Development and validation of monoclonal antibodies specific for *Candida albicans* Als2, Als9-1, and Als9-2

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**Abstract**

Fungal agglutinin-like sequence (Als) cell-surface glycoproteins, best characterized in *Candida albicans*, mediate adhesive and aggregative interactions with host cells, other microbes, and abiotic surfaces. Monoclonal antibodies (MAbs) specific for each *C. albicans* Als protein are valuable reagents for gaining insight into Als protein localization and function. This manuscript describes development and validation of MAbs specific for *C. albicans* Als2, as well as for *C. albicans* Als9-1 and Als9-2, two protein variants produced from the ALS9 locus. Native *C. albicans* ALS9 expression levels were not sufficiently high to produce detectable Als9 protein on the wild-type cell surface so MAb validation required production of overexpression strains, each featuring one of the two ALS9 alleles. An anti-Als2 MAb was raised against an N-glycosylated form of the protein immunogen, as well as an Endoglycosidase H-treated immunogen. The MAb raised against the N-glycosylated immunogen proved superior and immunolabeled *C. albicans* yeast cells and germ tubes, and the surface of *Candida dubliniensis* and *Candida tropicalis* yeasts. Als2 was visible on *C. albicans* yeast cells recovered from a murine model of oral candidiasis, demonstrating Als2 production both in vivo and in vitro. These new MAbs add to the collection of anti-Als MAbs that are powerful tools to better understand the role of Als proteins in *C. albicans* biology and pathogenesis.

**Introduction**

Agglutinin-like sequence (Als) proteins are large, cell-surface glycoproteins that function in adhesive and aggregative interactions [reviewed in 1]. *Candida albicans* has eight distinct ALS loci (ALS1 to ALS7, ALS9); many species in the Saccharomycetales have at least one ALS gene [2]. Als proteins have a secretory signal peptide at the N-terminal end and a GPI anchor addition site at the C-terminal end that direct their processing and modification via the secretory pathway to a final localization linked to β-1,6-glucan in the fungal cell wall [3]. The structure of the Als adhesive N-terminal (NT) domain was solved by x-ray crystallography and features a peptide-binding cavity that is required for ligand recognition [4, 5]. Als adhesive interactions involve host cells, other microbes, and protein-coated surfaces such as denture or catheter...
materials [1]. Als aggregative interactions are mediated by an amyloid-forming region that is part of the N-terminal domain [6]. Although the general NT-domain structure appears to be conserved among Als proteins across a wide variety of fungal species [2], sufficient sequence variation exists to use the NT domain to raise monoclonal antibodies (MAbs) that specifically recognize individual Als proteins.

Previous reports documented use of \textit{Pichia pastoris}-produced Als NT protein immunogens to raise murine MAbs against several of the \textit{C. albicans} Als proteins [7–10]. Here, we report development and validation of anti-Als MAbs that augment the collection. Protein immunogens included \textit{C. albicans} Als2 [11, 12], as well as Als9-1 and Als9-2, two proteins that are 84% identical in the NT domain, produced from the \textit{C. albicans} \textit{ALS9} locus [7, 13, 14]. The \textit{ALS9} alleles are widespread among the \textit{C. albicans} clades [13]. Because the Als9-2 NT domain functions in adhesion to cultured human vascular endothelial cells but the Als9-1 NT domain does not [14], availability of specific anti-Als9-1 and anti-Als9-2 MAbs will benefit future analyses. This report also features initial use of the new MAbs to provide further insight into the dynamics of the \textit{C. albicans} Als family.

**Materials and methods**

**Monoclonal antibodies**

Methods for raising anti-Als MAbs were published previously [7] and are reproduced briefly here for the reader’s convenience. CUG-codon-corrected \textit{C. albicans} Als NT domain fragments were cloned into pPIC3 and transformed into \textit{Pichia pastoris} GS115 (Invitrogen). Sequences from coordinates 1 to 984 of \textit{ALS2} (GenBank accession number AF024582), \textit{ALS9-1} (AY269423), and \textit{ALS9-2} (AY269422) were used to direct synthesis of hexa-His-tagged Als NT domain fragments (amino acids 18–328) that were secreted using their own signal sequence. Proteins were purified from culture supernatant using ammonium sulfate precipitation and His-Trap column chromatography (GE Healthcare). Proteins were dialyzed in phosphate-buffered saline and concentrations determined using the Micro BCA Protein Assay kit (Thermo Scientific). Endoglycosidase H (Endo H; Roche) was used according to manufacturer’s instructions to remove the N-linked carbohydrate.

Proteins were used to immunize BALB/c mice intraperitoneally [7]. Splenic lymphocytes were fused with murine myeloma tumor cells (SP2/0) and selected in hypoxanthine aminopterin thymidine medium. Enzyme-linked immunosorbent assays were used to screen for antibody production and for specificity of the antibody for the immunogen and none of the other Als proteins [7]. Western blotting was used for additional confirmation of MAb specificity [7]. The MAb raised against the glycosylated (non-Endo-H-treated) Als2 immunogen was named 2-C6. The anti-Als2 MAb raised against the deglycosylated Als2 immunogen was named 2-C7. Anti-Als9 immunogens were both Endo H-treated prior to immunizing the mice. Anti-Als9-1 was named 9-C4; anti-Als9-2 was named 9-A7. All MAbs described here were IgG1 with a kappa light chain as determined using the Monoclonal Antibody Isotyping Kit (Pierce).

**Biacore analysis of MAb-antigen binding affinity**

A Biacore 3000 SPR instrument with version 4.1 control software was used for protein binding kinetics and affinity measurements. The CM5 sensor chip, amine coupling kit, rabbit anti-mouse Fc antibody (RamFc), glycine solutions (10 mM, pH 1.5 and pH 2.0), sodium acetate (10 mM, pH 5.0), 0.5 N NaOH, and 10% Surfactant P20 were purchased from GE Healthcare (Piscataway, NJ). HBS-EP running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.0005% Surfactant P20) was made in-house. Als protein fragments were made and purified as described by Coleman et al. [7].
The CM5 chip was docked in the Biacore 3000, normalized with 70% glycerol and cleaned twice with 0.5 N NaOH by injection at 20 μl/min flow rate for 30 sec before ligand surface preparation. RamFc was covalently immobilized to the CM5 surface by performing the standard amine coupling procedure, increasing surface response units (RU) by approximately 5,000 RU. Anti-Als MAb (50 μg/ml) were then captured to the surfaces by manual injection at a flow rate of 5 μl/min until about 350 RU net increase was achieved. Control flow cells had no anti-Als MAb.

