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(54) Title: ANTIBODIES FOR PREVENTING OR TREATING CANDIDIASIS

(57) Abstract: The invention relates to a single heavy chain variable domain antibody (VHH) that binds Ece1p, and/or a peptide derived therefrom, from C. albicans. The invention further relates to VHH that recognize cell wall proteins of C. albicans hyphae, such as Als3p. The invention further relates to a bi- or multi-specific antibody comprising such VHH and to mono- or bispecific antibody comprising such VHH fused on gene level to the Fc part of human antibodies and to methods and means for producing such VHH. The invention further relates to an antibody of the invention, for use in diagnostic applications and for use as a medicament. The invention further relates to single heavy chain variable domain antibodies that bind to adhesins of C. albicans and related pathogenic fungi. The invention further relates to an antibody of the invention, for use in diagnostic applications or for use as a medicament. The invention further relates to a microbicide comprising an antibody of the invention, and to a pharmaceutical composition comprising an antibody of the invention. Finally the invention relates to food grade lactic acid bacteria that express VHH against Ece1p or adhesins.

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Title: Antibodies for preventing or treating Candidiasis.

Field: The invention relates to the field of microbial infections. More specifically, the invention relates to single heavy chain variable domain antibodies (VHH) which bind cell wall-associated proteins, or secreted proteins, such as Ece1p and peptides derived therefrom.

1. BACKGROUND OF THE INVENTION

Candida spp. are fungal commensals which reside on epithelial surfaces of the skin and the whole digestive tract of most humans. Colonisation by *Candida* spp. is usually harmless but in case of a person treated with antibiotics, a person with a compromised immune system, or a person who have undergone surgery, they can turn into invasive pathogens. In particular *C. albicans* present in the intestine are often translocated into the bloodstream (Allert et al., 2018. mBio 9: e00915-18). Moreover, vulvovaginal candidiasis is estimated to occur in 50-75% of women in their childbearing years. Biofilm formation of *Candida albicans* on implanted medical devices is also a considerable source of candidiasis.

Candida spp. frequently become invasive in immunocompromised people (estimated 400.000 cases per year), disseminate through the bloodstream and attack the whole body with mortality rates of 46 – 75% (Brown et al.,2012. Science Transl Med 4:165rv13). Due to high prevalence and mortality rates *Candida* spp. are receiving increased scientific attention.

C. albicans is the most prevalent of the species causing invasive *Candida* infections worldwide (about 50% of all cases), and invasive *C. albicans* infection is the third most common hospital-acquired infection. (Yapar, 2014. Ther Clin Risk Manag 10: 95–105). One of the main contributors to virulence of *C. albicans* is its ability to switch from a budding yeast form to invasive filamentous growth (Mayer et al., 2013. Virulence 4: 119–128) and the expression and secretion of Ece1p by hyphae.

Recently it was shown that *C. albicans* secretion of Ece1p results in the release of a cytolytic pore-forming peptide toxin (candidalysin) during

filamentous growth (Moyes et al., 2016. Nature 532: 64-68). Before this discovery, the secretion of pore-forming cytolytic toxins among pathogenic fungi had just been described as Nep1-like proteins for necrotrophic plant pathogens, with a first description in *Fusarium oxysporum* (Oome and Van den Ackerveken, 2014. MPMI 27: 1081–1094).

Candidalysin is a 31 amino acid polypeptide that is released post-translational from the Ece1p precursor protein [Figure 1] by proteolytic cleavage during secretion via the Golgi apparatus (Richardson et al., 2018. mBio 9: e02178-17). Candidalysin is structurally related to the main component of honeybee venom, the pore forming 26 amino acid peptide toxin melittin. The 3D structures of candidalysin as well as melittin comprises two alpha-helices [Figure 2], connected by a proline at position 14, while the C-termini of candidalysin and melittin are basic (Raghuraman and Chattopadhyay, 2007. Biosci Rep 27: 189–223).

Secretion of candidalysin by *C. albicans* was found to be limited to filamentous growth and was shown to be obligatory for causing damage as well as provoking innate immune responses in mucosal animal and cell culture infection models (Moyes et al., 2016. Nature 532: 64-68; Richardson et al., 2018. mBio 9: e02178-17; Verma et al., 2017. Science Immunology 2, eaam8834).

Because candidalysin was identified to be the main virulence factor during tissue infections and for translocation of *C. albicans* to the blood stream, it represents an attractive target for raising antibodies for diagnostic or therapeutic purposes.

2. BRIEF DESCRIPTION OF THE INVENTION

Phage-display allows for targeted selection of antigen specific antibodies from antibody libraries and has been successfully applied to isolate detection and neutralisation antibodies for toxins off different origins (e. g. *Clostridium botulinum* or *C. dificile* toxins and ricin (Kuhn et al., 2016. Proteomics Clin Appl 10: 922-948). *Candida albicans* immune lama VHH libraries (the variable domains of the heavy-chains of heavy-chain only antibodies from camelids) were generated by immunisation of yeast and filamentous whole

cells of *C. albicans*. Ece1p, a precursor protein from which candidalysin is produced and released after proteolytic cleavage in the Golgi apparatus, is highly expressed during filamentous growth (Birse et al., 1993. Inf Immunity 61: 3648-3655; Moyes et al., 2016. Nature 532: 64-68; Richardson et al., 2018. mBio 9: e02178-17). Thus, we reasoned that the hyphal cells used for immunisation may comprise Ecep1, candidalysin and other Ece1p derived peptides. As a result, the VHH libraries were screened for VHH molecules that bind Ece1p and peptides derived from Ece1p, including candidalysin [see Figure 3]. As this approach worked quite well, VHH against other cell wall protein peptides and cell wall proteins were also selected in a similar set up.

Therefore, the invention provides a single heavy chain variable domain antibody (VHH) that binds to a cell wall component and/or secreted protein, such as Ece1p, and/or a peptide derived therefrom, of *Candida* spp., preferably of *C. albicans*. A preferred VHH antibody according to the invention binds and neutralizes a cytolytic pore-forming peptide toxin, termed candidalysin.

It is shown herein that indeed heavy chain only, VHH antibodies can be raised in llamas against Ece1p, candidalysin and other Ece1p derived peptides and these VHH antibodies can be selected using phage-display. Moreover, it is shown that these VHH antibodies can neutralize Ece1p derived peptides such as candidalysin and have great potency for therapeutical use.

A preferred VHH antibody according to the invention comprises CDR1, CDR2 and CDR3 amino acid sequences as depicted in Figure 4A, preferably CDR1, CDR2 and CDR3 amino acid sequences from an individual VHH antibody. A further preferred VHH antibody according to the invention comprises amino acid sequences as depicted in Figure 4B and/or Figure 4C.

A further preferred VHH antibody according to the invention binds to a non-candidalysin peptide and which antibody reduces or eliminates mammalian cell lysis that is induced by Ece1p derived peptides such as candidalysin.

A preferred VHH antibody that binds to a cell wall component of a Candida spp., binds a protein of the Als-, Hpw- and Yff/Hyr family of adhesin

molecules of *Candida* spp. preferably *C. albicans*, most preferably to adhesin Als3.

A VHH antibody of the invention may be fused to an immunoglobulin Fc region or functional part thereof, preferably derived from IgGl, IgG2, IgG3, IgG4), more preferably derived from IgG1. Said Fc region or functional part thereof preferably is human or a humanized lama Fc or functional part thereof.

The invention further provides a bi- or multi-specific antibody, such as a tri-specific antibody, comprising a VHH antibody according to the invention. Said bi- or multi-specific antibody preferably comprises at least two VHH antibodies that are non-competing and non-interfering VHH antibodies.

Said bi- or multi-specific antibody preferably comprises at least a VHH that recognize a hyphae specific cell wall protein, and a VHH that recognizes ECE1 and an ECE1 derived peptide such as candidalysin.

A further preferred bi- or multi-specific antibody protects *Galleria mellonella* against death after infection with *C. albicans*.

The invention further provides a nucleic acid encoding an antibody according to the invention. The invention further provides a method for producing an antibody, the method comprising expressing the nucleic acid of the invention in a relevant cell and recovering the thus produced antibody from the cell.

The invention further provides the antibody according to the invention for use in diagnostic applications.

The invention further provides the antibody according to the invention for use as a medicament.

The invention further provides an antibody according to the invention, for use in a method of prophylactic treatment of an immune compromised person that may become infected with *C. albicans*.

The invention further provides the antibody according to the invention for use in a method for treatment of an individual infected with a *Candida* spp.

The invention further provides a microbicide or apheresis device comprising an antibody according to the invention.

The invention further provides a pharmaceutical composition comprising the antibody according to the invention,

The invention further provides a medical device, comprising a coating that prevents adhesion and biofilm formation of *C. albicans* hyphae, said coating preferably comprising one or more antibodies of the invention.

Further provided is a host cell, preferably a lactic acid bacterium, expressing a VHH antibody of the invention. Said lactic acid bacterium can be used as a delivery system of these VHH to the gastro-intestinal tract to neutralize Ece1p, candidalysin and other factors causing translocation of *C. albicans* to the bloodstream, and/or a lactic acid bacterium that expresses VHH on its surface that may block adhesion of *Candida* spp., especially *C. albicans*, to the mucosal layer.

3. FIGURE LEGENDS

Figure 1. A cartoon representation of Ece1p and the amino acid sequences of the peptides derived from Ece1p by the action of Kex2 and/or Kex1. The peptides - fused to FLAG - used for the capturing of VHH during selection are indicated below the Ece1p-derived peptides.

Figure 2. The 3D structure of Ece1p determined with the bio-informatics tool i-Tasser and the 3D model for candidalysin is superimposed on the 3D structure of Ece1p. The Arg 81 and Arg 93 residues indicate the N- and C-termini of candidalysin, whereas the arrows indicate the α-helices of candidalysin.

Figure 3. Amino acid sequences of FLAG-tagged candidalysin peptides used for the selection of anti-Ece1p and anti-candidalysin VHH. A FLAG tag comprises the amino acid sequence DYKDDDDK.

Figure 4. Amino acid sequences of VHH antibodies. (A) Amino acid sequences of the CDR regions of the anti Ece1p and anti-candidalysin VHH of the F1 subfamily and of the H1 subfamily. (B) Complete amino acid sequences of the F1 and H1 KQREL subfamilies of anti Ece1p and candidalysin VHH. Alternative amino acid residues are indicated in brackets after an amino acid residue. (C) Amino acid sequences of anti-Als3 and anti-

Als4 antibodies. Alternative amino acid residues are indicated in brackets after an amino acid residue.

Figure 5. Dose response ELISA with VHH CAL1-F1 (A) and -H1 (B) on affinity captured candidalysin.

Figure 6. Results of ELISA with VHH-F1 (A) and VHH-H1 (B) on recombinant Ece1p. Shown is the absorbance at (490-655) nm of ELISA test samples in dependence on the VHH concentration for the VHHs CAL1-F1 (A) or CAL1-H1 (B) as a measure for binding to a candidalysin (CL), candidalysin with C-terminal (CLF) or N-terminal (FCL) FLAG tag and recombinant Ece1p (rEce1p) all of which had been coated to a polystyrene plate overnight.

Figure 7. SDS page and Western blots with recombinant Ece1p and VHH CAL1-F1 and VHH CAL1-H1.

Figure 8. Immunofluorescence of *C. albicans* during infection of epithelial cells; stained with anti-candidalysin CAL1-F1. Fluorescence is indicated by white arrows.

Figure 9. Immunofluorescence of human epithelial cells exposed to candidalysin – staining with anti-candidalysin VHH CAL1-F1 and CAL1-H1. Note the remarkable difference between these VHH. IF samples were prepared, fixed with formaldehyde and stained with anti-candidalysin VHH CAL1-F1 and CAL1-H1.

Figure 10. Cell damage of human epithelial cells through *C. albicans* or candidalysin and the inhibition of this damage by VHH CAL1-F1 and CAL1-H1. Shown is the damage in the presence of VHHs in relation to the damage detected in samples without VHHs. TR146 epithelial cells were exposed either to *C. albicans* cells (A) or to 32 µM synthetic candidalysin (B) in presence of VHHs. As a measure of cell damage the lactate-dehydrogenase release was determined. Each value represents the average and standard deviation of three technical replicates.

Figure 11. Dose response ELISA with VHH CAW-3A8 on viable *C. albicans* yeast or hyphal cells.

Figure 12. Immunofluorescence microscopy of *C. albicans* deletion mutants as indicated in Table 1 with hyphae specific VHH CAW3A8. The cell

wall of *C. albicans* was stained with Calcofluor White Staining (Merck) and VHH CAW3A8 was detected by IgG-goat-anti-rabbit Alexa fluor 647 conjugate (far-red emission) (Thermoscientific) via IgG-rabbit-anti VHH. An overview of the deletion mutants is provided in Tables 2, 4, and 5.

