Data showed that exposure to Ca37 had no significant effect on fungal growth (Fig. 3a,b). It is possible that active yeast division could hamper the action of the antibody during growth; therefore, inhibition was also studied by incubating C. albicans with the mAb in Dubelcco’s phosphate buffer saline (DPBS) prior to counting the colony-forming units (CFUs) grown in agar plates. In this case, a significant reduction in C. albicans CFU numbers was observed, 10 μg/mL being the most effective mAb concentration, which achieved a maximum of 90% inhibition (P = 0.001 in the case of NCPF 3153 strain and P < 0.0001 in the case of CECT 13062) (Fig. 3c,d). Therefore, this concentration was used with the rest of the strains, achieving an inhibition of between 70 to 90%, while the immunoglobulin (Ig) G1 isotype control exhibited no effect (Fig. 3e). The effect of Ca37 on yeast germination was also analysed. Although only three of the five strains showed a significant inhibition, the germination of all strain was inhibited between 6 and 20% (Table 2).

Since Ca37 demonstrated inhibitory activity towards C. albicans, a possible synergy with current standard of care antifungal agents was investigated. The combined effects with antifungals that target different structures was explored: amphotericin B inhibits ergosterol synthesis and fluconazole targets cytochrome P450. Although the CECT 13062 strain was already susceptible to amphotericin B (The European Committee on Antimicrobial Susceptibility Testing-EUCAST breakpoints for antifungals, v. 9.0) the addition of the antibody reduced the minimum inhibitory concentration (MIC) value of amphotericin B to half, from 0.5 to 0.25 mg/L (Fig. 4a). In the case of fluconazole, the concentration of the antifungal needed to inhibit 50% of the growth of UPY 93–153 fluconazole-resistant strain was also reduced by half (from 32 to 16 mg/L) in the presence of 20 and 10 μg/mL of Ca37 (Fig. 4b). Fractional inhibitory concentration index (FICI) values were 0.55 for the combination of the anti-Adh1 with amphotericin B and 1 with fluconazole, both cases showing an additive effect.

**Effect of the Adh1 on fungal virulence, and effect of Ca37 antibody against the yeast in vivo.** The role of C. albicans Adh1 in virulence and the ability of the Ca37 antibody to inhibit this pathogen were tested in vivo using Galleria mellonella as an invertebrate infection model (Fig. 5). To establish the infection profile of an ADH1 mutant strain, larvae were initially infected with an ADH1 knockout strain, which was found to be less virulent, with prolonged survival compared to the DAY286 parental strain (P = 0.0198; Fig. 5a). The larvae infected with the ADH1 single allele revertant strain also showed an increased survival rate (P = 0.035) compared to the parental strain.

To study the inhibitory effect of the antibody, C. albicans (CECT 13062) cells were pre-treated with Ca37 before their inoculation. A fluconazole control and an IgG1 isotype control were also included (Fig. 5b). Data showed that the antibody was able to significantly reduce the mortality of C. albicans infected larvae (P = 0.0001),

| Spot no. | Accession no. | Protein name | Matching peptides | Coverage (%) | MASCOT score | Theor. pI/Mr | Exper. pI/Mr |
|----------|---------------|--------------|-------------------|--------------|--------------|-------------|-------------|
| 1        | P43067.1      | Alcohol dehydrogenase 1 | 5 | 16 | 368 | 6.02/37 | 5.1/46 |
| 2        | KGU32026.1    | Alcohol dehydrogenase 2 | 2 | 6 | 128 | 6.25/37 | 6.31/40.5 |

Table 1. LS/MS-MS identification of the protein spots detected by Ca37 monoclonal antibody in the cell wall-associated protein fraction western blots.

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the mean survival time being 4–5 days longer than the \textit{G. mellonella} group receiving non-treated yeast. The effect on the survival of the highest Ca37 concentration was only one half-day shorter than that of fluconazole. As expected, the IgG1 isotype control did not have any effect ($P = 0.3173$).