Als proteins were assayed for binding to their corresponding MAb for kinetics and affinity measurements. For each binding-assay cycle, a protein of known concentration was injected to flow cells with or without MAb (control surface) simultaneously at a 20 μl/min flow rate with 2 min association and various lengths of dissociation time using the kinject command. After the dissociation phase, the reaction surface was regenerated to remove antibody-antigen complex from the sensor chip surface by injecting 10 mM glycine, pH 1.75 for 30 sec. The Als2 (Endo H-treated) protein was an exception because it dissociated from the MAb surface completely after 10 min dissociation thus no glycine regeneration was used and no MAb recharge was needed between each binding cycle. The integrated fluidic cartridge (IFC) and injection needle were washed between assay cycles if glycine was used.

Analyte concentration series and dissociation time for each assay were: 35, 70, 140, 280, 560 and 1120 nM, and 5 min dissociation for the Als9-2 assay; 3.9, 7.8, 15.6, 31.3, 62.5 and 125 nM, and 5 min dissociation for Als9-1 assay; 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 μM, and 10 min dissociation for Als2 (Endo H-treated) assay; 16.9, 33.9, 67.5, 135, 271, 1843 nM, and 5 min dissociation for Als2 (glycosylated) assay. Running buffer instead of analyte was injected at the beginning, in the middle, and at the end of each analyte concentration series assay. The resulting buffer blanks were used to correct bulk shift and other instrument noise during data analysis. Each assay was repeated three times.

Biacore sensorgrams were analyzed using BIAevaluation software (version 4.1). Analyte binding curves subtracted from controls were trimmed and aligned by performing X- and Y-transformations. The curves were adjusted from blanks during Y-transformation. Curve fitting was conducted by using simultaneous $k_d$ and $k_a$ fitting with the 1:1 binding model with mass transfer. The resulting parameters from three replicates were used to produce the mean and standard deviation for each assay. Chi-square values were calculated for the average squared residual per data point to judge the closeness of fit of the data to the predicted model with Chi-square values ideally < 2.0.

**ALS2 promoter deletion and reintegration constructs**

Previous difficulty deleting the second *C. albicans* ALS2 allele from the SC5314 strain background led to attempts to control ALS2 expression by integrating inducible promoters in place of the remaining ALS2 promoter in strain 1443 (ALS2/als2Δ-ura3) [12]. Integration of the MAL2 promoter led to a strain with considerably decreased ALS2 expression [12]. The tetracycline-regulatable (Tet) promoter described by Nakayama et al. [15] was also considered for use. Attempts to construct a tetracycline-regulatable ALS2 allele were unsuccessful, but led to strain 2757, derived from strain 1443 [12]. Strain 2757 featured deletion of one ALS2 allele and integration of a disruption cassette in place of the ALS2 promoter upstream of the second ALS2 allele. The strain had nearly unmeasurable ALS2 transcription (see below). This section details construction of strain 2757 and its companion strain (2801) into which a functional ALS2 promoter was restored to drive expression of one ALS2 allele.

Restriction enzymes were purchased from Thermo Fisher Scientific or New England Biolabs and used according to manufacturer instructions. PCR screening of transformant
candidates used *Taq* polymerase (Thermo Fisher Scientific). PCR amplifications for cloning used *Pfu* (proofreading) or *Pfu* Turbo polymerase (Stratagene). Agarose gel purification of PCR products and restriction fragments used 0.7% agarose/Tris Acetate-EDTA gels (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). Gel fragments were excised and DNA purified using the GeneClean III kit (MP Biomedicals). Ligations used T4 DNA Ligase (Thermo Fisher Scientific) according to manufacturer instructions. Ligations were transformed into *Escherichia coli* TOP10 competent cells (Thermo Fisher Scientific) and selectively plated onto Luria Broth agar (per liter: 10 g tryptone, 10 g sodium chloride, 5 g yeast extract with 15 g Bacto agar to solidify the medium) supplemented with the appropriate antibiotic, typically ampicillin (Sigma-Aldrich) at 100 μg/ml. Yeast transformations used a spheroplast method [16]; transformants were selected on plates of synthetic complete medium without uridine (SC-Uri) [17] containing 1 M sorbitol. Transformants were grown in SC-Uri liquid medium and genomic DNA extracted using the MasterPure Yeast DNA Purification kit (Epicentre). Southern blotting used the Genius system (Roche). Accuracy of constructs was checked by Sanger DNA sequencing at the Roy J. Carver Biotechnology Center, University of Illinois Urbana-Champaign.

The region upstream of *ALS2* was amplified from *C. albicans* strain SC5314 [18] genomic DNA with primers 2TR-AKpnIF and 2TR-AXhoIR (Table 1). The 1004-bp fragment, which included the 1003 bp upstream of *ALS2* and the A of the *ALS2* ATG start codon, was designed to delete 1003 nt upstream of *ALS2* following transformation of the construction cassette. PCR products were digested with restriction enzymes *Kpn*I and *Xho*I, then agarose-gel purified. The fragment was cloned into vector p99-CAU1 [15] that had been digested with the same enzymes. The resulting vector was called p99-CAU1-flankingA.