Figure 13. Determination of various epitopes of the cell wall of *C. albicans* recognized by different VHH [compare also Tables 2, 4, 5] A. Overview of the various VHH that recognize different cell wall proteins [CWP]; B. Immunolabeling of *C. albicans* hyphae with VHH19. C. Immunolabeling of *C. albicans* yeast cells with VHH9. D. Immunolabeling of *C. albicans* yeast cells with VHH2. E. Immunolabeling of *C. albicans* hyphae with VHH15.

Figure 14. Protection of *Galleria mellonella* infection caused by *C. albicans* cells by [A] a single VHH; and [B] a mixture of VHHs.

Figure 15. Results of the damage inhibition assay induced with peptides. Confluent TR146 cells were grown and peptides (CaL = candidalysin in two concentrations) were added to damage the cells as measured by concentration of lactate dehydrogenase (LDH) in ng/ml. Full lysis indicates the LDH concentration measured when all TR146 were lysed and "medium" indicates no damage. White bars indicate the wells where no antibody was added and dotted bars where 2,5 μM of F1-H1 bihead was added. Depicted is the mean of three technical replicates.

Fig 16. Results of the damage inhibition assay induced with *Candida albicans* cells. Confluent TR146 cells were grown and yeast cells (WT cells or cells expressing EceI) were added to damage the TR146 cells as measured by concentration of LDH (ng/ml). White bars indicate the wells where no antibody was added and dotted bars where 2,5 μM of F1-H1 bihead was added and striped bars where 3,5 or 2,5 μM CalF1-CAW-3A8 bihead was added. Depicted is the mean of three technical replicates.

Figure 17. Explanation of increased capturing resulting in lower concentration of free candidalysin by biheads consisting of anti candidalysin VHH and anti adhesion protein Als3 compared to monohead combinations of F1 and H1

4. DETAILED DESCRIPTION OF THE INVENTION

4.1 Definitions

The term "antibody" as used herein, refers to an antigen binding protein comprising at least a heavy chain variable region (Vh) that binds to a target epitope. The term antibody includes monoclonal antibodies comprising immunoglobulin heavy and light chain molecules, single heavy chain variable domain antibodies, and variants and derivatives thereof, including chimeric variants of monoclonal and single heavy chain variable domain antibodies.

The term "VHH", as used herein, refers to single heavy chain variable domain antibodies devoid of light chains. Preferably a VHH is an antibody of the type that can be found in *Camelidae* which are naturally devoid of light chains or a synthetic and non immunized VHH which can be constructed accordingly.

The term "*Camelidae*", as is used herein, includes reference to Llamas such as, for example, *Lama glama*, *Lama vicugna* (*Vicugna vicugna*) and *Lama pacos* (*Vicugna pacos*), and to *Camelus* species including, for example, *Camelus dromedarius* and *Camelus bactrianus*).

The terms "family" and "sub-family", as are used herein refers to the origin of the selected VHH. It is known that VHH are encoded by specific VHH V-genes, which are about 20 genes that can be divided into 4 groups by their VH genes (Deschacht et al., 2010. J Immunol 184: 5696–704) and the fusion of these V-genes to the various D- and J-genes. Whereas the D-genes can encode a very large number of sequences, there are only 7 J-genes in the genomes of lamas (Achour et al., 2008. J Immunol 181: 2001–9). Here we define a family as a fusion product of one of the 3 V genes specific for VHH and the VH genes, and one of the 7 J genes. A sub-family includes the variability of CDR3 mainly encoded by D-genes. A family of VHH is thus defined as a particular combination of one of the 4 groups of V-genes and one of the 7 J-genes. When V- and J- genes are equal, differences in the CDR3 encoded by D-genes will result in different sub-families.

As described herein, the amino acid sequence and structure of a heavy chain variable domain, including a VHH, can be considered - without however being limited thereto - to be comprised of four framework regions or 'FR',

which are referred to in the art and herein as 'Framework region 1' or 'FRl'; as 'Framework region 2' or'FR2'; as 'Framework region 3' or 'FR3'; and as 'Framework region 4' or 'FR4', respectively; which framework regions are interrupted by three complementary determining regions or 'CDR' s', which are referred to in the art as 'Complementarity Determining Region l' or 'CDRl'; as 'Complementarity Determining Region 2' or 'CDR2'; and as 'Complementarity Determining Region 3' or 'CDR3', respectively.

The amino acid residues of heavy chain variable regions, including VHH, are numbered according to the general numbering of Kabat (Kabat, et al. (1991) Sequences of Proteins of Immunological Interest, 5th edition. Public Health Service, NIH, Bethesda, MD). For the purpose of this application, amino acid residues 26-32 of VHH are defined as CDR1, amino acid residues 52-58 of VHH are defined as CDR2, and amino acid residues 95-103 of VHH are defined as CDR3, with the amino acid residue numbering according to the Kabat numbering.

The term 'binding' as used herein in the context of binding between an antibody, preferably a VHH, and an epitope on Ece1p or peptides derived from Ece1p, such as candidalysin as a target, refers to the process of a non-covalent interaction between molecules. Preferably, said binding is specific. The terms 'specific' or 'specificity' or grammatical variations thereof refer to the number of different types of antigens or their epitopes to which a particular antibody such as a VHH can bind. The specificity of an antibody can be determined based on affinity. A specific antibody preferably has a binding affinity K_d for its epitope of less than 10^{-7} M, preferably less than 10^{-8} M, most preferable less than 10^{-9} M.

The term "Ece1p", as is used herein, refers to a 271 amino acid preproprotein which is expressed on an invasive form, termed hypha, of *C. albicans.* The hyphae-specific preprotein is cleaved by proteases, especially Kex2 and Kex1, into 9 peptides termed SP, Ece1-I, Ece1-II, Ece1-III, Ece1-IV, Ece1-V, Ece1-VI, Ece1-VII, and Ece1-VIII, of which Ece1-III is also termed candidalysin. Only some of these non-candidalysin peptides can be detected in hyphal culture supernatants. Their biological functions is unknown although there are indications that they enhance the lysis of mammalian cells

by candidalysin. An attractive hypothesis is that due to the non-candidalysin peptides, the probability that candidalysin penetrates the membranes of targets cells increases.

The term "Ece1p derived peptide", as is used herein, refers to the 9 peptides termed SP, Ece1-I, Ece1-II, Ece1-III, Ece1-IV, Ece1-V, Ece1-VI, Ece1-VII, and Ece1-VIII, that result from proteolytic cleavage of the precursor protein Ece1p. Kex2/Kex1 mediated hydrolysis of Ece1p is essential for the release of candidalysin and seven other peptides [see Fig 1].

The term "candidalysin", as used herein, refers to a 31 amino acid polypeptide that is released post-translational from a *Candida* Ece1p precursor by proteolytic cleavage during secretion from the Golgi apparatus (Richardson et al., 2018. mBio 9: e02178-17). A primary amino acid sequence of candidalysin is provided in Figure 1.

The term epitope or antigenic determinant refers to a part of an antigen that is recognized by an antibody. The term epitope includes linear epitopes and conformational epitopes. A conformational epitope is based on 3-D surface features and shape and/or tertiary structure of the antigen.

The term neutralizing antibody refers to an antibody that, when bound to an epitope, interferes with the normal activity of the Ece1p-derived peptides. For example, a neutralizing antibody interferes with cytolytic activity of candidalysin and/or reduces the function of the non-candidalysin peptides.

The term affinity refers to the strength of a binding reaction between a binding domain of an antibody and an epitope. It is the sum of the attractive and repulsive forces operating between the binding domain and the epitope. The term affinity, as used herein, refers to the dissociation constant, Kd.

The terms "strong interaction" and "strong binding" refers to the electrostatic interactions as is known to the skilled person.

The terms "weak interaction" and "weak binding " refers to the presence of hydrogen bonds and van der Waals interactions as are known to the skilled person.

The term microbicide refers to products that contain active components that block transfer of harmful living entities into the human body, in particular via the vagina and/or rectum.

The term "*Candida* mucosal infection", as is used herein, refers a severe mucosal infections that is associated with severe morbidity. A defining feature of this pathogenesis is the transition from yeast to invasive filamentous hyphae which damage mucosal epithelia and induce activation of the activating protein-1 (AP-1) transcription factor.

The term "lactic acid bacteria", as is used herein, refers to lactic acid bacteria that can survive for a considerable time in the gastro-intestinal tract or vagina and are able to produce VHH antibodies in that tract or the vagina that can neutralize *Candida* spp. The lactic acid bacteria are preferably modified in such a way that, when excreted in the environment, they can't survive.

The term "products that protect implanted medical devices against *Candidiases*" refers to sprays, gels and/or emulsions that contain VHH that block biofilm formation on these devices.

4.2 Blocking or neutralizing VHH

The invention relates to a specific class of antibodies, namely single heavy chain variable domain antibodies, or VHH antibodies, that are capable of binding to and neutralizing Ece1p-derived peptides including candidalysin and non-candidalysin peptides. These antibodies are termed "the Variable domains of the Heavy-chains of Heavy-chain only antibodies", or abbreviated VHH. The genes encoding heavy chain variable domain antibodies were isolated from lymphocytes of llamas that were immunized with hyphal *Candida albicans* cells that express high levels of Ece1p, the precursor protein of candidalysin and other peptides.

Because Ece1p is the precursor of candidalysin and other peptides we reasoned that in the rather complex immunogen that was used there was a reasonable probability that Ece1p, candidalysin and some of the other peptides were present. Consequently, we embarked on a strategy to select

VHH antibodies from the immune libraries that recognize Ece1p, candidalysin and other peptides derived form Ece1p.

Immune phage display libraries were generated from these animals at 43 days after the first injection. These libraries were used to select phages that bind to captured candidalysin. This resulted in the isolation of two families of VHH antibodies that showed binding and neutralization of candidalysin.

Further VHH antibodies that were isolated and showed binding to non-candidalysin Ece1p derived peptides, belong to 3 different families, notably the families containing V-genes characterized by the hall mark sequences KQREL, KEREG and KGLEW, as depicted in Figure 4. Only from the V-genes characterized by KEREF sequence no VHH antibodies were selected

A VHH according to the invention preferably comprises CDR1, CDR2 and CDR3 amino acid sequences as depicted in Figure 4a, or a derivative, for example an exchange for an amino acid that has a related side group as described in Rajpal et al., 2005 [Rajpal et al., 2005. PNAS 102: 8466–8471]. A further preferred VHH antibody comprises any one of the variable domain amino acid sequences as depicted in Figure 4b and/or 4C, or a derivative, for example a conservative derivative, thereof. A derivative preferably is more than 90% identical, more preferably more than 95% identical, more preferably more than 99% identical to the amino acid sequences depicted in Figure 4b and/or 4C. Said derivative preferably has 10 or less conserved amino acid exchanges, such as 5 conserved amino acid exchanges, 4 conserved amino acid exchanges, 3 conserved amino acid exchanges, 2 conserved amino acid exchanges or 1 conserved amino acid exchange. The term conserved amino acid exchange refers to an exchange for an amino acid that has a related side group as described in Rajpal et al., 2005.

The isolated anti-candidalysin VHH can be grouped into 2 families termed F1 and H1, according to the germ line V- and J-genes of which the isolated VHH have been derived by selection and maturation (see Fig 4b).

The invention further provides an antibody that effectively competes with an anti-candidalysin antibody of families F1 and H1 for binding to its epitope on candidalysin. The term effectively is used to indicate that the

competing antibody binds with substantially the same affinity to the same or nearly the same epitope, when compared to the antibody of the invention. The term substantially is used to indicate that the difference in affinity between an antibody of the invention and a competing antibody is preferably less than 10-fold, more preferred less than 5-fold, more preferred less than 2-fold, more preferred less than 1,5 fold. A preferred competing antibody is capable of effectively competing with an antibody of the invention when the competing antibody lowers the affinity of the observed binding of an antibody of the invention to its epitope about 2-fold using the same molar amount of competing antibody. Assays for measuring competition are known in the art and include, for example, competitive ELISA.

The same phage libraries were used to select VHH that recognize proteins present in the cell membrane or the cell wall of *C. albicans*. VHH that only bind to cell wall proteins that are only present in the hyphal form of *C. albicans* were selected. One of the VHH selected in this way proofed to recognize the protein Als3, an adhesin. Recently it has been shown that a vaccine-like product using a modified form of Als3 give some protection against candidiasis [Schmidt et al., 2012. Vaccine 30: 7594–600; Edwards et al., 2018. Clin Infect Diseases 66: 1928–1936], whereas blood of the animals treated with this vaccine-like product were able to prevent adhesion and biofilm formation on the surface of medical devices (Uppuluri et al., 2018. Frontiers Immunol 9: Article 1349).