\textbf{Figure 2.} Visualization of the Ca37 monoclonal antibody-mediated recognition of \textit{Candida albicans} yeast and hyphae through indirect immunofluorescence. \textit{C. albicans} NCPF 3153 strain incubated (a) for 1.5 h with the Ca37 monoclonal antibody, (b) for 1.5 h with the Ca37 monoclonal antibody after treatment with 50 mM sodium metaperiodate and (c) for 18 h with the Ca37 monoclonal antibody. \textit{C. albicans} ADH1 knockout strain incubated (d) for 1.5 h with the Ca37 monoclonal antibody after treatment with 50 mM sodium metaperiodate and (e) for 18 h with the Ca37 monoclonal antibody.
To test the therapeutic effect of Ca37, the mAb was injected immediately after infecting the larvae with *C. albicans*. As seen in Fig. 5c, *G. mellonella* treated with Ca37 or fluconazole survived a day longer than those larvae not treated with the mAb after *C. albicans* inoculation. The difference between non-treated larvae and larvae treated with Ca37 was statistically significant ($P = 0.0052$). Thus, showing that the mAb was able to inhibit the *C. albicans* infection in the infection model, an encouraging finding.

**Discussion**

Fungi present a particularly challenging pathogen to combat given that, like human cells, they are eukaryotic; and finding viable cellular targets that do not have adverse effects on the host is difficult. Proteins belonging to the cell wall of fungal pathogens have been studied as potential target for antifungal therapies, since this structure is not present in human cells. Alcohol dehydrogenase is a cytoplasmic enzyme, needed for glucose and ethanol processing, and plays an essential role in yeast metabolism; although, its role in the fungal cell wall is still unclear. It has been described previously as an antigen and adhesin\(^{12,16}\), but it may also play other roles yet to be elucidated.
In fact, other metabolic proteins such as enolase have also been found on the cell wall and studied as therapeutic targets and even as a vaccine. Recently, monoclonal antibodies have been studied as alternative therapies in order to overcome the toxicity of current antifungal agents and the emergence of resistance to them. In fact, one anti-\textit{Candida} mAb, named Mycograb, directed against heat shock protein 90, was developed, and showed a positive outcome in patients suffering from invasive candidiasis in combination with amphotericin B. Unfortunately, due to concerns over its safety for use in humans, its application was discouraged in Europe. This does not, however, mitigate the development of new mAbs against new or alternate fungal targets.\textit{C. albicans} Adh has recently been associated with a high immunogenic capacity and the conversion of monocytes into pro-inflammatory macrophages. In addition, Adh inhibition may play a role in the reduction of fungal proliferation. Therefore, an anti-Adh1 monoclonal antibody, Ca37, was developed against \textit{C. albicans} Adh1 protein and its effect on the viability of the yeast studied.

The mAb developed in this study specifically recognized Adh1 on yeast and Adh2 on hyphae CWP fraction. These two proteins share an 84% homology; therefore, the detection of these similar proteins is probably due to cross-reactivity. This result contrasts with those found by others, such as Pitarch et al., who found Adh1 in both yeast and hyphae CWP fraction, whereas Adh2 was detected in the cytosolic extract of hyphae. In this study, we found that neither Adh1 nor any other human protein was detected by Ca37 antibody, as observed by western blot analysis.

**Table 2.** Germination inhibition (in percentage) of five different \textit{Candida albicans} strains in the presence of 20 and 10 \(\mu\text{g/mL}\) of Ca37 monoclonal antibody. Statistically significant differences in comparison to control (no mAb added), \(P\) value for NCPF 3153 (10 \(\mu\text{g/mL}\)) was 0.02, for UPV 15–170 (20 \(\mu\text{g/mL}\)) 0.013 and for UPV 15–171 0.002 in the case of 20 \(\mu\text{g/mL}\) and 0.02 in the case of 10 \(\mu\text{g/mL}\). \(N = 3\) in all cases. All data were normally distributed, therefore one way ANOVA was used with Bonferroni’s (for homogenous variances) or Tamhane’s T2 (for non-homogenous) corrections for multiple comparisons. Alpha value was set to 0.05 at least. \(*P \leq 0.05\), \(**P \leq 0.01\).

**Figure 4.** Additive effect of Ca37 monoclonal antibody with antifungals. (a) CECT 13062 strain and amphotericin B and (b) UPV 93–153 strain and fluconazole were used. MIC values are marked in red. The grey scale indicates the rate of inhibition, being higher in the case of light grey (100 to 40% of inhibition) and lower in the dark one (20 to 0% of inhibition).
which may be related to the fact that Adh2 has been described to be expressed only in the stationary phase28,29.

not being great differences, our results show more reactivity in stationary phase of cells grown in some media,

compensate the absence of Adh1.