The 5’ end of the *ALS2* gene was amplified from SC5314 genomic DNA using primers 2TR-BNotIF and 2TR-BSstIR (Table 1). The 976-bp fragment included -6 to 970 of the *ALS2* coding region. PCR products were digested with restriction enzymes *Not*I-*Sst*I, agarose-gel purified, then cloned into p99-CAU1-flankingA that had been similarly digested. The resulting plasmid, 2685 Table 1. Primer sequences used in *C. albicans* strain constructions.

| Primer Name | Primer Sequence (5’–3’) |
|-------------|-------------------------|
| 2TR-AKpnIF  | CCC GGT ACC TGA TTT ATC TAC GGA GAT AGC |
| 2TR-AXhoIR  | CCC CTC GAG AGT CTT GTC TGG TTT GGT TIG |
| 2TR-BNotIF  | CCC GCG GCC GCT TTC AAA TGC TTT TAC AAT TTG |
| 2TR-BSstIR  | CCC GAG CTC AAC CGT TAG AAT TAG CTT CAC |
| URA-XhoIF   | TTT CTC GAG GTG AAT TGT AAT ACG ACT CAC |
| URA-SmaIR   | TTT CCC CGG GAG GTC GAC GGT ATC ATG AAG C |
| ALS2upF     | CCC CCT AGG GAT GTT TGC ATT ACG CTT TGG |
| ALS2upR     | CCC CTC GAG CCA TAA GGT TGT TGA AAA CAT C |
| 2pRC-HindF  | CCC GAG CTC CAA GTG TTA AAT AGC CTA CAG |
| 2pRC-SstIIF | CCC GCG CCC AAC AAA CCA GAC AAG ACT C |
| 2pRC-SstIR  | CCC GAG CTC CAG CAG TAG TAC TGT CAG TAA CTT |
| 9LA-XhoF    | CCC CTC GAG ATG CTA CCA CAA TCT CTA TGG |
| ALS9XbalR   | CCC TCT AGA TTA AAT AAA CAA AAA TAA TAT TGT GAC C |
| 9SA-XhoF    | CCC CTC GAG ATG CTT CCA CAA TCC ATG TTA |
| 9BglR       | CCC AGA CTC TTA AAT AAA CAA AAA TAA TAT TGT |
| RT-ALS9F    | CAT CAT TGG TGT CTA CAA CAG CTG |
| RT-ALS9R    | GAA CCC TTT GTT TCT GAA TAT GGA |

https://doi.org/10.1371/journal.pone.0269681.t001
(p99-CAU1-2TR) encoded (in linear order) upstream ALS2 sequences, URA3, the tetracycline-regulatable promoter, and the 5\' ALS2 region.

The URA3 gene in plasmid 2685 was replaced with a copy of URA3 flanked by direct repeats so that the Ura marker could be recycled when integrating a wild-type copy of the ALS2 promoter. The URA3 fragment flanked by direct repeats (URA3-dpl200) was amplified by PCR from plasmid pDDB57 [19] using primers URA-XhoIF and URA-SmaIR (Table 1). PCR products were digested with XhoI and SmaI, agarose gel purified and GeneCleaned as described above. The fragment was cloned into XhoI-SmaI-digested plasmid 2685. The resulting plasmid was named 2717.

Plasmid 2717 was digested with restriction enzymes SmaI and NotI to remove the tetracycline promoter from the plasmid. After agarose gel purification, the fragment was treated with Pfu polymerase to create blunt ends. The blunt ends were religated and the vector transformed into E. coli TOP10. The resulting plasmid was named 2756 and contained the ALS2 promoter deletion cassette. The cassette was released with KpnI-SstI digestion, agarose gel purified and GeneCleaned. Approximately 10 μg of purified cassette was transformed into C. albicans strain 1443 spheroplasts. Transformants were selected on SC-Uri plates with 1 M sorbitol.

Transformants were streaked on SC-Uri plates for purification and grown in liquid SC-Uri for genomic DNA extraction. Restriction-enzyme-digested genomic DNA was Southern blotted with the ALS2 promoter fragment amplified using primers ALS2upF and ALS2upR (Table 1). A strain that was lacking the ALS2 promoter and that had the same growth rate as the control strain (CAI12) [20] was identified and named 2757. Zhao et al. [16] described the method for growth rate analysis.

A different transformation cassette was constructed to reintegrate the wild-type ALS2 promoter into strain 2757. An ALS2 upstream flanking fragment was amplified from SC5314 genomic DNA using primers 2pRC-HindF and 2TR-AXhoIR (Table 1). PCR products were digested with HindIII and XhoI then agarose gel purified and GeneCleaned. The fragment was cloned into HindIII-XhoI-digested plasmid pUL [16]. An ALS2 promoter and partial coding region fragment was amplified from SC5314 genomic DNA using primers 2pRC-SstIIF and 2pRC-SstIR (Table 1). The PCR product was digested with SstII-SstI, agarose gel purified and GeneCleaned, then cloned into the growing vector that was digested with the same enzymes. The resulting plasmid contained the ALS2 promoter reintegration cassette that was released with HindIII-SstI digestion.

C. albicans strain 2757 was plated on agar medium containing 5-fluoroorotic acid to select for excision of the URA3 marker [21]. Resulting colonies were analyzed by Southern blot as described above. The ura` strain was named 2796. The ALS2 promoter reintegration cassette was transformed using the spheroplast method and transformants selected as described above. Strain 2801 was verified as correct using Southern blotting and demonstrated to have the same growth rate as CAI12 [16].

Construction of ALS9 overexpression strains

Information presented here details construction of strain 3299 in which ALS9-1 expression is driven by the TPI1 promoter; the construct was integrated into the C. albicans ALS9 locus. Details are also provided for construction of strain 2965 in which ALS9-2 expression is driven by the TPI1 promoter with the construct integrated at the RP10 locus. Overexpression strains were needed to produce sufficient cell-surface quantities for Als9-1 or Als9-2 to validate specificity of the anti-Als9 MAb described here. General methods for PCR amplification, restriction enzyme digestion, transformation, and screening of transformants were presented above.