Further VHH that were selected in this way proofed to recognize the protein Als4, which also is an adhesin. In the literature, blocking of mainly HWP1, Als3 and SSa1 are mentioned as an option to reduce or even block an interaction between hyphae and the extracellular matrix of epithelial cells. See, for example, da Silva Danta et al., 2016. Current Opinion Microbiol 34: 111-118). However, it is now surprisingly shown in the examples that also antibodies such as VHH against Als4 are providing protection. A more commonly used route to select VHH against proteins can also be followed. Recently Allert et al. [Allert et al., 2018. mBio 9: e00915-18] found 8 proteins of which 6, notably Prn4p, Npr2p, Aaf1p, Hma1p, Tea1p, Pep12p, may play a role in the pathogenicity of *C. albicans* hyphae. As the whole genome of *C.*

albicans has been determined [WGS data available at www.candidagenome.org], this information can be used to find the DNA and the corresponding amino acid sequence of Hma1p.

Subsequently, the HMA1 gene with a FLAG-tag-encoding sequence linked to it, may be synthesized and cloned and expressed in *E. coli* using standard procedures. FLAG-tagged Hma1p may subsequently be purified using an anti FLAG antibody column. The Hma1p may then be captured on a microtiter plate coated with anti FLAG antibody as described above and phage selections may be carried out using the above indicated phage libraries as described in detail herein above.

4.3 De-immunization and humanization

A heavy chain variable domain antibody is small, does not or hardly aggregate and have a short half life when administered, for example, to humans. Therefore, VHH hardly induce an immune response after administration to humans. However, de-immunization and/or humanization may be required for use of the antibodies of the invention in pharmaceutical compositions. De-immunization is a preferred approach to reduce the immunogenicity of antibodies according to the invention. It involves the identification of linear T-cell epitopes in the antibody of interest, using bioinformatics, and their subsequent replacement by site-directed mutagenesis to non-immunogenic sequences or, preferably human sequences. Methods for de-immunization are known in the art, for example from WO098/52976, which is herein incorporated by reference.

A further preferred approach to circumvent immunogenicity of antibodies according to the invention when applied to humans involves humanization. Various recombinant DNA-based approaches have been established that are aimed at increasing the content of amino acid residues in antibodies that also occur at the same of similar position in human antibodies while retaining the specificity and affinity of the parental non-human antibody. Most preferred are amino acid residues that occur in antibodies as they are encoded by genomic germ line sequences. Humanization may include the construction of VHH-human chimeric antibodies, in which the VHH

binding regions are covalently attached, for example by amino acid bonds, to one or more human constant (C) regions. These methods rely on analyses of the antibody structure and sequence comparison of the non-human and human antibodies in order to evaluate the impact of the humanization process into immunogenicity of the final product.

4.4 Neutralization of candidalysin

Neutralization of candidalysin is measured by determining the loss of cell damage after incubation of cells with candidalysin or, preferably, with a *C. albicans* species, preferably with hyphae of a *C. albicans* species, in the presence and absence of an antibody according to the invention. For this, candidalysin and/or cells of *C. albicans,* are mixed with the antibody under appropriate conditions and then provided to an indicator cell culture, preferably comprising epithelial cells, that is sensitive to candidalysin. The loss of cell damage is brought about by interference by the bound antibody with any one of the steps leading to damage of the indicator cells, including membrane permeabilization, inward current concomitant with calcium influx, and activation of the activating protein-1 (AP-1) transcription factor. An antibody is preferably assayed for neutralization as a soluble antibody fragment.

4.5 Antibodies comprising an anti-Ece1p directed VHH antibody

The invention further provides a VHH antibody that binds and neutralizes Ece1p and Ece1p-derived peptides such as candidalysin. A preferred antibody is a bispecific or multispecific antibody such as a tri-specific antibody, comprising at least one neutralizing anti-candidalysin VHH antibody. Said bispecific or multispecific antibody preferably comprises means for eliminating candidalysin and/or other Ece1p-derived peptides, via antibody-dependent cell-mediated cytotoxicity (ADCC) routes and/or complement dependent cytotoxicity (CDC) routes.

In a preferred embodiment, said antibody comprises an immunoglobulin Fc region or functional part thereof of an immunoglobulin heavy chain. The Fc region or functional part thereof is preferably derived from IgG1, IgG2,

IgG3, IgG4, IgM, IgD, IgA or IgE. It is further preferred that the Fc region or part thereof is a human Fc region or part thereof similar to VHH-Fc fusion antibodies as described for anti HIV VHH [McCoy et al., 2014. Retrovirology 11: 83]. Said camelid Fc region or part thereof preferably is humanized. The single heavy chain variable domain is preferably connected to a Fc region or functional part thereof via a hinge region. A preferred hinge region is the hinge region of a camelid or human immunoglobulin heavy chain molecules from IgG1, IgG2, IgG3, IgG4, IgM, IgD, IgA or IgE, most preferred from IgG1. A hinge region of a camelid immunoglobulin heavy chain molecule preferably is humanized.

A preferred part of an Fc region is the region comprising the C2 domain, the C3 domain, or the C2 and C3 domains of IgGs, preferably IgG1 or IgG3, most preferably C2 and C3 domains of human IgG1.

4.6 Neutralization of the combined cell lysing effect of candidalysin and non-candidalysin Ece1p derived peptides

A further preferred antibody is a bi- or multivalent antibody comprising a neutralizing single heavy chain variable domain according to the invention. Said bi- or multivalent antibody preferably is a bispecific or multispecific antibody comprising two or more different single heavy chain variable domains that recognize different epitopes on Ece1p or on an Ece1p-derived peptide such as candidalysin and/or another, non-candidalysin peptide derived from Ece1p. A bi- or multispecific antibody preferably comprises two or more single heavy chain variable domains of which one binds candidalysin, and a second one binds another non-candidalysin peptide derived from Ece1p. Non-limiting examples can be provided of combinations of different anti-Ece1p VHH antibodies, directed against candidalysin and/or against a non-candidalysin peptide derived from Ece1p. Any combination of these antibodies can be used in a bi- or multispecific antibody to generate functional bi-heads or even tri-heads that can be used in research, for imaging, or as therapeutics.

Also biheads formed from VHH that only recognize non-candidalysin peptides [e.g. F1-F6b- B2-F5a] are included as they may have superior

binding properties or biological functionalities. To develop further diagnostic and research tools, biheads may be constructed that recognize both Ece1p, candidalysin or other peptides derived from Ece1p and VHH recognizing markers specific for hyphae of *Candida* spp.

A preferred bihead or multi-head such as trihead comprises an anti-candidalysin antibody and an antibody against a membrane-anchored target. Said membrane-anchored target preferably is a protein such as adhesion molecule, preferably Als 3 and/or Als4. As is indicated in Figure 17, said anti-membrane-anchored target antibody will localize the bihead or multi-head such as trihead on the *Candida* membrane, allowing it to effectively and specifically capture a candidalysin molecule after transport through the *Candida* membrane, before its diffusion and entering the extracellular matrix of the mammalian target cell, thereby lowering the concentration of the candidalysin molecule at the membrane of the mammalian target cell. Without being bound by theory, it is thought that a minimal concentration of candidalysin is required at the membrane of the mammalian target cell in order for multimerisation of candidalysin which is required to generate pores in the membrane of the mammalian target cell. Effectively capturing candidalysin will reduce the concentration below the minimally required concentration for multimerisation.

A preferred linker group is a linker polypeptide comprising from about 10 to about 50 amino acid residues, preferably from 10 to about 30 amino acid residues, most preferred about 15 amino acid residues.

Some preferred examples of such amino acid sequences include gly-ser linkers, for example of the type $(Gly_x\ Ser_y)_z$, such as, for example $(Gly_4\ Ser)_3$ or $(Gly_3Ser_2)_3$, as described in WO 99/42077, which is hereby incorporated by reference, and the GS30, GS15, GS9 and GS7 linkers described in, for example, WO 06/040153 and WO 06/122825, both of which are hereby incorporated by reference, as well as hinge-like regions, such as the hinge regions of naturally occurring heavy chain antibodies or similar sequences (such as described in WO 94/04678; which is hereby incorporated by reference). A preferred bihead or multihead comprises at least two non-competing and non-interfering monospecific anti- candidalysin VHH

antibodies , or an anti- candidalysin VHH antibody and a second VHH antibody directed against a non-candidalysin peptide derived from Ece1p.

4.7 Methods to improve the affinity of the selected VHH by in-vitro constructed combinations of CDRs.

In spite of good affinities of the selected VHH, to achieve optimal effectivity of any of the VHH based constructs it may be necessary to improve the binding of the VHH to its cognate epitope further more. Although several routes for achieving this can be followed we have used the method described by Rajpal et al., 2005 [Rajpal et al., 2005. PNAS 102: 8466–8471], although in the construction of these libraries we took measures to keep the canonical folds of the CDRs close to that of the selected members of the F1 family. The constructed combinatorial CDRs for VHH belonging to F1 family are given in Figure 4A. A similar approach can be followed for VHH of the H1 family and the VHH that recognize the non-candidalysin peptides.

Another method to improve the binding of the selected VHH towards Ece1p, candidalysin or non-candidalysin peptides is the direct screening approach. An immune library contains 10^4 to 10^5 genes encoding different VHH, low through-put selections only result in a small number of VHH. High through put screening on functionality, defined as reducing the lysis of mammalian cell by Ece1p, candidalysin or non-candidalysin Ece1p derived peptides, in combination with deep sequencing has been proven to be successful in the hands of the applicants [McCoy et al 2014, PLOS Path 10, e1004552]. Another route that may be applied to enlarge the number of VHH that neutralize Ece1p, candidalysin of non-candidalysin Ece1p peptides is the so-called family approach, which capitalize on the information on amino acid sequence of the C-terminus of the VHHs showing biological functionality [Koh et al., 2010. J Biol Chem 285: 19116-19124].

4.8 Methods to prevent translocation of Candida spp.

It has been shown that *C. albicans* can translocate from mucosal surfaces into the bloodstream. In particular translocation of the intestinal population of *C. albicans* results in candidases [Allert et al., 2018, mBio 9:

e00915-18]. Methods to prevent translocation of *C. albicans* hyphae in the intestine by using specially selected – food grade - lactic acid bacteria that protect the epithelial layers of the intestine by interacting with surface proteins of epithelial cells and simultaneously secrete VHH that can neutralize the main factor causing translocation, ECE1 and its derivative candidalysin. In this way there is a more or less continuous delivery of neutralizing VHH at the place where they are mostly required, close to the epithelial cells of the intestine and vagina, will take place.

A preferred host cell for expression of anti-*Candida* VHH in general, preferably anti-*C. albicans* VHH, in particular anti candidalysin VHH, are lactic acid bacteria [Pant et al., 2006. J Infect Diseases 194: 1580–8; Hultberg et al., 2007. BMC Biotechn 7: 58; Marcotte et al., 2008. Future Virol 3: 327-341; Anderson et al., 2016. Infection Immunity 84: 395-]. These bacteria can be cultivated at large scale and supplied as food ingredient to potential patients or immune compromised persons. The live lactic acid bacteria will produce the protective VHH in, for example, the intestine and/or vagina. In particular, lactic acid bacteria that are common inhabitants of the vagina can be used to prevent candidiasis by exposing anti adhesin VHH on there surfaces. Anti Ece1p, anti-candidalysin and/or anti Als3p VHH can also be taken up in a gel or emulsion that can be used to protect the epithelial cells of the vagina against necrosis caused by *C. albicans* hyphae and products that are produced by the *C. albicans* hyphae.

For this, said lactic acid bacteria preferably are food grade lactic acid bacteria that are frequently used in fermented food products. Said lactic acid bacteria preferably are generally recognised as safe (GRAS). After transformation of these bacteria with vectors that contain an appropriate promotor, a signal sequence followed by a nucleotide sequence encoding the VHH gene, and a terminator sequence or a sequence encoding a protein that is present in the cell wall of these bacteria, in order to anchor the VHH on the cell wall of lactic acid bacteria. thereby blocking the interaction of hyphae to epithelial cells, and then a terminator sequence, these transformed lactic acid bacteria are able to produce VHH that can neutralize candidalysin, and/or

VHH that block adhesion of *Candida* species such as *C. albicans* to the mucosal layer of the intestine or vagina.

Said lactic acid bacteria can be cultivated at large scale and supplied as food or food ingredient to potential patients or immune compromised persons. These still living lactic acid bacteria will produce the protective VHH in the intestine and vagina. In particular lactic acid bacteria that are common inhabitants of the vagina can be used to prevent candidiasis by exposing anti adhesin VHH on there surfaces. Anti Ece1p, anti-candidalysin, and/or anti Als3p VHH can also be taken up in a gel or emulsion that can be used to protect the epithelial cells of the vagina against necrosis caused by *C. albicans* hyphae, and/or by products produced by the hyphae.