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of the epitope is partially obscured by carbohydrates, and considering the high immunogenicity recently

prior removal of carbohydrates, suggesting that these molecules prevent mAb binding. However, after an incubation of 18 h the antibody was able to bind both fungal morphologies, although not uniformly. This may indicate that the epitope is partially obscured by carbohydrates, and considering the high immunogenicity recently described for this protein the hypothesis seems plausible25. On the other hand, the ADH1 null mutant was recognized by the antibody in spite of its lack of Adh1 protein. The ability of the Ca37 to bind hyphae is not surprising, as we found that Adh2 is present in the CWP fraction of that morphology. However, the unexpected recognition of the yeasts would need further investigation to elucidate whether Adh2 might be overexpressed in order to compensate the absence of Adh1.

The activity of the mAb against C. albicans could not be observed on actively growing yeasts, but a small inhibition of 20% in germination was found. This contrasts with a recent study26 showing a decreased hyphal formation by an ADH1 null mutant. However, in this article, the authors suggest that the inhibition of hyphal formation is due to a decreased intracellular ATP content and the suppression of a signalling pathway controlling various virulence factors, which would be related to the cytosolic role of the Adh1, whereas Ca37 antibody will presumably act only upon Adh1 protein present in the cell wall. On the contrary, when the yeast was incubated with the mAb in DPBS before inoculation onto Sabouraud Dextrose agar (SDA) plates, the inhibition percentages reached 70 to 90% compared to the control. Other authors have achieved similar rates of inhibition against C. albicans.
using monoclonal antibodies (63%, 74–92% and 55–100%), but the mAb concentration used was between 2.5 to 10 times higher than the concentration tested in this study31–33.

As the use of antifungal drugs at high doses can lead to hepatic and nephrotoxicity, synergistic ability between Ca37 and commonly used antifungals was also tested. It was found that the MIC values for amphotericin B and fluconazole were reduced to half in the strains used. Therefore, the antibody showed an additive effect, which might be of high relevance for clinicians, reducing high dose-associated adverse effects of antifungal drugs. It was shown that ADH1 is upregulated in yeast treated with fluconazole34, which could explain the observed effect of the mAb. In the case of the fluconazole-resistant strain, this effect was not enough to reduce the in vitro MIC to a therapeutically successful concentration (2 mg/L or less35). However, the antibody might render some strains with intermediate susceptibility more sensitive to the action of this antifungal. Nevertheless, this concept needs to be tested and confirmed. Other studies have already shown that a combination of a mAb and an antifungal drug can enhance the survival of mice infected with Candida1.

In the in vivo assessment, it is worth noting that the fungal density used was much higher than the one used for the in vitro tests, but larvae possess hemocytes and antimicrobial peptides that help them overcome infections36, and to survive to high inoculums of C. albicans (10⁴ yeast/larva)37. In these assays, the importance of the Adh1 for fungal virulence was shown, as G. mellonella larvae infected with a C. albicans ADH1 null mutant strain exhibited prolonged survival in comparison to the larvae infected with the parental strain. The group treated with the single allele revertant strain also lived longer than the group infected with the parental strain but it was slightly more pathogenic than the null mutant strain. In this strain, only one allele of the ADH1 gene was reintroduced into the knockout mutant38, which may account for the failure to reach parental strain pathogenicity and fall as an intermediate. Song et al.39 also found that Adh1 is important for C. albicans pathogenicity, as they showed that survival of larvae, but also mice infected with an ADH1 null mutant, was increase in comparison to the infected with the wild type strain.

Then, the ability of Ca37 to increase larvae survival was explored, both as pre-treatment of the yeasts before infection and as treatment for larvae. In both cases, an increase of G. mellonella survival was observed, by 5 days in larvae injected with pre-treated yeasts, and one day in mAb treated larvae after C. albicans inoculation, compared to larvae injected with the control fungus. This enhanced survival rate was similar to that observed with fluconazole treatment in both cases. It may also be that a higher or additional dose of the mAb may provide additional protection as a therapeutic. In the future, it would be interesting to study the additive effect of Ca37 with fluconazole, to determine whether a lower dose of drug could be protective in combination with the mAb, or whether G. mellonella infected with a fluconazole resistant strain could be recovered in the presence of Ca37.