The ALS9-1 overexpression construct was built in plasmid 1105 [22] which was derived from CIp10 [23]. ALS9-1 was amplified from a cloned, sequence-verified copy in plasmid
Amplification used primers 9LA-XhoF and ALS9XbaIR (Table 1). The PCR product was digested with XhoI-BglII and cloned into similarly digested plasmid 1105. The XhoI site in plasmid 1105 was downstream of the TPI1 promoter; the BglII site was downstream of the RP10 sequence. The resulting vector (3297) was linearized with BstZ17I, which cut at nt 884 of the ALS9-1 coding region. The linearized plasmid was transformed into C. albicans CAI4 [24]. The correct construct (3299) had ALS9-1 expression driven by the TPI1 promoter, terminated with the native ALS9-1 termination signal, followed by URA3, the ampicillin resistance gene and ColEI origin from Clp10 integrated at the ALS9-1 locus.

Plasmid 1499, which consisted of ALS9-2 cloned into the pCRBlunt vector (Invitrogen), was used as the template for PCR amplification of ALS9-2 with primers 9SA-XhoF and 9BglR (Table 1). The fragment was digested with XhoI-BglII, gel purified, and cloned into plasmid 1105 [22] that had been digested with the same enzymes. Transformants were screened by PCR with primers RT-ALS9F and RT-ALS9R (Table 1). The resulting plasmid was named 2920. Plasmid 2920 was linearized with NcoI which cut in the RP10 sequence. Transformation of the linearized plasmid into C. albicans strain CAI4 directed the overexpression construct to the RP10 locus. The correct transformant was identified by Southern blotting and named 2965.

**Immunolabeling C. albicans cells**

Methods for immunolabeling C. albicans cells were described by Coleman et al. [7]. Endo H treatment of cells aided immunolabeling visualization of Als proteins. For Endo H treatment, C. albicans cells were recovered from culture by centrifugation and washed twice in DPBS. Cells were resuspended in 50 mM potassium acetate, pH 5.0 and 1 × proteinase inhibitor cocktail (Roche catalog number 11836153001) in a total volume of 0.3 ml. Endo H (0.05 unit; Roche catalog number 11088726001) was added and the tube placed in a 360-degree rotator for 5 h at 37˚C. Following incubation, cells were washed twice with DPBS and fixed with 3% paraformaldehyde for 10 min. Fixed cells were washed twice with DPBS and stored in DPBS at 4˚C until used for immunolabeling.

For some experiments, the C. albicans cell surface was covalently labeled with Alexa Fluor 594 according to the method of Coleman et al. [8]. Labeling of the inoculum cells prior to in vitro culture growth was used to distinguish them from cells of subsequent generations in the culture flask population.

**Murine model of oral candidiasis**

The murine model of oral candidiasis, and recovery and preservation of C. albicans cells from the murine infection, were described by Coleman et al. [10]. Use of animals was reviewed and approved by the University of Illinois Institutional Animal Care and Use Committee under protocol number 11197. Mice were euthanized by pentobarbital sodium injection. All efforts were made to minimize animal suffering.

**Real-time RT-PCR assays**

Real-time RT-PCR analysis was used to measure the effect of ALS2 deletion on ALS4 expression. Primers and a detailed method were reported in Zhao et al. [12]. Absolute quantification of transcript copy number was also described in that report.

**Bioinformatics resources**

The Candida Gene Order Browser (cgob.ucd.ie) [25] was used to detect C. albicans ALS gene orthologs as identified by synteny (conserved chromosomal location) in other Candida
species. Sequence alignments and calculation of percent identity used Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo) [26]. Oh et al. [2] lists the amino acid sequences for the protein immunogens used to raise the various MAbs, as well as the corresponding N-terminal domain sequences across the Als family in *C. dublindiensis*, *C. tropicalis*, and other closely related fungal species.

**Results**

**Monoclonal antibody specificity and characteristics**

Endo H-treated Als9-1 (amino acids 18–328) and Als9-2 (amino acids 18–328) were used as immunogens to raise specific mouse MAbs. Specificity of each MAb for its respective immunogen was demonstrated by Western blotting (Fig 1A). MAbs 9-C4 and 9-A7 were used to immunolabel the surface of wild-type *C. albicans* cells grown under various combinations of conditions of growth medium (YPD, RPMI 1640), temperature (30˚C, 37˚C), time (early, log-phase, saturated cultures), and Endo H treatment without success. However, surface labeling of *C. albicans* strains that overexpressed one of the two ALS9 alleles was successful, further validating the specificity of MAb 9-C4 for Als9-1 and MAb 9-A7 for Als9-2 (Fig 1B). Detection of Als9-2 on the *C. albicans* overexpression strain surface required Endo H treatment of the cells prior to immunolabeling. Biacore measurement of antigen-antibody binding kinetics for anti-Als9-1 (9-C4) provided a dissociation constant (K\(_D\)) of 32 ± 6 nM (Chi-square = 0.3 ± 0.2). The K\(_D\) value for anti-Als9-2 (9-A7) was 71 ± 14 nM (Chi-square = 1.1 ± 0.9).

An anti-Als2 MAb was raised against the glycosylated form of the immunogen. This MAb was named 2-C6 and had a K\(_D\) of 8.2 ± 0.8 nM (Chi-square = 1.1 ± 0.6). MAb 2-C6 recognized both the glycosylated (G) and Endo H-treated (De) forms of Als2 on a Western blot and did not react with other Als protein fragments, indicating its specificity for Als2 (Fig 2A). MAb 2-C6 immunolabeling was visible on the surface of *C. albicans ALS2/ALS2* yeast cells and germ tubes but was not detected on strain 2757 which produced very little ALS2 transcript (Fig 2B). Reintegration of the wild-type ALS2 promoter to restore ALS2 transcription yielded strain 2801, for which anti-Als2 MAb 2-C6 labeling was not visible. Quantitative real-time PCR analysis of these strains was conducted using yeast cells grown in YPD for 16 h at 37˚C. Means and standard deviations of ALS2 transcript copy number from three data points on two separate days were 28615 ± 3510 for strain CAI12, 33 ± 11 for strain 2757, and 3130 ± 777 for strain 2801. Therefore, although ALS2 transcription increased in strain 2801, reintegration of the ALS2 promoter did not restore wild-type transcription levels and did not produce sufficient cell-surface protein for immunolabeling visualization.