Said lactic acid bacteria preferably are provided as food, or food-like products or as a drink. Said lactic acid bacteria will bind to receptors of various mucosae of the gastro-intestinal tract and secrete the neutralizing VHH at the site where *Candida* spp. may translocate from mucosal surfaces of the gastro-intestinal tract into the bloodstream.

Finally the invention provides products containing VHH that block the adhesion of *C. albicans* hyphae to implanted medical devices thereby preventing biofilm formation of these *C. albicans* hyphae and their production of candidalysin.

4.9 Methods to produce neutralizing antibodies comprising a VHH

An antibody according to the invention, for example a single heavy chain variable domain or an antibody comprising a single heavy chain variable domain, may be produced using antibody producing prokaryotic cells or eukaryotic cells, preferably mammalian cells such as CHO cells or HEK cells, or fungi, most preferably filamentous fungi such as *Aspergillus awamori,* or yeasts such as *Saccharomyces cerevisiae* or *Pichia pastoris.* An advantage of an eukaryotic production system is that folding of the protein results in proteins that are more suitable for treating a human individual. Moreover, eukaryotic cells often carry out desirable post translational modifications that resemble posttranslational modifications that occur in mammalian cells.

Production of VHH in filamentous fungi is preferably performed as described by Joosten et al. (2005). J Biotechnol 120:347–359, which is included herein by reference. A preferred method for producing VHHs in *Saccharomyces cerevisiae* is according to the method as described by v. d. Laar et al, (2007), Biotech Bioeng 96, 483-494; or Frenken et al. (2000). J Biotechnol 78:11–21, which are included herein by reference. Another preferred method of VHH production is by expression in *Pichia pastoris* as described by Rahbarizadeh et al. (2006) J Mol Immunol 43:426–435, which is included herein by reference.

A further preferred method for production of therapeutic VHH comprises expression in mammalian cells such as a fibroblast cell, a Chinese hamster ovary cell, a mouse cell, a kidney cell, a retina cell, or a derivative of any of these cells. A most preferred cell is a human cell such as, but not limited to, Hek293 and PER.C6. A further preferred cell line is a cell line in which alpha-(1,6)-fucosyltransferase has been inactivated, for example the ΔFUT8 CHO cell line, as described in Yamane-Ohnuki et al 2004, Biotechnol. Bioeng 87, 614-622. It was found that antibodies that are produced in ΔFUT8 cells enhance the ADCC route.

An antibody according to the invention is preferably produced by the provision of a nucleic acid encoding said antibody to a cell of interest. Therefore, the invention further provides a nucleic acid encoding an antibody according to the invention. Said nucleic acid, preferably DNA, is preferably produced by recombinant technologies, including the use of polymerases, restriction enzymes, and ligases, from the single heavy chain variable domains that were isolated from the immunized animal, as is known to a skilled person. Alternatively, said nucleic acid is provided by artificial gene synthesis, for example by synthesis of partially or completely overlapping oligonucleotides, or by a combination of organic chemistry and recombinant technologies, as is known to the skilled person. Said nucleic acid is preferably codon-optimised to enhance expression of the antibody in the selected cell or cell line. Further optimization preferably includes removal of cryptic splice sites, removal of cryptic polyA tails and/or removal of sequences that lead to unfavourable folding of the mRNA. The presence of an intron flanked by

splice sites may encourage export from the nucleus. In addition, the nucleic acid preferably encodes a protein export signal for secretion of the antibody out of the cell into the periplasm of prokaryotes or into the growth medium, allowing efficient purification of the antibody.

The invention further provides a vector comprising a nucleic acid encoding an antibody according to the invention. Said vector preferably additionally comprises means for high expression levels such as strong promoters, for example of viral origin (e.g., human cytomegalovirus) or promoters derived from genes that are highly expressed in a cell such as a mammalian cell (Running Deer and Allison (2004) Biotechnol Prog 20: 880–889; US 5888809). The vectors preferably comprise selection systems such as, for example, expression of glutamine synthetase or expression of dihydrofolate reductase for amplification of the vector in a suitable recipient cell, as is known to the skilled person.

The invention further provides a method for producing an antibody, the method comprising expressing a nucleic acid according to the invention in a prokaryotic or eukaryotic cell and recovering the thus produced antibody from the cell cultures. The nucleic acid, preferably a vector comprising the nucleic acid, is preferably provided to a cell by transfection or electroporation. The nucleic acid is either transiently, or, preferably, stably provided to the cell. Methods for transfection or electroporation of cells with nucleic acid are known to the skilled person. A cell that expresses high amounts of the antibody may be selected. This cell is grown, for example in roller bottles, in fed-batch culture or continuous perfusion culture.

The invention additionally provides a host cell comprising a nucleic acid or vector according to the invention. Said host cell may be grown or stored for future production of an antibody according to the invention.

4.10 Product compositions and administration of an antibody

The invention further relates to a product or composition containing or comprising at least one neutralizing anti-candidalysin antibody as described herein, and/or a VHH antibody against another, non-candidalysin peptide that is derived from Ece1p. Therefore, the invention provides an antibody

according to the invention for use as a medicament. The antibodies of the invention are preferably used for prophylactic administration or therapeutic administration in both humans and other animals that are infected with *C. albicans*. Thus, the antibodies according to the invention are administered to high-risk individuals in order to lessen the likelihood of developing *Candida* infection related diseases such as candidiasis and invasive candidiasis, in these individuals or to lessen the severity of the disease or administered to subjects already evidencing active *C. albicans* infection.

The administration of an antibody of the invention is preferably provided in an effective amount. An effective amount of an antibody of the invention is a dosage large enough to produce the desired effect in which the symptoms of the *Candida* infection are ameliorated or the likelihood of infection is decreased. A therapeutically effective amount preferably does not cause adverse side effects, such as hyperviscosity syndrome, pulmonary edema, congestive heart failure, and the like. Generally, a therapeutically effective amount varies with the subject's age, condition, and sex, as well as the extent of the disease in the subject and can be determined by one of skill in the art. The dosage may be adjusted by the individual physician or veterinarian in the event of any complication. A therapeutically effective amount may vary from about 0.01 mg/kg to about 500 mg/kg, preferably from about 0.1 mg/kg to about 200 mg/kg, most preferably from about 0.2 mg/kg to about 20 mg/kg, in one or more dose administrations daily, for one or several days. Preferred is administration of the antibody for 2 to 5 or more consecutive days in order to avoid "rebound" of *Candida* infections from occurring.

The antibodies of the invention can be administered by injection or by gradual infusion over time. The administration of the antibodies preferably is parenteral such as, for example, intravenous, intraperitoneal, or intramuscular. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions,

including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

A preferred prophylactic use of an anti-Ece1p VHH antibody, or of a VHH antibody directed against an Ece1p-derived peptide such as candidalysin, according to the invention is provided by a microbicide. The term microbicide refers to a product that is used topically, preferably vaginally or rectally, to prevent infection. A microbicide offers the potential for women to protect themselves and their sexual partners from *Candida* infections. For other anti-infective use, they may also be applied to the skin, mucous membranes, and orally. Preferred microbicides are inexpensive, affordable, stable at ambient temperature, preferably at temperatures above 35 °C, more preferably at temperatures above 40 °C, compatible and active after mixture with cosmetically acceptable formulations, non-toxic and non-damaging to vulvar, vaginal, cervical, penile or other epithelium. A microbicide preferably further comprises a base or carrier, such as a foam, cream, wash, gel, suppository, ovule, lotion, ointment, film, tablet, foaming tablet, tampon, vaginal spray, aerosol, or other base or carrier as would be apparent to a skilled person. Said microbicide can be coupled to, or included in, a support, for example a vaginal ring, for providing sustained protection against *Candida* species, as described, for example in Wahren et al. 2010 (Wahren et al. 2010. J Transl Med 8: 72).

Many medical devices require antimicrobial coatings to prevent the transfer of external bacteria and/or fungi into the body environment. Antimicrobial coatings must provide active resistance to microbe absorption and transference. In order to supply this microbial resistance, antimicrobial coatings often have release functions when exposed to bodily fluids in order to fight such infectious bacteria as *E.coli* and *Staphylococcus*, which they do by minimizing toxicity in organic tissue.

The invention further provides products that protect medical devices against *Candidiases*, prior to implantation. These products include sprays and gels emulsions that comprise VHH according to the invention that block biofilm formation on medical devices.

Such biofilm-blocking VHH preferably include Als3p, Hma1p and Aaf1p.

The invention further provides a composition comprising an antibody according to the invention, preferably at least one antibody according to the invention. Said composition preferably is a pharmaceutical composition. A pharmaceutical composition preferably comprises a pharmaceutically acceptable carrier. A carrier, as used herein, means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The term "physiologically acceptable" refers to a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts buffers, stabilizers, solubilizers, and other materials which are well known in the art.

4.11 In vitro and in vivo detection and imaging tools based on VHH that recognize Ece1p, candidalysin, non-candidalysin Ece1p derived peptides, and/or proteins that are present in the hyphal form of C. albicans

An anti-Ece1p or anti-Ece1p-derived peptide VHH antibody, such as an anti-candidalysin VHH antibody of the invention may be labeled by a variety of means for use in diagnostic applications. There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, infra red dyes, near Infra Red [nIR] dyes, and bioluminescent compounds. For non invasive diagnosis, radioisotopes may be bound to the VHH or the VHH-Fc fusion product either directly or indirectly by using an intermediate functional group. Intermediate functional groups which are often used to bind radioisotopes which exist as metallic ions are the

bifunctional chelating agents such as NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) and DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) and derivatives thereof. Typical examples of metallic ions which can be bound to the anti- Ece1p and/or anti-Als3p VHH antibodies, such as VHH antibodies directed against an Ece1p-derived peptide such as candidalysin, of the invention are .sup.111In, .sup.97Ru, .sup.67Ga, .sup.68Ga. .sup.72As, .sup.89Zr and .sup.201Tl. The antibodies of the invention can also be labeled with an infrared dye or with a paramagnetic isotope for purposes of in vivo diagnosis, as in, for example, magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT), computed tomography(CT) or photo-acoustic imaging. Although many methods exist they are all characterized that the VHH used for imaging have an extended C-terminus containing at least the amino acid sequence A-C-A-A directly after the common terminus of VHH [VTVSS]. An extension at the C-terminus that contains the A-C-A-A amino acid sequence enables directed coupling of any imaging agent at the SH of the Cys residue. Beside the standardization of the imaging agent, the biggest advantage of labeling at the A-C-A-A amino acid sequence is that this ensures that the imaging label is at least 20 A, preferably even 30 A, away of the antigen binding site. Therefore, such directionally labelled VHH retain full functionality.

The antibodies of the invention may further be used *in vitro*, for example, in immunoassays in which they can be utilized for detection of antigens in liquid phase or bound to a solid phase carrier. The antibodies in these immunoassays can be detectably labeled in various ways. Examples of types of immunoassays which can utilize the antibodies of the invention are competitive and non-competitive immunoassays. The assays either comprise a direct or an indirect format and include radioimmunoassay (RIA) and the sandwich assay. *C. albicans* cells present in biological fluids and tissues can be detected by the antibodies of the invention. A sample can be a liquid such as urine, saliva, cerebrospinal fluid, blood serum or the like; a solid or semi-solid such as tissues, feces, or the like; or alternatively, a solid tissue such as those commonly used in histological diagnosis.

5. Examples

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Example 1

Materials and methods

Immunogens

For creation of *Candida albicans* SC5314 (C. a.) immune VHH libraries, two llamas (namely llama 7 and llama 11) were immunised with both heat or formaldehyde inactivated *C. albicans* SC5314 yeast, germ and hyphae cells. Filamentation of *C. albicans* was induced by incubation of yeast cells at 37 °C in RPMI-1640 (pH 7) supplemented with 165mM MOPS and 1.36 mM N-acetyl-glucosamine with shaking for 3 h (germ-tubes) or statically for 24 h (hyphae). Before immunization, the fungal cells were inactivated with either 4% formaldehyde or by incubation for 2 h at 90 °C.

Immunization

According to the standard subcutaneous immunization schedule both llamas received full immunogen doses of formaldehyde and heat inactivated cells on day 0, 14, 28 and 35. The immune response was verified by ELISA with llama sera from the day 0, 28 and 43 against coated *Candida albicans* SC5314 yeast, germ-tube and hyphae cells.

Library creation

From B-cells, RNA samples of the llamas from day 43 post immunisation the *Candida albicans* SC5314 immune VHH libraries #7 and #11 have been created. After generation of cDNA, the VHH coding sequences were cloned into phagemid pUR8100 and transformed to *Escherichia coli* TG1 with an estimated diversity of $2.4*10^7$ clones for each library.