Despite the promising results showed in this study, several questions should be addressed in the future. It would be interesting to deepen into the role of the Adh2 in virulence by constructing an ADH2 and a double ADH1/ADH2 null mutant, to elucidate whether the lack of Adh1 is compensated by Adh2, to study the effect of the Ca37 mAb in opsonization and phagocytosis, and to confirm the protective effect of the Ca37 mAb in a mice model.

In conclusion, the results presented here show that the Ca37 mAb, developed against Adh1 protein, is able to inhibit the growth of C. albicans in vitro, and to improve the survival of G. mellonella in vivo. Moreover, it acts in an additive way with amphotericin B and fluconazole, leading to a reduction of the MIC by half compared to the control. Therefore, although further research is needed to corroborate its efficacy in animal models and define its mechanism/s of inhibition, this study, together with studies of other authors, indicates that C. albicans Adh1 may represent an interesting therapeutic target and, as it does not bind to human Adh1 protein, that the Ca37 mAb is a candidate for further in-depth studies as an alternative and safe treatment.

**Methods**

**Microorganisms and human cell line culture.** Nine Candida albicans strains were used in this study: NCPF 3153, a reference strain; CECT 13062 strain, isolated from systemic candidiasis; UPV 93–153, a fluconazole resistant strain; UPV 15–170, UPV 15–171 and UPV 15–172 strains, isolated from oral candidiasis and kindly provided by Cristina Marcos-Arias from the Department of Immunology, Microbiology and Parasitology of the University of the Basque Country (UPV/EHU); and DAY286, ADH1 knockout and revertant strains, obtained as described in Muhkherjee et al.22. In addition to C. albicans, other three Candida species were used, C. parapsilosis ATCC 22019, C. glabrata ATCC 90030 (kindly provided by Dr. Dolores Moragues from the UPV/EHU) and C. auris CJ-197, isolated form an outbreak in La Fe University and Polytechnic Hospital (Valencia), which was a kind gift from Dr. Javier Pemán. The use of clinical strains from patients that had previously signed an anonymous informed consent form was approved by the Ethics Committee of the University of the Basque Country. All isolation methods were carried out in accordance with relevant guidelines and regulations.

For obtaining yeast cells, an overnight culture was inoculated with 10⁵ blastospores/mL in SDB and grown at 37°C with agitation. Yeasts were always grown in this medium unless otherwise stated. For supporting the growth of the DAY286 and its derived ADH1 knockout and revertant strains, histidine (100 μg/mL) was added to SDB. Hyphae were obtained by incubating yeast cells in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) for 4h at 37°C with agitation.

THP-1 human monocyte cell line were obtained from the American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% FBS and L-glutamine (2 mM), penicillin (10000 U/mL), streptomycin (10 μg/mL), amphotericin B (25 μg/mL) and β-mercaptoethanol (0.05 mM), in a 5% CO₂ atmosphere at 37°C. Subcultures were made when cell density reached 10⁶ viable cell/mL.

**Production of Adh1 recombinant protein and anti-Adh1 (Ca37) monoclonal antibody.** The C. albicans CECT 13062 ADH1 gene was cloned into pETBlue-2 vector (Novagen, Madison, WI, USA) and introduced into Escherichia coli Turner (DE3) pLac cells (Novagen). Protein expression was induced by addition of
1 mM IPTG and purification was performed by affinity chromatography (HisTrap FF crude kit, GE Healthcare, Chicago, IL, USA). Finally, the obtained Adh1 was treated with Polymyxin B for endotoxin removal.

The production of the anti-Adh1 Ca37 mAb was carried out in BALB/c mice by the hybridoma production technology at Abystek Biopharma Company (Technology Park, Zamudio, Spain), using purified Adh1 for the immunization process. Briefly, splenocytes were fused with Sp2/0 myeloma cells and the most reactive clones were selected by enzyme-linked immunosorbent assay (ELISA). The antibodies were purified by protein G chromatography. For this study, an IgG1 isotype-producer hybridoma was selected.