Another attempt to raise a specific anti-Als2 MAb used the Endo H-treated form of the immunogen, resulting in MAb 2-C7. Binding kinetic analysis for MAb 2-C7 showed a K\(_D\) of 13 ± 0.8 nM (Chi-square = 0.17 ± 0.01). Although MAb 2-C7 recognized the surface of wild-type *C. albicans* cells, it also produced a slight background on strain 2757 indicating the presence of a cross-reactive epitope. Because MAb 2-C6 did not produce the background immunolabeling of strain 2757, it was considered the preferred MAb and used in subsequent studies.

ALS4 transcription was a focus for analysis in the newly created *C. albicans* strains because previous work suggested that ALS4 expression increased when ALS2 expression was compromised [12]. Real-time RT-PCR analysis showed that ALS4 was overexpressed by 1.9 ± 0.4-fold (mean and standard deviation of three separate experiments) in strain 2757 compared to CAI12 for cells grown for 16 h in YPD at 37˚C.
Fig 1. Specificity of anti-Als9-1 and anti-Als9-2 for their respective proteins. (A) Western blotting of purified NT domain fragments (amino acids 18 to 328 for each) from Als9-1 and Als9-2. The silver-stained polyacrylamide gel validated equal loading for the proteins (left panel). Western blots (middle and right panels) of identical sets of lanes were also prepared and exhibited specificity of each MAb for its antigen. (B) Immunolabeling of ALS9 overexpression strains with anti-Als9-1 MAb 9-C4 or anti-Als9-2 MAb 9-A7. Immunolabeling did not produce visible signals for wild-type C. albicans so overexpression strains were constructed. Each produced protein under control of the constitutive TPII promoter. Labeling only of cells that expressed the allele used to produce the antigen further validated specificity of the anti-Als9 MAbs. Endo H treatment of the ALS9-2 overexpression cells was required to see a positive immunolabeling signal with MAb 9-A7. The ALS9-1 overexpression strain did not require EndoH treatment to see its positive signal after immunolabeling with anti-Als9-1 MAb 9-C4. Scale bars in each figure = 10 μm.
Fig 2. Western blotting and immunolabeling of *C. albicans* cells to demonstrate specificity of anti-Als2 MAb 2-C6 for Als2. (A) Purified N-terminal fragments of Als2 (amino acids 18–328) untreated (G) or treated (De) with EndoH, Als3 (amino acids 18–329), and Als4 (amino acids 18–329) were separated by polyacrylamide gel electrophoresis and silver stained (left panel; 0.5 μg of each protein). 250 ng of each protein were run in additional lanes of the polyacrylamide gel, transferred to a Hybond-P PVDF membrane (Amersham) and Western blotted with the anti-Als2 MAb 2-C6 (raised against the glycosylated form of the protein). MAb 2-C6 recognized both the glycosylated and Endo H-treated forms of Als2, but did not recognize Als3 or Als4, consistent with the conclusion that MAb 2-C6 was specific for Als2. (B) Additional evidence for its Als2 specificity was secured by using anti-Als2 MAb 2-C6 to immunolabel the surface of ALS2/ALS2 strain CAI12 and strain 2757 which has near-zero ALS2 expression. Immunolabeling only of the wild-type cell surface suggested specificity of the MAb. The immunolabeling pattern of yeast was punctate, and only visible on a fraction of the cells in a 16-h culture. Labeling of the germ tube surface for cells grown at 37°C for 1 h in RPMI medium was diffuse. Scale bars in each image indicate 10 μm.

https://doi.org/10.1371/journal.pone.0269681.g002
Anti-Als2 immunolabeling of Candida cells

Because wild-type ALS9 transcription levels were too low to visualize Als9 cell-surface protein with the anti-Als9 MAbs, additional experimentation was not pursued. The ability to see anti-Als2 2-C6 immunolabeling prompted initial exploration of Als2 localization on C. albicans and other closely related species.

MAb 2-C6 immunolabeled the surface of some C. dubliniensis CD36 cells grown in YPD medium for 16 h at 30˚C (Fig 3A). Immunolabeling of C. tropicalis strain ATCC 201380 was...
even stronger and more-consistent, suggesting that MAb 2-C6 recognizes cross-reactive epitopes on these species that are closely related to *C. albicans*. MAb 2-C7, raised against the Endo H–treated form of Als2, did not cross-react with the *C. dubliniensis* or *C. tropicalis* strains tested above. MAb 2-C6 immunolabeled *C. albicans* yeast cells recovered from a murine model of oral candidiasis (Fig 3B) documenting *ALS2* transcription *in vivo*.

Localization of Als2 was variable among *C. albicans* cells from the same culture, as well as influenced by culture conditions. For example, on some germ tubes in Fig 3C, Als2 was displayed diffusely along the germ tube length, while on others, Als2 was focused at the junction between mother cell and germ tube. Alexa Fluor 594 labeling of inoculum cells was used to distinguish them from daughter cells as the culture population expanded. Fig 3D showed that in some yeast cells, Als2 was localized intensely at the mother cell-bud junction and that immunolabeling was more associated with the mother cell than the bud. Additional exploration is required to better understand the general trends noted in this initial study. Availability of the anti-Als2-specific MAbs will facilitate such inquiries.

**Discussion**

MAbs specific for *C. albicans* Als9-1 (9-C4) and Als9-2 (9-A7) that are capable of distinguishing between the two main variants produced by the *ALS9* locus [13, 14] were raised in mice. Two other MAbs, raised against the glycosylated (2-C6) and Endo H–treated (deglycosylated; 2-C7) form of the Als2 NT domain [7], were also created. While each of the anti-Als2 MAbs are suitably specific for Als2 in ELISA screening, MAb 2-C6 is preferred because of its negative background when immunolabeling *C. albicans* strain 2757 (Fig 2). Previous work described specific anti-Als MAbs including 1-B2 (anti-Als1) [8], 3-A5 (anti-Als3) [7], 4-A1 (anti-Als4) [10], 5-A5 (anti-Als5) [7, 9], and 6-A1 (anti-Als6) [7]. All anti-Als hybridomas listed here were deposited in the Developmental Studies Hybridoma Bank (University of Iowa; https://dshb.biology.uiowa.edu) so that they are available to the research community. These new MAbs complete the collection for the Als family in *C. albicans*, except Als7.