Candidalysin, the main target of VHH selection

The target of the selections described here was Ece1p, in particularly the Ece1p-derived peptide candidalysin, a cytolytic peptide toxin secreted by the opportunistic pathogen *Candida albicans* which has been first described by Moyes et al., 2016 (Moyes et al., 2016. Nature 532: 64-68). Candidalysin's

C-terminus is hydrophobic, while its N-terminus is hydrophilic and basic. Due to the hydrophobicity, it was expected that handling of the peptide during selection might be difficult. Therefore, the phage-display selections were conducted on immobilised candidalysin to keep the peptide in a solution and in a natural conformation, as described (Verheesen and Laeremans, 2012. Methods Mol Biol 911: 81-104). The immobilisation of candidalysin was realised by addition of a FLAG tag to the peptide and employing affinity capture with an anti-FLAG antibody (monoclonal mouse-anti-FLAG M2, Sigma F3165). The conformational restriction of the peptide or steric hindrance of VHH binding due to the addition of a FLAG tag could not be predicted, therefore variants with C- or N-terminal FLAG tag were synthesized along with natural candidalysin. The 31-mer natural Candidalyins peptide was obtained from Peptide Protein Research Ltd. (Funtley, UK). The FLAG-tagged variants were obtained from Caslo (Kongens Lyngby, Denmark). Their sequences are given in Figure 3. The selections were done using 4 different candidalysin containing peptides. Remarkably the selection using peptides with the FLAG sequence [DYKDDDDK] at the N- or C- Terminus resulted in the selection of superior VHH.

Non-candidalysin Ece1p derived peptides

Besides candidalysin additional capture selections were conducted on non-candidalysin Ece1p derived peptides using similar methods. The peptides had been selected due to their abundance in hyphal supernatants, as detected by LC-MS. Most fragments of the expected non-candidalysin Kex2/Kex1 cleavage products of Ece1p could be detected in hyphal supernatants. Therefore these fragments were chosen for VHHs selections. Additionally selections for non detectable peptides of Kex2/Kex1 cleavage have been conducted. Analogous to candidalysin, variants of all peptides were fused C- or N-terminally to a FLAG tag. All peptides were synthesised by Peptide Protein Research Ltd. (Funtley, UK). The non-candidalysin peptides are specified in Table 1. The numbering of the peptides refers to the position of the corresponding Kex2/Kex1 processing product of Ece1p when counting from the C- to N-terminus.

Table 1. Non-candidalysin peptides from ECE1p

name	amino acid sequence	weight (kDa)
FLAG-P5a	DYKDDDDK-DGANDDVANAVVRLPEIVA	2.9
P5a-FLAG	DGANDDVANAVVRLPEIVA-DYKDDDDK	2.9
FLAG-P5b	DYKDDDDK-VATGVQQSIENAK	2.3
P5b-FLAG	VATGVQQSIENAK-DYKDDDDK	2.3
FLAG-P6a	DYKDDDDK-DGVPDVGLNLVANAPR	2.6
P6a-FLAG	DGVPDVGLNLVANAPR-DYKDDDDK	2.6
FLAG-P6b	DYKDDDDK-LISNVFDGVSETVQQAK	2.8
P6b-FLAG	LISNVFDGVSETVQQAK-DYKDDDDK	2.8
FLAG-P7a	DYKDDDDK-DGLEDFLDELLQRLPQLIT	3.2
P7a-FLAG	DGLEDFLDELLQRLPQLIT-DYKDDDDK	3.2
FLAG-P7b	DYKDDDDK-SAESALKDSQPV	2.2
P7b-FLAG	SAESALKDSQPV-DYKDDDDK	2.2

Bacterial strain Escherichia coli TG1

For phage display and production of VHHs, the amber suppressor *Escherichia coli* (*E. coli*) strain TG1 (Agilent Technologies) with the following genotype was used: K-12 [F′ traD36 proAB lacIqZ ΔM15] supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5(rK– mK–)

Vectors for phage display and VHH production

One vector was used for phages display and VHH production (pUR8100 derivative of pHEN1 with C-terminal c-myc and hexahistidine tag fusions to the gene insert; Hoogenboom et al., 1991. Nucleic Acids Res 19: 4133-4137). Two other vectors were exclusively used for VHH production (pMEK219 and pMEK222, which are derivatives of pUR8100 without gene III, while in pMEK222 the c-myc- tag is substituted for a FLAG tag). All vectors contain the bacteriophage F1 and the bacterial MB1 origin of replication as well as an ampicillin resistance gene coding for ß-lactamase. In all 3 vectors, the inserted gene expression is under control of the *lacZ* promotor. Gene inserts are N-terminally fused to a *pelB* leader sequence for translocation of the gene product to the periplasm for all three vectors. A c-myc tag (EQKLISEEDL) followed by a hexahistidine tag (HHHHHH) are fused to the C-terminus of gene inserts in pUR8100 and pMEK219. Instead of a myc-tag, pMEK222 contains a FLAG tag (DYKDDDDK), followed by a hexahistidine tag. In pMEK219 and pMEK222 the coding sequences are followed by two ochre stop codons (TAA) for termination of translation. In pUR8100, an amber stop

codon (TAG) is present after the coding sequences, after which the M13 phage gene III has been inserted that codes for the M13 hull protein III.

Phage production and purification

For phage production from whole libraries 50 mL 2× YT Medium (Carl Roth GmbH + Co. KG, Karlsruhe, Germany; hereinafter Roth) supplemented with 2% glucose (Roth) and 100 µg/mL ampicillin (Roth) were inoculated with the glycerol stocks of the libraries #7 or #11 to reach a starting OD600nm of 0.05. The cultures were incubated at 37°C and 200 rpm (Series 25 Incubator Shaker, New Brunswick Scientific) until they had reached an OD600nm of 0.5, then 7.5 mL of each culture were infected with 5 µL helper phage (VCSM13, Stratagene, titer: 1.2*10E13 pfu/ul). After an incubation of 30 min at 37°C without shaking the *E. coli* TG1 cells were sedimented by centrifugation (5 000 × g, 10 min) and the supernatants were discarded. Following phage production by incubation overnight at 37°C and 200 rpm (Series 25 Incubator Shaker, New Brunswick Scientific) the cell pellets were resuspended in 50 mL 2× YT Medium (Roth) supplemented with 100 µg/mL ampicillin (Roth) and 25 µg/mL kanamycin (Roth). The next morning the bacterial cells were sedimented by centrifugation (7 000 × g, 10 min) and 40 mL of each supernatant were transferred into an aqueous solution containing 200 g/L PEG6000 (Sigma-Aldrich, Saint Louis, MO) and 2.5 M NaCl (Roth) for precipitation of secreted phage particles. After incubation for 60 min on ice the precipitated phages were sedimented by centrifugation (7 000 × g, 15 min). The supernatants were removed and the precipitated phage were resuspended in 1 mL PBS (NaCl 8g/L; KCl 0.2 g/L; KH2PO4 0.2 g/L, Na2PO4 1.44 g/L; pH7.4; all of Roth) followed by centrifugation for 5 min at 17 000 × g. The resulting supernatants were transferred to 250 µL aqueous solution containing 200 g/L PEG6000 (Sigma-Aldrich) and 2.5 M NaCl (Roth) for phage precipitation. After incubation for 30 min on ice the precipitated phages were sedimented by centrifugation (5 min at 17 000 × g). The supernatants were removed and the precipitated phages were resuspended in 1 mL PBS followed by a final centrifugation step (5 min at 17 000 × g) to remove any residual bacteria and storage of the resulting supernatants,

which contain the purified phages, at 4 °C until further use in phage-display selections.

Phage productions from glycerol stocks of phage outputs from selections were carried out the same way with the following modifications until infection with helper phage: 5 mL 2× YT Medium (Roth) supplemented with 2% glucose (Roth) and 100 µg/mL ampicillin were inoculated with 75 µL of a selection output glycerol stock and incubated at 37°C and 200 rpm (Series 25 Incubator Shaker, New Brunswick Scientific) until they had reached an OD600nm of 0.5. Then 1 µL of helper phage (VCSM13, titer: 1.2*1013) was added to each sample. The remaining steps were as described for the phage production from libraries.

Phage display capture selection on peptides

To select phages that bind to non-candidalysin peptides, an additional step has been performed. This step was necessary as selections on small – FLAG tagged peptides may result in false positive results because the selected phages recognize the capturing antibody, in this case the anti FLAG antibody, but not the peptides of interest. The additional step was to substract from the original phage libraries all phages that have affinity to the capturing antibody, in particular phages that bind to the paratope of this capturing antibody. After removal of these phages according to methods known to a person skilled in the art, the subtracted phage libraries can be used to select phages that bind specifically to a non-candidalysin peptide.

Adsorption of peptides to hydrophilic or hydrophobic surfaces for phage-display selections remains problematic because adsorption itself might be inefficient or restrain the peptides in non-natural conformations. To keep the peptides in solution, selections on antibody captured peptide were performed in accordance to Verheesen and Laeremans, 2012 (Verheesen and Laeremans, 2012. Methods Mol Biol 911: 81-104). In brief: The wells of flat bottom hydrophilic polystyrene 96-well plates (Nunc Maxisorp #442404, Thermo Scientific) were coated with 100 µL/well of 7,5 µg/mL mouse-anti-FLAG M2 antibody (#F3165, Sigma) in PBS overnight at 4°C. After three washes with PBS and blocking with 4% dried skimmed milk (Marvel) in PBS (4% MPBS) for 1 h the wells were incubated at RT with 100 µL/well of 5 µM peptide

solution in 1% MPBS on a platform shaker (Titramax 100, Heidolph Instruments) at 900 rpm (control wells were incubated solely with 1% MPBS). Subsequently, the peptide solutions were removed from the anti-flag coated wells and 100 µL/well of pre-incubated phage mixes were added to the corresponding wells with captured peptides or control wells followed by incubation for 1 h at room temperature with shaking at 900 rpm on the platform shaker. The pre-incubated phages were obtained by diluting purified phages produced from two libraries (#7, #11) 1:5 in 2,5% MPBS and pre-incubated for 30 min at room temperature on a rotating shaker at 20 rpm (Loopster basic, IKA). For the 2nd-round of selection the input phages, which had been derived from the output phages of the 1st-round of selection, were diluted 1:5 in 12,5% mouse serum (v/v) in 2,5% MPBS and pre-incubated for 30 min at room temperature on a rotating shaker at 20 rpm.

After incubation, wells were washed thirteen times with PBS-TWEEN® 20 (0,05%, v/v, Serva Electrophoresis) followed by 10 min incubation on a platform shaker at 900 rpm and ten more washes with PBS-TWEEN® 20 ending with another 10 min of incubation on the platform shaker at 900 rpm. Then the wells were washed three times with PBS to remove TWEEN. For elution of the bound phages, 100 µL/well of 0.1 M triethanolamine-solution (TEA) was added followed by incubation for 15 min on the platform shaker at 900 rpm. For neutralisation these elutions were transferred to 50 µL/well of 1 M Tris/HCl (pH 7.5). From the neutralised phages from the elutions (outputs) and from the excess pre-incubated phage mixes (inputs) serial 1:10 dilutions were prepared in PBS of which 10 µL were used to infect 90 µL of a fresh *E. coli* TG1 culture with an OD600nm of 0.5 (culture prepared by a 1:100 dilution of a fresh overnight culture in 50 mL 2×YT medium, growth at 37°C and 200 rpm for 105 min, Series 25 Incubator Shaker, New Brunswick Scientific) for phage in- and output quantification. Besides a negative control for TG1 without any treatment controls for PBS, Tris/HCcl (NB) and TEA with Tris/HCl (2:1, ENB) were prepared of which 10 µL were transferred to 90 µL of the same E. coli TG1 culture to ensure that the culture or the buffers had not been contaminated. After 30 min of incubation of the infected *E. coli* TG1 samples at 37°C without shaking 5 µL of each dilution sample were

spotted on LB/agar/glucose2%/ampicillin100 µg/ml plates (Roth) and incubated overnight at 37°C.

Of note, for each selection sample 75 µL of the neutralised phages from the elutions were used to infect 500 µL of the same *E. coli* TG1 culture for storage of the phage outputs. After 30 min of incubation of the infected E. coli TG1 samples at 37°C without shaking 5 µL of each dilution sample were spotted on LB/agar/glucose2%/ampicillin100 µg/ml plates (Roth) and incubated overnight at 37°C. To the phage output samples 5 mL of 2×YT medium supplemented with 2% glucose and 100 µg/ml ampicillin were added and the phage output samples were incubated overnight at 37°C and 200 rpm (Series 25 Incubator Shaker, New Brunswick Scientific). The next morning glycerol stocks were made of the phage output samples by transfer of 1 ml culture to 0,5 ml 60% glycerol (Roth v/v) and stored at -80°C. The growth of the spotted dilution samples was documented by photographs and based on the number of colonies formed by the different dilutions of output phages, the number of eluted phages from each output and input was calculated.