In order to avoid antibody aggregates, the mAb was treated using a thermal-cycling method as in Sadavarte and Ghosh. Infrared and filtered before use.

**Growth curves of yeast cells in different media.** *C. albicans* CECT 13062 yeast cells where incubated at 10° cell/mL in four different media; minimal medium supplemented with 0.5% glucose, 1% glucose and 2% glucose and in SDB. Minimal medium was composed of yeast nitrogen base (1.34%) and Biotin (0.02%). The yeasts were incubated at 37°C and the 600 nm absorbance (Nanophotometer, Implen, Munich, Germany) was measured each hour. Absorbance data was translated into cell density. Yeast cells were harvested by centrifugation at 10,000 xg for 10 min at the middle of the exponential phase (8 h since the beginning of the incubation) and at the stationary phase (27 h since the beginning of the incubation). Yeast cell pellets were stored at −20°C until further use.

**Protein extraction, electrophoresis (SDS-PAGE and 2-DE), western blotting and Liquid chromatography–mass spectrometry (LC-MS/MS) identification.** Protein extraction was performed as described by Pitarch et al. Briefly, yeast or hyphal cells were mixed with glass beads and disrupted using a Millimix 20 BeadBeater cell disruptor (Technika, Zeleznik, Slovenia) for 20 min at 30 Hz. Then, they were centrifuged in order to separate the cytosolic fraction (supernatant) from the cell wall (pellet). This pellet was treated with SDS extraction buffer for 10 min at 100°C, then centrifuged and the supernatant was collected to obtain the CWP fraction. In the case of THP-1 cells, the followed procedure was similar, but with slight changes. Specifically, they were disrupted only for 2 minutes in the BeadBeater, centrifuged and only the soluble fraction was recovered for analysis.

For the SDS-PAGE, protein samples were loaded into 10% acrylamide gels and run at 70 mA, 100 W and 200 V for 45 min in a MiniProtean II (Bio-Rad, Hercules, CA, USA), using the Page Ruler Plus as protein standard (Thermo Fisher Scientific, Waltham, MA, USA). For 2-DE, the protocol described by Pellon et al. was followed, but using 7 cm long Immobiline Drystrip gels (GE Healthcare) for the isoelectric focusing (IEF), under the following conditions: rehydration for 12 h, 500 V for 2000 Vhr, 1000 V for 3000 Vhr, 5000 V for 10 000 Vhr, and 5000 V for 40000 Vhr. The proteins were then separated by SDS-PAGE. For protein identification, 2-DE gels were stained with Coomassie Brilliant Blue G250.

To carry out the WB, proteins were transferred to Amersham Hybond-P PVDF membranes (GE Healthcare) for 1 h at 400 mA. Then, WB was performed using 30 µg/mL of Ca37 mAb at 37°C for 1.5 h as primary antibody and an anti-mouse IgG-HRP diluted 1:100,000 for 45 min as secondary. Membranes were developed using the ECL prime (GE Healthcare) in a G:BOX Chemi system (Syngene, Cambridge, United Kingdom). All the incubation steps were carried out at room temperature unless otherwise indicated. The analysis of 2-DE and WBs was carried out by image analysis using ImageMaster 2D Platinum Software (GE Healthcare).

The selected protein spots were manually excised from Coomassie Brilliant Blue G250 stained gels and identified by LC-MS/MS in the proteomics service of the UPV/EHU, SGIker, as described in Pellon et al.

**Indirect immunofluorescence.** Fungal cells were used for detection using Ca37 by IIFs. They were carried out on cells treated with sodium metaperiodate (50 mM) to remove cell wall carbohydrates, and on non-treated cells. Then, 100 µg/mL of Ca37 mAb at 37°C for 1.5 h or at 4°C for 18 h were added as primary antibody, and an anti-mouse IgG-FITC (4 µg/mL) diluted in PBS containing 0.05% (w/v) Evans Blue and 0.05% (v/v) Tween 20 for 30 min at 37°C as secondary. Cells were observed under a fluorescent Eclipse Ni microscope (Nikon, Tokio, Japan), and images taken with a Zyla sCMOS camera (Andor, Belfast, UK).