Attempts to raise a MAb specific for *C. albicans* Als7, using the NT domain (amino acids 19–332) produced in *P. pastoris* [7] were unsuccessful; none of the resulting antibodies were specific for Als7 when assayed by ELISA. Despite the impressive number of *ALS7* alleles that have been documented, Als7 is predicted to have a minor presence on the *C. albicans* surface due to low expression levels of the gene [27]. TaqMan measurement of *ALS7* expression levels suggest they are as low as *ALS6* expression levels in standard *in vitro* culture conditions [28].

In fact, previous work used the anti-Als6 MAb 6-A1 as a negative control in adhesion assays since its interaction with the cell surface could not be visualized by immunofluorescent microscopy [7]. It is expected that an anti-Als7 MAb would require an *ALS7* overexpression strain to visualize surface protein, much like the *ALS9* overexpression strains described here. The need for *ALS9* overexpression strains to visualize Als9 was unanticipated. For example, measurement of *ALS* transcription levels suggested similar results for *ALS2* and *ALS9* in the diploid *C. albicans* [28]. It is possible that the haploid dosage of *ALS9-1* and *ALS9-2* essentially halved protein abundance, dropping it below the detection limit for immunolabeling and fluorescent microscopy.

Work with the entire *C. albicans* anti-Als MAb collection demonstrated that it is easiest to visualize Als proteins produced from the most-highly-expressed ALS genes, confirming a positive association between transcriptional activity and protein abundance across the family. For example, Als3 is readily apparent on the length of *C. albicans* germ tubes and hyphae, consistent with strong *ALS3* expression during hypha formation [7]. Als1 covers the surface of yeast cells except bud scars and is localized proximal to the mother yeast as a germ tube emerges [8].
The burst of ALS1 expression that accompanies release of cells from a saturated culture into fresh growth medium results in strong Als1 production on the first daughter yeast, as well as on some of the granddaughters. Als1 persistence on the surface of these yeast cells during subsequent doublings leads to a heterogeneous anti-Als1 immunolabeling population in a culture flask. A similar heterogeneous cell population was observed here for anti-Als2 immunolabeling (Fig 2) although the kinetics of ALS2 gene expression and protein production remain to be defined.

Als2 was detectable on the C. albicans cell surface using anti-Als2 MAb 2-C6 although its distribution varied among individual cells and incubation conditions (Figs 2 and 3). Rare Als2-positive yeast cells were recovered from a murine model of oral candidiasis (Fig 3). Anti-Als1 immunolabeling of C. albicans cells in vivo were noted with MAbs against some of the other proteins produced from highly expressed ALS genes. For example, anti-Als3 immunolabeled C. albicans hyphae in a formalin-fixed, paraffin-embedded mouse kidney section as well as hyphae dissected from kidney tissue [7]. Als1 and Als4 were visualized on C. albicans yeast and hyphae dissected from a mouse kidney and fungal cells recovered from a murine model of oral candidiasis [10]. Compared to the limited localization of Als1 and Als4 at the mother yeast-germ tube junction on hyphae in vitro, protein was detected over greater lengths of hyphae in vivo, suggesting differences in Als protein production in vivo compared to a culture flask.

Little is known about Als2, relative to some of the other proteins in the C. albicans Als family. Fanning et al. [29] demonstrated that ALS2 (as well as ALS1 and ALS4) is repressed by the catalytic protein kinase A subunit Tpk1: expression of these ALS genes increases approximately 5- to 20-fold in a tpk1/tpk1 C. albicans strain. ALS2 overexpression makes C. albicans hypersensitive to caspofungin and results in significantly decreased cell-wall depth compared to wild-type cells. Dissection of the contributions of the Als proteins to adhesion, cell wall integrity, and cell wall structure suggested that Als2 functions in all three capacities.

Previous work indicated difficulty with deleting the second ALS2 allele to create a null C. albicans strain [12], a result echoed by Fanning et al. [29] and consistent with an essential role for Als2 in cell wall biogenesis. In the previous study [12], the maltose-inducible MAL2 promoter was placed upstream of the intact ALS2 allele in a als2/ALS2 heterozygous strain. Under non-inducing conditions, the resulting strain had ALS2 expression of 0.36 ± 0.21 fold-change compared to a wild-type control [12]. Growth in the presence of maltose showed as much as a 21-fold increase in ALS2 transcription compared to the wild-type strain. ALS4 expression increased by 3.2-fold in the PMAL2-ALS2 strain under non-inducing conditions suggesting compensatory regulation between the loci. In the present study, strain 2757 showed considerably decreased ALS2 expression and a concomitant increase in ALS4 expression, essentially confirming the previous result. Measurable ALS2 expression in strain 2757 indicates that it is not zero, so a true als2/als2 null has yet to be constructed. Despite the compensatory increase in ALS4 expression, results from Fanning et al. [29] demonstrated that Als4 does not affect caspofungin sensitivity or cell-wall depth so Als2 and Als4 do not fully overlap functionally.

The anti-Als2 MAb 2-C6 was notable because of its cross-reactivity with C. dubliniensis and C. tropicalis. Another anti-Als MAb, anti-Als4 4-A1, recognized the surface of C. dubliniensis [10]. Although detailed and accurate sequences for the ALS loci are now available for C. albicans, C. dubliniensis [30], and C. tropicalis [28], the information does not shed much light on the basis for cross-reactivity of anti-Als2 2-C6 or anti-Als4 4-A1 with C. dubliniensis and/or C. tropicalis. For example, the C. dubliniensis positional ortholog of C. albicans ALS2 is Cd64800, a gene for which an identical copy is present at another locus (Cd65010) [25, 30]. TaqMan assays cannot distinguish between these identical loci which produce extremely high expression levels in cells grown under standard in vitro culture conditions [28].
immunogen region (amino acids 18–328) [7]. C. albicans Als2 is 71% identical to the C. dubli-
niensis proteins. The C. albicans Als2 immunogen is also 74% identical to Cd64210, the protein
predicted from the ALS1 positional ortholog Cd64210 which is also expressed at a level that
would promote immunolabeling detection of the encoded protein [28]. It is unclear whether
either of these proteins is responsible for the cross-reactivity of anti-Als2 MAb 2-C6.