Preparation of master plates

Master plates were prepared by plating 100 µl of 10-5 dilutions of glycerol stocks from successful selection outputs onto LB/agar/glucose2%/ampicillin100 µg/ml plates (Roth) followed by incubation overnight at 37°C. Single colonies were used to inoculate 80 µL 2×YT medium supplemented with 2% glucose and 100 µg/ml ampicillin within the wells of 96-well polystyrene v-bottom microplates (#651161, Greiner Bio-One). Besides two negative control wells without bacteria (wells H6, H12) a control well with an E. coli TG1 clone that carries a pUR8100 plasmid with an irrelevant VHH (well H5) and plasmid control with an E. coli TG1 clone that carries an empty pUR8100 plasmid without any VHH (well H11) were incorporated into the layout of the master plate. Freshly prepared master plates were incubated overnight at 37°C then 40 µl/well of 60% glycerol were added. Master plates were stored at -80°C.

Preparation of periplasmic extracts

The pUR8100 phagemid's expression cassette contains a N-terminal pelB-leader sequence fused in frame within the open reading frame encoding

VHH. Therefore, VHH expressed in *E. coli* TG1 via pUR8100 is translocated to the periplasm. Periplasmic extracts of induced *E. coli* TG1 cultures were prepared from master plates for screening for antigen binding VHHs by ELISA. A copy plate of the master plate was prepared by transferring 1 µL of each well of the master plate to the corresponding wells of 96-well polystyrene v-bottom microplates (# 651161, Greiner Bio-One) which had been filled with 80 µL 2×YT medium supplemented with 2% glucose and 100 µg/ml ampicillin (Roth). After incubation overnight at 37°C, 10 µl of each well of the copy plate were transferred to the respective wells of a polypropylene 96-well 2mL v-bottom assay block (Costar 3960, Corning Inc.) which had been filled with 900 µl /well 2×YT medium supplemented with 0.1% glucose and 100 µg/ml ampicillin (Roth) and pre-warmed to 37°C. The 96-well assay block was incubated for 3.5 h at 37°C and 200 rpm (Series 25 Incubator Shaker, New Brunswick Scientific), followed by addition of 100 µl/well of a 10 mM IPTG (Thermo Scientifc) solution in 2×YT-medium (Roth) for induction. The incubation was continued overnight at room temperature on vibrating platform shaker (Titramax 100, Heidolph Instruments) at 1350 rpm. The next morning the 96-well plate was centrifuged at 4 400 x g for 10 min resulting in sedimentation of the bacterial cells. The supernatants were discarded and the pellets were resuspended in 120 µl/well PBS. After incubation of the 96-well plate for at least 30 min at -80°C the suspensions were thawed in a water bath at room temperature. The completely thawed supsensions were centrifuged at 4 400 x g for 10 min then the supernatants/ periplasmic extracts were transferred to a 96-well polystyrene v-bottom microplates (# 651161, Greiner Bio-One) and stored at -20°C.

Periplasmic extract ELISA

For ELISAs with the periplasmic extracts derived from master plates, the wells of a flat bottom hydrophilic polystyrene 96-well microplates (Nunc Maxisorp #442404, Thermo Scientific, Waltham, MA) were coated with either 50 µL/well of 3.75 µg/mL mouse-anti-FLAG M2 antibody (#F3165, Sigma) in PBS for peptide capturing or with 50 µL/well 5 µg/mL mouse IgG in PBS for detection of mouse IgG binders. The plates were incubated overnight at 4°C. The next morning, after two washes with PBS, blocking was performed with

200 µL/well of 4% dried skimmed milk (Marvel, New York, NY) in PBS (4% MPBS) for 1 h at room temperature with shaking at 900 rpm on a vibrating platform shaker (Titramax 100, Heidolph Instruments GmbH & CO. KG, Schwabach, Germany). Then the blocking solution was discarded and the anti-FLAG M2 antibody coated wells were incubated with 50 µL/well of 5 µM peptide solution in 1% MPBs for 1 h at room temperature with shaking at 900 rpm on the platform shaker (mouse IgG coated wells were incubated solely with 1% MPBS). After two washes with PBS 50 µL/well of a 1:2 dilution of the periplasmic extracts in 1% MPBS were added to all coated wells (the layout of the master plate was conserved) followed by incubation for 1 h at room temperature and 900 rpm on the platform shaker. Afterwards the wells were washed three times with PBS-Tween 20 (0,05%, v/v , Serva Electrophoresis, Heidelberg, Germany) and incubated with 50 µL/well of a 1:5000 dilution of rabbit-anti-myc tag antibody (Jackson Immunoresearch, Cambridgeshire, UK) in 1% MPBS for 1 h at room temperature with shaking at 900 rpm on the platform shaker, followed by three washes with PBS-Tween 20 (0,05%, v/v). Then all coated wells were incubated with 50 µL/well of a 1:5000 dilution of donkey-anti-rabbit IgG (H+L)-horseradish peroxidase conjugate antibody (# 711-035-152, Jackson Immunoresearch) in 1% MPBS for 1 h at room temperature with shaking at 900 rpm on the platform shaker. Another three washes with PBS-Tween 20 (0,05%, v/v) were performed followed by three washes with PBS. Then 50 µL/well of a 0,8 g/L o-phenylenediamine (Alfa-Aesar, Haverhill, MA) solution (chromogenic horseradish peroxidase substrate, aqueous solution with 50 mM Na_2HPO_4 and 25 mM citric acid, Roth) freshly supplemented with 0,03% H_2O_2 (v/v, Sigma) were added to all coated wells and the microplates were incubated in the dark at room temperature for 25 min. After addition of 25 µL/well 1M H_2SO_4 (Roth) to stop the turnover of the enzyme substrate the absorbances at 490 nm (signal) and 655 nm (background) were determined with a microplate spectrophotometer (Multiskan Go, Thermo Scientific).

Plasmid purifications and DNA sequencing

Plasmid purification were performed with fresh overnight cultures of selected clones which had grown in 5 mL LB (Roth) medium supplemented

with 2% glucose (m/v, Roth) and 100 µg/mL ampicillin (Roth) at 37°C and 200 rpm (Series 25 Incubator Shaker, New Brunswick Scientific). For plasmid purification NucleoSpin Plasmid EasyPure Kit (#740727.250; Macherey-Nagel, GmbH & Co. KG, Düren, Germany) was used according to the manufacturer's instructions. DNA sequencing of VHHs was conducted by the company Macrogen (Amsterdam. The Netherlands) and sequencing samples were prepared with purified plasmids and the M13rev primer according to Macrogen's recommendations.

VHH production and purification

Fresh overnight cultures prepared from glycerol stocks of selected clones which had been grown in 2×YT medium supplemented with 2% glucose (w/v) and 100 µg/ml ampicillin at 37°C and 200 rpm (Series 25 Incubator Shaker, New Brunswick Scientific) were diluted 1:100 in 800 ml 2×TY with 0.1% glucose (w/v) and 100 µg/ml ampicillin (prewarmed to 37°C) an incubated under the same conditions as the overnight culture until they had reached an OD600nm between 0.6 and 0.9. Then the cultures were induced by addition of IPTG to a final concentration of 1 mM and incubation was continued for 4 h. After sedimentation (7 000 x g, 15 min) the supernatants were discarded and the bacterial pellets were resuspended in 30 mL PBS and stored at least 30 min at -80°C. Then the suspensions were thawed in a water bath at room temperature and centrifuged at 6 500 x g for 10 min. The resulting supernatants (periplasmic extracts) were collected, mixed with 750 µL/sample of ddH2O and PBS washed iminodiacetic acid agarose/Co2+ beads (Roth, #1235.2) for Immobilized metal ion affinity chromatography (IMAC) purification and incubated on a rotating shaker at 20 rpm (Loopster basic, IKA-Works, Staufen im Breisgau, Germany) for 30 min at room temperature. Then the beads were collected by centrifugation (700 x g, 2 min) and washed one time with 50 mL PBS-Tween 20 (0,05%, v/v , Serva Electrophoresis) followed by two washes with 50 mL PBS (pH 7.4) and resuspension in 10 mL PBS. The bead suspensions were transferred into chromatography columns (Poly Prep, # 731-1550, BioRad, Hercules, CA) and rinsed with 10 mL PBS. Two pre-elutions were performed with 1 mL of 15 mM imidazole in PBS and 400 µL of 150 mM imidazole in PBS which both were discarded. Then three

subsequent elution fractions were collected by rinsing the column with 200 µL of 150 mM imidazole in PBS, 600 µL of 150 mM imidazole in PBS and 400 µL of 150 mM imidazole in PBS. The first and the last elution were combined. The two resulting fractions per sample were dialysed in a regenerated cellulose membrane (MWCO: 3.5 kDA, #5015-19, Membrane Filtration Products Inc., Seguin, TX) against PBS two times for 1 h at room temperature and one time overnight at 4°C to deplete imidazole. The absorbances of the dialysed fractions were measure at 280 nm spectrophotometrically (Multiskan Go, Thermo Scientific) and with the VHH clone specific extinction coefficients the concentrations of the fractions were calculated. On a case-by-case basis the two fractions of a sample were combined. Purity of the VHHs was verified by SDS-PAGE gel electrophoresis.

SDS-PAGE

IMAC purified VHHs were analysed for their purity with the help of discontinuous sodium dodecylsulphate-polyacrylamide gelelectrophoresis (SDS-PAGE). Separation and stacking gel solutions had a concentration of 15% (pH 8.8) or 5% (pH 6.8) acrylamide, respectively. Discontinuous SDS-PAGE mini gels with a thickness of 0,75 mm were made by polymerization of separation and stacking gel solutions within the Mini-PROTEAN 3 Multi-Casting Chamber (BioRad). The composition of the running gel was as follows: 13.5 mL ddH2O, 15 mL 1.5 M TRIS (pH 8.8), 30 mL 30% acrylamide, 600 µL 10% SDS, 600 µL 10% ammonium persulfate (APS) and 60 µL tetramethylethyleendiamine (TEMED). The composition of the stacking gel was as follows: 20.5 mL ddH2O, 3.75 mL 1 M TRIS (pH 6.8), 5 mL 30% acrylamide, 300 µL 10% SDS, 300 µL 10% APS and 30 µL TEMED. After mixing samples with loading buffer (4x stock: 80 mm TRIS, 33% glycerol (v/v), 6.7% SDS (v/v) , 0,3 M DTT, 0,25% ß-mercaptoethanol (m/v), 0,01% bromophenol blue (m/v), 0.01% orange G (m/v)) and loading samples to the stacking gel, separation was achieved by using a PowerPack basic (Bio-Rad) at 80 V constant until the samples had passed the stacking gel, then 180 V and separation was continued until the dye front had reached the end of the gel. For staining of gels PAGEblue protein staining solution (Thermo Scientific) was used according to the manufacturer's instructions.

Dose-response ELISA

For determination of the apparent affinity of the VHH to candidalysin or non-candidalysin Ece1p peptides, dose-response ELISAs against the M2 antibody captured FLAG-tagged peptide variants were performed. The dose-response ELISAs were conducted in the same way as the periplasmic extract ELISA with the following modifications: The VHHs were not tested against mouse IgG (an anti-FLAG M2 antibody control was incorporated). In addition dose-response ELISAs were conducted with both candidalysin variants (N- or C-terminal FLAG tagged), candidalysin without a tag and recombinantly produced Ece1p (rEce1p),which were coated directly to the wells of a microplate overnight at 4 °C (application of 100 µL/well of a 2.5 µM peptide solution in PBS). Instead of periplasmic extracts serial 1:10 dilutions of IMAC purified VHH were applied (dilution range from 10000 nM – 0,01 nM in 1% MPBS, application of 100 µL/well). Besides rabbit-anti-myc tag antibody (Jackson Immunoresearch, Cambridgeshire, UK) also polyclonal rabbit-anti-VHH (QVQ, dilution 1:1000 in 1% MPBS) was used as a secondary antibody.All volumes added to wells of the microplates were doubled.

Subcloning of VHH coding DNA sequences

After production, purification and testing in dose-response ELISA selected VHH sequences were subcloned from vector pUR8100 to the production vector pMEK219, which yields higher production levels and purity due to the lack of pIII fusion products. Therefore, purified pUR8100 plasmids, which contained the sequences of interest were sequentially digested with the restriction enzymes SfiI and Eco91I (both from Thermo Scientific) according to the manufacturer's instructions. Digested VHH inserts were separated from the vector by DNA-gelelectrophoresis (1% agarose (Roth),TBE buffer (Roth), 80V constant) and extracted from the gel with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, # 740609.250) according to the manufacturer's instructions. Digested VHH insert was ligated with pMEK219 vector hydrolysed with the same restriction enzymes by preparing samples with T4-DNA ligase (Thermo Scientific) according to the manufacturer's instruction. The ligation samples were transformed into chemically competent E. coli TG1 cells with the heat-shock method. In short, thawing of

calcium competent cells on ice, addition of 5-10 µL ligation sample, incubation for 5 min on ice, heat shock for 2 min at 42°C in a water bath. incubation for 5 min on ice and spread on LB/agar/glucose2%/ampicillin100 µg/ml plates for growth overnight at 37°C. The next day single colonies were analysed by colony PCR with the M13 primer set for the presence of an insert. Plasmid purifications were performed for the positive clones and plasmid samples with M13rev primer were sent for sequencing to verify the success of the subcloning efforts.