**Inhibitory effect of Ca37 monoclonal antibody on fungal growth and germination.** For testing the effect of the antibody on the growth of *C. albicans*, 100 µL of 1.5 x 10³ yeast/mL in SDB were added to each well of a 96-well plate. SDB alone was used as blank and *C. albicans* without mAb was included as a growth control. Ten microliters of the antibody were added in a range from 10 µg/mL to 1 pg/mL. The plates were incubated at 37°C and the absorbance at 600 nm measured every 10 min for 24 h (Synergy HT, BioTek, Winooski, VT, USA). Absorbance data from three different experiments were used and translated into densities.

To test the ability of the mAb to inhibit non-actively growing yeast, the procedure described by Manglani et al. was followed. Briefly, 1.5 x 10³ yeast/mL were pre-incubated in DPBS for 18 h at 37°C at 120 rpm with the same range of antibody concentrations mentioned above. Then, 100 µL of each treatment were inoculated onto SDA plates, in triplicate, so that the CFUs could be counted.

To study the effect of the Ca37 on *C. albicans* germination, suspensions of yeasts at 1.5 x 10³ yeast/mL were incubated in RPMI 1640 supplemented with 10% FBS, alone or with 20 or 10 µg/mL of mAb for 4h at 37°C and 120 rpm. Then, cells were fixed with 10% formalin saline and the percentage of germinated yeast was estimated by counting with an inverted optical microscopy.

**Measure of Minimal Inhibitory Concentration (MIC).** The effect of Ca37 mAb in combination with amphotericin B and fluconazole was studied following the guidelines of EUCAST using *C. albicans* CECT 13062 and fluconazole-resistant UPV 93–153 strains, and 20, 10, 2, 1 and 0 µg/mL concentrations of antibody.
The plates were incubated for 24 or 48 h at 37 °C, and absorbance recorded at 530 nm. The FICI was calculated using the derived MICs according to Bardbari et al.19.

Insect infection studies. In this study G. mellonella infection model was used. Sixth-instar larvae (Grubco Company, Fairfield, OH, USA or Bichosa and Mundo Gusano, Vitoria, Spain) were injected with 10^6 cells of C. albicans in 10 μL PBS through the last right pro-leg. Sixteen larvae were used per group, and two negative control groups were included: non-injected and PBS injected group. Larvae were incubated at 37 °C and checked daily over 7 days44. At least two independent experiments were performed (n ≥ 32).

Initially, DAY286, ADH1 knockout and an ADH1 revertant strain were used, following the procedure described above, to study the effect of the absence of the ADH1 gene on yeast virulence. To observe the effect of the mAb, yeasts were treated for 18 h at 37 °C with Ca37 (20 and 10 μg/mL), a non-specific IgG1 isotype control from murine myeloma (Sigma-Aldrich, 10 μg/mL), fluconazole (13.86 μg/mL) or DPBS before using the treated C. albicans to inoculate G. mellonella. Conversely, we also studied the therapeutic effect of Ca37 by infecting larvae with C. albicans in the last right pro-leg followed by provision of 10 μL of the mAb (12 mg/kg), fluconazole (14 mg/kg) or PBS in the last left pro-leg. Once all the larvae were infected, C. albicans (pre- and not pre-treated) cells were inoculated into SDA in order to obtain a CFU count and make sure that 10^6 cells/larva had been provided to all the groups.

Statistical analysis. All data are presented as mean ± standard error. The statistical analyses were carried out using IBM SPSS statistics 22 (Professional Statistic, Chicago, IL, USA). Normality of data was checked using the Shapiro Wilks test and homogeneity of the variance using the Levene test. In the case of the G. mellonella survival curves, Mantel-Cox test in GraphPad Prism software was used. Alpha value was set at 0.05, and the exact P values for each statistical analysis are showed in the text or figure legends.

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Author contributions
A.A. has participated in design of the study, acquisition and data analysis and drafting of the article. L.A.F., A.P., L.M.S. and have participated in data acquisition. I.B. has participated in data acquisition and analysis. A.R., M.A.G., and B.B.F. have participated in conception and design of the study. E.M., F.L.H. and A.R.G. have participated in conception and design of the study, and data analysis. All authors have critically revised the article and approved the final version.

Competing interests
The authors declare no competing interests.

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