In C. tropicalis, CtrALS3786 is the positional ortholog of C. albicans ALS2 [25]. However,
CtrALS3786 expression levels are not particularly high and the two proteins share only 50%
identity [2, 28]. C. albicans ALS4 does not have a predicted positional ortholog in the C. tropi-
calis genome and its protein immunogen sequence is only approximately 50% identical to the
same region of the C. tropicalis proteins. Several of the C. tropicalis ALS genes are highly
expressed under typical in vitro culture conditions (e.g. CtrALS2293, CtrALS3882-1, CtrALS1028)
and may produce the protein that responsible for the cross-reactive immunolabeling signals [28].

Much remains to be learned about Als2 and other proteins in the C. albicans Als family.
The availability of MAbs to specifically recognize individual proteins, and even proteins pro-
duced by allelic variants such as ALS9-1 and ALS9-2, will aid future investigations into Als pro-
tein function and diversity of the Als family.

Supporting information
S1 Raw images. Original gel and blot images for Figs 1A and 2A.
(PDF)

Acknowledgments
We thank Liping Wang and Rachel Breitenfeld of the University of Illinois Immunological
Resource Center, part of the Roy J. Carver Biotechnology Center, for producing anti-Als2 and
anti-Als9 MAbs. We thank Liping Wang for conducting the Biacore measurements of anti-
body affinity. Sanger DNA sequencing was completed by the DNA Services Laboratory of the
Roy J. Carver Biotechnology Center. Vectors for constructing the ALS2 promoter deletion cas-
tette were a gift from Hironobu Nakayama. C. albicans strains CAI4 and CAI12 were a gift
from William Fonzi (Georgetown University). Plasmid pDD57 was a gift from Aaron Mitchell
(Columbia University).

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References
1. Hoyer LL, Cota E. Candida albicans agglutinin-like sequence (Als) family vignettes: a review of Als protein structure and function. Front Microbiol. 2016; 7: 280. https://doi.org/10.3389/fmicb.2016.00280 PMID: 27041205
2. Oh S-H, Schliep K, I森hower A, Rodriguez-Bobadilla R, Vuong VM, Fields CJ, et al. Using genomics to shape the definition of the Agglutinin-Like Sequence (ALS) family in the Saccharomycetales. Front Cell Infect Microbiol. 2021; 11: 794529. https://doi.org/10.3389/fcimb.2021.794529 PMID: 34970511
3. Kapteyn JC, Hoyer LL, Hecht JE, Müller WH, Andel A, Verkleij AJ, et al. The cell wall architecture of Candida albicans wild-type cells and cell wall-defective mutants. Mol Microbiol. 2000; 35: 601–611. https://doi.org/10.1046/j.1365-2958.2000.01729.x PMID: 10672182
4. Salgado PS, Yan R, Taylor JD, Burchell L, Jones R, Hoyer LL, et al. Structural basis for the broad specificity of host-cell ligands by the pathogenic fungus Candida albicans. Proc Natl Acad Sci USA. 2011; 108: 15775–15779. https://doi.org/10.1073/pnas.1103496108 PMID: 21896717
5. Lin J, Oh S-H, Jones R, Garnett JA, Salgado PS, Rushnakova S, et al. The peptide-binding cavity is essential for Als3-mediated adhesion of Candida albicans to human cells. J Biol Chem 2014; 289: 18401–18412. https://doi.org/10.1074/jbc.M114.547877 PMID: 24802757
6. Lipke PN, Garcia MC, Alsteens D, Ramsook CB, Klotz SA, Dufrene YF. Strengthening relationships: amyloids create adhesion nanodomains in yeasts. Trends Microbiol. 2012; 20: 59–65. https://doi.org/10.1016/j.tim.2011.10.002 PMID: 22099004
7. Coleman DA, Oh S-H, Zhao X, Zhao H, Hutchins JT, Vernachio JH, et al. Monoclonal antibodies specific for Candida albicans Als3 that immunolabel fungal cells in vitro and in vivo and block adhesion to host surfaces. J Microbiol Methods. 2009; 78: 71–78. https://doi.org/10.1016/j.mimet.2009.05.002 PMID: 19427882
8. Coleman DA, Oh S-H, Zhao X, Hoyer LL. Heterogeneous distribution of Candida albicans cell-surface antigens demonstrated with an Als1-specific monoclonal antibody. Microbiology 2010; 156: 3645–3659. https://doi.org/10.1099/mic.0.043851-0 PMID: 20705663
9. Zhao X, Oh S-H, Coleman DA, Hoyer LL. ALSS1, a newly discovered gene in the Candida albicans ALS family, created by intergenic recombination: analysis of the gene and protein, and implications for evolution of microbial gene families. FEMS Immunol Med Microbiol. 2011; 61: 245–257. https://doi.org/10.1111/j.1574-695X.2010.00914.x PMID: 21208290
10. Coleman DA, Oh S-H, Manfra-Maretta SL, Hoyer LL. A monoclonal antibody specific for Candida albicans Als4 demonstrates overlapping localization of Als family proteins on the fungal cell surface and highlights differences between Als localization in vitro and in vivo. FEMS Immunol Med Microbiol. 2012; 64: 321–333. https://doi.org/10.1111/j.1574-695X.2011.00914.x PMID: 22106872
11. Hoyer LL, Payne TL, Hecht JE. Identification of Candida albicans ALS2 and ALS4 and localization of Als proteins to the fungal cell surface. J Bacteriol. 1998; 180: 5334–5343. https://doi.org/10.1128/JB.180.20.5334-5343.1998 PMID: 9765564
12. Zhao X, Oh S-H, Yeater KM, Hoyer LL. Analysis of the Candida albicans Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. Microbiology 2005; 151: 1619–1630. https://doi.org/10.1099/mic.0.27763-0 PMID: 15870470
13. Zhao X, Pujol C, Soll DR, Hoyer LL. Allelic variation in the contiguous loci encoding Candida albicans ALS5, ALS1 and ALS9. Microbiology 2003; 149: 2947–2960. https://doi.org/10.1099/mic.0.26495-0 PMID: 14523127
14. Zhao X, Oh S-H, Hoyer LL. Unequal contribution of ALS9 alleles to adhesion between Candida albicans and human vascular endothelial cells. Microbiology 2007; 153: 2342–2350. https://doi.org/10.1099/ mic.0.2006/005017-0 PMID: 1760078
15. Nakayama H, Mio T, Nagahashi S, Kokado M, Arisawa M, Aoki Y. Tetracycline-regulatable system to tightly control gene expression in the pathogenic fungus Candida albicans. Infect Immun. 2000; 68: 6712–6719. https://doi.org/10.1128/IAI.68.12.6712-6719.2000 PMID: 11083786
16. Zhao X, Oh S-H, Cheng G, Green CB, Nuessen JA, Yeater K, et al. ALS3 and ALS8 represent a single locus that encodes a Candida albicans adhesin; functional comparisons between Als3p and Als1p. Microbiology 2004; 150: 2415–2428. https://doi.org/10.1099/mic.0.26943-0 PMID: 15256583
17. Hicks JB, Herskovitz I. Intercorversion of yeast mating types. I. Direct observations of the action of the homothallism (HO) gene. Genetics 1976; 83: 245–258. https://doi.org/10.1093/genetics/83.2.245 PMID: 17248712
18. Gillum AM, Tsay EY, Kirsch DR. Isolation of the Candida albicans gene for orotidine-5'-phosphate decarboxylase by complementation of S. cerevisiae ura3 and E. coli pyrF mutations. Mol Gen Genet. 1984; 198: 179–182. https://doi.org/10.1007/BF00328721 PMID: 6394964
19. Wilson RB, Davis D, Enloe BM, Mitchell AP. A recyclable Candida albicans URA3 cassette for PCR product-directed gene disruptions. Yeast 2000; 16: 65–70. https://doi.org/10.1002/(SICI)1097-0061(20000115)16:1<65::AID-YEA508>3.0.CO;2-M PMID: 10620776