DNA agarose gel electrophoresis

Agarose gels were prepared by dissolving 1% agarose (w/v) in 0,5x TBE buffer (Roth) and adding 0.4 µg/mL ethidium bromide. Samples were mixed with 6x DNA loading dye (Thermo Scientific) before application. Separation was achieved with constant voltage of 5-8 V per cm distance between cathode and anode of the gel chamber. The running buffer was 0,5x TBE buffer and separated bands were visualized by exposition to UV radiation on a transilluminator.

Colony PCR

Colony PCRs were performed to identify VHH insert carrying clones after subcloning from pUR8100 to pMEK219. PCR samples were composed of a colony sample resulting from transformation and 6.95 µL ddH2O, 1 µL 10x Dream Taq green buffer (Thermo Scientific), 1 µL 2 mM dNTP mix, 0.5 µL 5 µM M13 forward primer (5'-GTAAAACGACGGCCAG-3'), 0.5 µL 5 µM M13 reverse primer (5'-CAGGAAACAGCTATGAC-3') , 0.05 µL Dream Taq DNA polymerase (5 U/µL, Thermo Scientific). PCR conditions were 5 min 95°C/29 cycles of 30 s 95°C - 30 s 55°C - 45 s 72°C/ 10 min 72°C. PCR products were analyzed by DNA agarose gelelectrophoresis, amplicon size of a positive clone was expected to be about 700 bp. Colony samples were grown in parallel in 2×YT medium supplemented with 2% glucose and 100 µg/ml ampicillin at 37°C. Positive clones were prepared for plasmid purifications for DNA sequencing.

Preparation of calcium competent E. coli TG1 cells

4 mL of an overnight cultures of *E. coli* TG1 in 2×YT were used to inoculate 400 mL of fresh (prewarmed to 37°C) 2× YT medium. The culture

was grown at 37°C while shaking until it reached an OD600nm between 0,3 and 0,6. Then the culture was chilled on ice for 20 min. All solutions and vessels used had been pre chilled to 4°C and were stored on ice during the whole procedure. Cells were collected by centrifugation in centrifuge tubes at 4°C, 3000 × g for 15 min. The Supernatant was discarded and cells were resuspended in 400 mL 100 mM MgCl2 followed by centrifugation at 4°C, 2000 × g for 15 min. Again the supernatant was discarded; the cells were resuspended in 200 mL 100 mM CaCl2 and incubated on ice for 25 min. Then cells were collected by centrifugation at 4°C, 2000 × g for 15 min. The supernatant was discarded and the pellet was resuspended in 50 mL of 85 mM CaCl2 with 15% glycerol followed by the final centrifugation step at 4°C, 2000 × g for 15 min. After that the supernatant was discarded and the pellet was resuspended in 2 mL of 85 mM CaCl2 with 15% glycerol. Finally the suspension was divided into aliquots in 1,5 mL reaction tubes and stored at -80°C until use.

Recombinant expression and purification of Ece1 in E. coli

N-terminally tagged Ece1p was expressed in *E. coli*. IMAC purification was performed with the help of a N-terminal hexa-histidine tag, which had been fused to the Ece1 protein coding gene fragment.

Recombinant expression and purification of a candidalysin bihead VHH construct in yeast

The candidalysin bihead Cal-F1-10 GS linker - Cal-H1 gene was ordered via Twist Bioscience (San Francisco, USA). The construct was ordered as a non-cloned gene with flanking cloning sites for restriction enzymes SacI and BstEII. The F1-H1 VHH gene fragment was restricted with these enzymes and after cleaning of the restricted DNA the gene was ligated in the pYQVQ11 (Heukers et al., 2019. Antibodies 8: 26), which provides the VHH with a C-terminal C-Direct tag containing a free thiol (cysteine) and an EPEA (Glu, Pro, Glu, Ala) purification tag (C-tag, Thermo Fisher Scientific).

The pYQVQ11 plasmid containing the F1-H1 gene was transformed through heat-shock into the *Escherichia coli* strain DH5α. The colonies were allowed to grow overnight at 37 °C on LB agar plates supplemented with 2% glucose and 100 µg/ml ampicillin.

Six transformants were collected from the plate and grown again overnight at 37 °C with a rotation speed of 140 RPM in LB medium supplemented with 2% glucose and 1:2000 ampicillin for DNA isolation. Prior to DNA isolation the plasmids of the transformants were controlled by multiplying the VHH gene with PCR using yeast forward and reverse primers annealing on pYQVQ11. The PCR product was analysed on 1% agarose gel to check for the presence of the F1-H1 gene.

After assuring that the transformants indeed contained the desired gene, DNA was isolated from two of the transformants using the nucleospin plasmid easypure kit (Machery Nagel). The isolated DNA was send for sequencing to verify the correct insert was ligated into the vector. The sequencing results were aligned against the initial F1-H1 gene sequence and analysed and only when sequence was correct the next step was performed.

The isolated plasmid DNA was transformed into *Saccharomyces cerevisiae* strain VWK18. Prior to transformation, yeast cells were prepared by growing the cells for 24 hours at 30 °C with a rotation speed of 140 RPM in Yeast Peptone medium supplemented with 2% glucose. The cells were collected by centrifugation at 5000 RCF for 10 minutes. The DNA together with the transformation mix (50% (w/v) PEG3350, 1M LiAc, 5 mg/ml Fish sperm carrier DNA) and yeast cells transformed through the heat-shock method transforming the plasmid DNA into the yeast cell. The VWK18 strain was grown for 2 – 4 days on a selective minimal medium plate (Yeast Nitrogen Base agar supplemented with 2% glucose) to form colonies.
To test the production of the bihead two transformants are inoculated in Yeast Nitrogen Base medium supplemented with 2% glucose lacking leucine and grown for 24 hours at 30°C with a rotation speed of 140 RPM, thereby selecting for yeast cells with the pYQVQ11 which contains the gene for leucine which is essential for the yeast to grow. The overnight culture is transferred to a rich medium where the culture was diluted 10 times in Yeast Peptone medium supplemented with 2% glucose and 1% galactose. The culture was grown for 64 hours at 30°C with a rotation speed of 140 RPM. The optical density at 600 nm of the culture was measured, where the desired optical density of > 20 was reached. The supernatant was collect and filter

sterilized after centrifugation at 5000 RCF for 15 minutes. The produced VHH is excreted by the yeast cell and is present in the supernatant of the culture. The supernatant was analysed on SDS-PAGE for the presence of VHH at 30kDa

DNA was isolated out of the two yeast transformant clones using the nucleospin plasmid easypure kit. The two transformant clones are analysed on genetic scale by sequencing using yeast forward and reverse primers. The sequencing results were aligned against the initial F1-H1 gene sequence and analysed.

The VHH was purified using the tag containing the EPEA sequence and affinity chromatography, on the CaptureSelect™ C-tag column. The purified VHH was buffer exchanged to PBS by desalting membrane. The functionality of the bihead was tested using ELISA.

Recombinant expression and purification of a candidalysin bihead VHH construct in yeast

The candidalysin bihead F1-CAW3A8 gene was ordered via Twist Bioscience. The construct was ordered as a non cloned gene with cloning sites for digestive enzymes SacI and BstEII. The VHH gene was ligated in the desired plasmid pYQVQ11.

The pYQVQ11 plasmid containing the F1-CAW3A8 gene was transformed through heat-shock into the Escherichia coli strain DH5α. The DH5α strain was grown overnight at 37 °C on LB agar plates supplemented with 2% glucose and 100 µg/ml ampicillin.

Six transformants are collected from the plate and grown again overnight at 37 °C with a rotation speed of 140 RPM in LB medium supplemented with 2% glucose and 1:2000 ampicillin for DNA isolation.

DNA was isolated out of two of the transformants using the nucleospin plasmid easypure kit. The isolated DNA was send for sequencing. The sequencing results were aligned against the initial F1-CAW3A8 gene sequence and analysed for correct insert.

The isolated plasmid DNA was transformed into the *Saccharomyces cerevisiae* strain VWK18 (Sagt et al., 1998. Appl Environ Microbiol 64: 316-324). Prior to transformation, yeast cells were prepared by growing the cells

for 24 hours at 30 °C with a rotation speed of 140 RPM in Yeast Peptone medium supplemented with 2% glucose. The cells were collected by centrifugation at 5000 RCF for 10 minutes and resuspended in a transformation mix (50% (w/v) PEG3350, 1M LiAc, 5 mg/ml Fish sperm carrier DNA), together with the DNA. Yeast cells were transformed through heat-shock method. The VWK18 strain was grown for 2 – 4 days on a selective minimal medium plate to form colonies (Yeast Nitrogen Base agar supplemented with 2% glucose).

Two transformant clones were inoculated in Yeast Nitrogen Base medium supplemented with 2% glucose and grown for 24 hours at 30°C with a rotation speed of 140 RPM. The overnight culture was transferred to a rich medium, in which the culture was diluted 10 times in Yeast Peptone medium supplemented with 2% glucose and 1% galactose. The culture was grown for 64 hours at 30°C with a rotation speed of 140 RPM. The optical density at 600 nm of the culture was measured, where a desired optical density of > 20 was reached. The supernatant was collected and filter sterilized after centrifugation at 5000 RCF for 15 minutes. The produced VHH was excreted by the yeast cell and was present in the supernatant of the culture. The supernatant was analysed on SDS-PAGE for the presence of VHH at 30kDa.

DNA was isolated out of the two yeast transformant clones using the nucleospin plasmid easypure kit. The two transformant clones were sequenced and aligned against the initial F1-CAW3A8 gene sequence.

The VHH was purified using affinity chromatography on a CaptureSelect™ C-tag column. The purified VHH was desalted by the use of a desalting membrane. The desalted pure VHH was analysed on SDS-PAGE for the presence of VHH at 30 kDa and the absorbance at 280 nm wavelength was measured for the determination of VHH concentration. As the bihead F1-CAW3A8 VHH also contains the VHH F1 which recognizes Ece1-III-K cells, similar binding properties were expected in dose response ELISA using Ece1-III-K cells.

Western blot

Recombinant Ece1p (rEce1) samples were separated by discontinuous SDS-PAGE (5% or 15% acrylamide for stacking or running gel, respectively).

Separation was conducted at 60 V for 15 min followed by 80 V for 15 min and 100 V for 30 min. After equilibrating the membranes in blotting buffer (25 mM TRIS, 192 mM glycine, 10% methanol, pH 8.5), transfer from the SDS-PAGE gel protein to PVDF membranes (Roche) was conducted at 2.5 mA/cm2 for 30 min with the Owl semi-dry electroblotter (Thermo Scientific) according to the manufacturer's instructions. All PVDF membrane incubations were performed on a rocking platform at room temperature. PVDF membranes were blocked in PBS containing 4% dry milk powder (4% MPBS) for 1 h. Subsequent incubations were performed in 1% MPBS with 1 μM VHH raised against candidalysin (termed CAL1-F1 and CAL1-H1), polyclonal rabbit-anti-VHH purified antibody (QVQ, dilution 1:1000) and goat-anti-rabbit HRP conjugate IgG (#7074S, dilution 1:3000, Cell Signaling Technologoy, Danvers, MA). Finally the membranes were washed 3 times with PBS and signal detection was achieved with ECL Plus Western Blotting substrate (Thermo Scientific) according to the manufacturer's instructions using a CCD chemiluminescence detector (Vilber Lourmat) and exposure time of 6 min.

The steps described herein above to select VHH against ECE1 or peptides derived form ECE1 can be followed to select VHH against non ECE1p proteins present in the cell wall of hyphae of *C. albicans.* This is described in more detail in Example 2.

TR146 cell culture

Human squamous cell carcinoma TR146 cells (ECACC #10032305) were routinely grown in 20 mL DMEM/F-12 (Gibco, Thermo Scientific) with 10% FBS (Gibco, Thermo Scientific, heat inactivated) in 75 cm2 tissue culture flasks with vented caps (#83.3911.002, Sarstedt, Nümbrecht, Germany) at 37°C, 5% CO_2 until they reached confluency. Confluent TR146 cultures were washed with PBS and dry adherent cells were released by addition of 2 mL Accutase (#ACC-1B, Capricorn Scientific GmbH, Ebsdorfergrund, Germany) and incubation for 15 min at 37°C, 5% CO_2. Accutase activity was inhibited by addition of 2 mL DMEM/F-12 with 10% FBS, cells were counted in a hemocytometer and diluted appropriately DMEM/F-12 with 10% FBS for routine culture (1:10-1:2) or experimental application.