20. Porta A, Ramon AM, Fonzi WA. PRR1, a homolog of Aspergillus nidulans palF, controls pH-dependent gene expression and filamentation in Candida albicans. J Bacteriol. 1999; 181: 7516–7523. https://doi.org/10.1128/JB.181.24.7516-7523.1999 PMID: 10601209

21. Boeke JD, LaCroute F, Fink GR. A positive selection for mutants lacking orotidine-5’-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol Gen Genet. 1984; 197: 345–346. https://doi.org/10.1007/BF00330984 PMID: 6394957

22. Green CB, Zhao X, Hoyer LL. Use of green fluorescent protein and reverse transcription-PCR to monitor Candida albicans Agglutinin-Like Sequence gene expression in a murine model of disseminated candidiasis. Infect Immun. 2005; 73: 1852–1855. https://doi.org/10.1128/IAI.73.3.1852-1855.2005 PMID: 15731087

23. Murad AM, Lee PR, Broadbent ID, Bareille CJ, Brown AJ. Clp10, an efficient and convenient integrating vector for Candida albicans. Yeast 2000; 16: 325–327. https://doi.org/10.1002/1097-0061(20000315)16:4<325::AID-YEA538>3.0.CO;2-# PMID: 10669870

24. Fonzi WA, Irwin, MY. Isogenic strain construction and gene mapping in Candida albicans. Genetics 1993; 134: 717–728. https://doi.org/10.1093/genetics/134.3.717 PMID: 8349105

25. Maguire SL, ÓhEigeartaigh SS, Byrne KP, Schröder MS, O’Gaora P, Wolfe KH, et al. Comparative genome analysis, and gene finding in Candida species using CGOB. Mol Biol Evol. 2013; 30: 1281–1291. https://doi.org/10.1093/molbev/mst042 PMID: 23486613

26. Madeira M, Park YM, Lee J, Buso N, Gur T, Madhusoodanan N, et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res. 2019; 47: W636–W641. https://doi.org/10.1093/nar/gkz268 PMID: 30976793

27. Zhang N, Harrex AL, Holland BR, Fenton LE, Cannon RD, Schmid J. Sixty alleles of the ALS7 open reading frame in Candida albicans: ALS7 is a hypermutable contingency locus. Genome Res. 2003; 13: 2005–2017. https://doi.org/10.1101/gr.1024903 PMID: 12952872

28. Oh S-H, Isenhower A, Rodriguez-Bobadilla R, Smith B, Jones J, Hubka V, et al. Pursuing advances in DNA sequencing technology to solve a complex genomic jigsaw puzzle: the agglutinin-like sequence (ALS) genes of Candida tropicalis. Front Microbiol. 2021; 11: 594531. https://doi.org/10.3389/fmicb.2020.594531 PMID: 33552012

29. Fanning S, Xu W, Beaurepaire C, Suhans JP, Nantel A, Mitchell AP. Functional control of the Candida albicans cell wall by catalytic protein kinase A subunit Tpk1. Mol Microbiol. 2012; 86: 284–302. https://doi.org/10.1111/j.1365-2958.2012.08193.x PMID: 22882910

30. Jackson AP, Gamble JA, Yeomans T, Moran GP, Saunders D, Harris D, et al. Comparative genomics of the fungal pathogens Candida dubliniensis and Candida albicans. Genome Res. 2009; 19: 2231–2244. https://doi.org/10.1101/gr.089750.109 PMID: 19745113