Candida albicans strains and culture

The *Candida albicans* strains used in this study are indicated in Table 2. *C. albicans* was routinely grown on YPD agar at 30°C. For overnight cultures YPD broth was inoculated with a colony sample and incubated at 30°, 200 rpm on a platform shaker for 12-16 h.

Immunofluorescence

10^5 oral epithelial cells (TR146 cells, ECACC #10032305) derived from a confluent culture were grown to confluence on round 12 mm coverslips (Roth, #P231.1) in 1 mL DMEM/F-12 with 10% FBS in 24-well flat clear bottom plates (Techno Plastic Products #92424) at 37°C, 5% CO_2 for 2-3 days. After one wash with PBS, TR146 cells were infected with $2*10^4$ yeast cells of an overnight culture of either *C. albicans* M2057 (ece1Δ/Δ) or *C. albicans* M1477 (isogenic wildtype) in 1 mL of DMEM/F-12. For candidalysin exposure, 1 mL of DMEM/F-12 containing candidalysin at sublytic concentrations (16-0.25 µM) were added to TR146 cells instead of yeast cell suspensions. After 6 hours of incubation at 37°C, 5% CO2 samples were washed three times with PBS and fixed with HistoFix (4% buffered formaldehyde, Roth) overnight at 4°C. After three PBS washes samples were blocked with 1% BSA in PBS for 1 h at RT with gentles agitation. Subsequent incubation steps with antibodies were performed in 0.5% BSA. Application volume was 150 µL, all incubations steps were performed for 1 h at RT with gentle agitation on a platform shaker followed by three washes with PBS. The staining protocol after blocking was as follows: Incubation with 1 µM VHH. Incubation with rabbit-anti-VHH IgG polyclonal serum (20 µg/mL, QVQ). Incubation with goat-anti-rabbit Alexa fluor 488 plus conjugate (polyclonal IgG, 5 µg/mL, Invitrogen, Thermo Scientific, ref A32731, lot SH251139) in the dark. After staining the samples were mounted onto microscopy slides with 6 µL ProLong Gold antifade mountant (Invitrogen, Thermo Scientific) incubated overnight at RT in the dark and stored at -20°C until analysis by fluorescence microscopy.

Neutralization assay

$2*10^4$ TR146 cells, derived from a confluent culture were grown to confluence in 200 µL DMEM/F-12) with 10% FBS in the inner wells of 96-well flat clear bottom plates (Techno Plastic Products #92696) at 37°C, 5% CO2 for 1-2 days. The outer wells were filled with PBS. After one wash with PBS,

the cells were infected with $2*10^4$ cells of an overnight culture of either *C. albicans* M2057 (ece1 Δ/Δ) or *C. albicans* M1477 (isogenic wildtype) in 200 µL of DMEM/F-12. Additionally TR146 cells were exposed to 32 µM candidalysin in 200 µL of DMEM/F-12. Before application to TR146 all samples were preincubated for 1 h at 37°C, 5% CO2.

For neutralization assays, the *C. albicans* suspensions or candialysin solutions were pre-incubated with serial dilutions of the VHHs CAL1-F1 or CAL1-H1. After 24 h of incubation at 37°C, 5% CO2 supernatants of the TR146 samples were collected after centrifugation (10 min, 250 ×g). As a measure of cell damage the lactate-dehydrogenase (LDH) release in relation to untreated controls was determined in the supernatants using the Cytotoxicity Detection Kit (LDH, #11644793001, Roche, Mannheim, Germany) according to the manufacturer's instructions.

Results:

A total of 2 sub-families of VHHs that bind Ece1p and peptides derived from Ece1p such as candidalysin, were isolated from libraries created by immunisation of llama's with various morphological forms of *C. albicans* cells, especially hyphae cells. Two sequential rounds of phage display selections were conducted with *Candida albicans* SC5314 immune VHH libraries #7 and #11 on a candidalysin peptide that was either N-terminally (FCL) or C-terminally (CLF) fused to a FLAG protein tag.

During selection on FCL and CLF the peptides have been kept in solution by capture with mouse monoclonal anti-FLAG M2 antibody (Sigma)) which had been coated to polystyrene.

The results of the first round of selection showed significant differences in phage outputs of selections on FCL, CLF or M2 antibody only for library 7 and 11. Outputs for library 7 were slightly higher than for library 11, PBS control outputs were lower than selection outputs. Enrichment of antigen binding phages was confirmed as phage outputs of the 2nd-round for library 7 of selections where roughly 100-1000 times higher than in the 1st-round.

Control selections on anti-FLAG M2 antibody without peptide showed that the phage outputs for selection on captured candidalysin were about 10

times higher than for the corresponding M2 antibody only controls and 10 000 times higher than the PBS controls.

From the outputs of the 2nd-round of selection a master plate was generated and screened by periplasmic extract ELISA for binders to captured candidalysin.

M2 binder elimination for identification of relevant clones was performed because the outputs for 2nd round selections on M2 antibody were at best 10-times lower than captured candidalysin selection outputs, therefore a high proportion of M2 antibody binders was expected.

Sequencing of relevant clones led to identification of two unique VHH sequence families, termed CAL1-F1 and CAL1-H1 (see Figures 4 A, B). After subcloning to production vectors pMEK219 and pMEK222 and recombinant production in *E. coli* followed by IMAC purification both VHHs were characterised.

For estimation of the binding affinities of the VHHs for M2 antibody captured FLAG tagged candidalysin or for the natural coated candidalysin dose-response ELISAs were performed. The apparent affinities in dose-response ELISA differed greatly between captured flag tagged candidalysin and coated natural candidalysin. Both VHHs showed milimolar affinities for captured flag tagged candidalysin. Apparent affinities for coated natural candidalysin where in the micromolar range for CAL1-F1 and nanomolar for CAL1-H1 (see Figure 5).

Binding of the VHHs to recombinant ECE1p was confirmed by dose-response ELISA and Western-blot on recombinantly produced ECE1p (see Figures 6 and 7).

SDS-PAGE as well as Western-blot results show bands with an apparent lower molecular weight than expected for rECE1p (ca. 29 kD) at 25, 15, 10 and below 10 kD (only visible in Western-blot). Analysis of recombinant Ece1p by SDS-PAGE always resulted in bands with an apparent weight of ca. 25, 15 or 10 kDa. See Figure 7.

Immunofluorescence experiments during infection of host cells highlight Ece1p/candidalysin in the tips of *C. albicans* hyphae. VHH CAL1-H1 and CAL1-H1 were both shown to bind to candidalysin and recombinant Ece1p in

ELISA. Therefore we tested if it was possible to localise candidalysin or Ece1p during infection of epithelial cells by *C. albicans* employing the VHHs in immunofluorescence microscopy. For this approach TR146 epithelial cells were exposed to *C. albicans*, after 6 h of incubation the samples were fixed with formaldehyde and subsequently stained for immunofluorescence microscopy. Using CAL1-H1 as a primary antibody no specific signal could be detected by fluorescence microscopy as it was the case for all immunofluorescence experiments conducted with the Ece1 double deletion mutant. Application of CAL1-F1 resulted in stained tips of the hyphal projections of filamentous *C. albicans* cells (see Figure 8).

Exposure of TR146 cells to candidalysin followed by immunofluorescence staining resulted in similar staining patterns of the surfaces of the epithelial cells for both VHHs (see Figure 9). The signal intensity was candidalysin concentration dependent and generally higher for CAL1-H1 compared to CAL1-F1 at equimolar candidalysin concentrations.

Both VHHs protect from candidalysin mediated cell damage. For investigating protective properties of the VHHs damage inhibition assays with human TR146 epithelial cells were performed. TR146 cells were exposed for 24 hours to 10^5 *C. albicans* cells per mL or 32 µM synthesised candidalysin in presence of the VHHs CAL1-F1 or CAL1-H1. As a measure of TR146 cell damage the lactate dehydrogenase release was determined in relation to epithelial cells exposed to the fungus or the toxin in absence of VHHs. The results for *C. albicans* induced damage are shown in Figure 10A and indicate that both VHHs protect in a concentration dependent manner against *C. albicans* induced cell lysis. Both VHHs seem to abolish *C. albicans* induced cell damage at a concentration of 32 µM. While CAL1-H1 is able to reduce cell damage caused by the fungus by approximately 80% at a concentration of 8 µM, CAL1-F1 offers similar protection at a concentration of 4 µM and generally reduces damage better than CAL1-H1 at equimolar concentrations. The results for candidalysin induced cell damage are shown in Figure 10B. While 32 µM CAL1-F1 does not offer significant protection from cell damage induced by 32 µM candidalysin, CAL1-H1 nearly abolishes cell damage, even at a concentration of 2 µM.

The results for specific bi-heads are indicated in Figures 15 and 16. As is clear from these figures, the biheads provide protection against peptide-mediated lysis and against cell-mediated lysis by specific capturing candidalysin.

Example 2 Selection and partial characterization of VHH that recognize proteins involved in adhesin of C. albicans to cells or medical devices

Pathogenic fungi express a number of adhesins to bind to mammalian cells, in particular epithelial cells of the intestine or vagina. Subsequently these pathogenic fungi can secrete cytolytic peptides that destroy the cells to which they adhere. Blocking the adhesion of hyphae of *C. albicans* to these epithelial cells will contribute significantly to prevent translocation to the blood stream. Adhesins can be grouped in three families: the Als, Hwp and Iff/Hyr families (de Groot et al., 2012. Eukaryotic Cell 12: 470–481). Although their sequences are quite different they have more or less the same molecular architecture. The method described below can be followed to select any adhesion.

Als3 is a multifunctional cell surface protein of *C. albicans* which plays a major role in adhesion and invasion of host cells. Als3p is exclusively expressed at high levels during hyphal growth. Als3p enables iron acquisition from host cell ferritin. (Liu and Filler, 2011. Eukaryotic Cell 10: 168–173).

Materials and methods

Preparation of *Candida albicans* cell wall antigens and phage-display selections with *C. albicans* SC5314 immune VHH libraries

Hyphae and germ-tube cells of clinical *Candida albicans* strains (inc. SC5314) were prepared as described here for the *C. albicans* SC5314 immune library creation. For further experiments, viable cells were either used immediately or fixed by overnight incubation in 4% formaldehyde at 4°C. Three types of antigen preparations of these cells were used for phage display selections employing the *C. albicans* immune libraries 7 and 11: (1) Whole viable or formaldehyde inactivated cells, (2) purified cell walls derived from

viable cells, or (3) supernatants of lyticase (Sigma) or zymolyase (Zymo Research) treated viable or formaldehyde inactivated cell suspensions. Purified cells walls were prepared according to Kapteyn et al. 2000. Mol Microbiol 35: 601-611, with modifications. Cells were mechanically broken in 10mM Tris-HCl (pH 7.5 by using zirconia beads and cell homogenizer (FastPrep-MP Biomedicals). The cell debris containing cell walls, plasma membranes and cytoplasm were washed 5 times with 1 M NaCl., followed by three boiling steps at 100°C for 10 min in 500 mM Tris-HCl buffer [pH 7.5], 2 % [w/v] SDS, 0.3 M ß-mercaptoethanol, and 1 mM EDTA, and resuspended in 500 mM Tris-HCl buffer [pH 7.5] containing 2% [w/v] SDS, 0.3 M ß-mercaptoethanol, and 1 mM EDTA). Finally the cell walls were lyophilised and stored at -80°C until further use. Lyticase/zymolyase treatment of whole viable or formaldehyde inactivated cells was conducted by addition of 5-200 units of lyticase/zymolyase to a cell sample containing $2*10^6 - 2*10^8$ cells in 10-70 mM sodium acetate buffer or potassium phosphate buffer (pH 5-8) and incubation at 30-37°C for 30-180 min. Supernatants of treated cell samples were collected after centrifugation (4000-5000 x g, 5 min) and stored at 4°C until use in phage display selections

Phage display selections were performed on samples of these antigen preparations or recombinantly produced proteins in PBS which had been coated overnight at 4°C or for 2 h at RT to flat bottom hydrophilic polystyrene 96-well plates (Nunc Maxisorp #442404, Thermo Scientific). The phage display selections on the coated antigens were performed as described in Example 1. In addition to phage display selections on coated viable or formaldehyde inactivated *C. albicans* cells, these antigen preparations have been employed for selections on cells in suspension. Phage-display selections on cells in suspension were performed according to Romero-Martinez et. al., 2007. FEMS Immunol Medical Microbiol 50: 77–85, with modifications. For each selection sample cell pellets containing 2*106 - 2*108 viable or formaldehyde inactivated *C. albicans* cells were resuspended in 250-400 µL 10 % fish gelatin or 2% BSA and incubated for 1 h at room temperature on a rotation incubator for blocking. Phage (sub)-libraries, which had been produced as described in the methods, were diluted 1:5 in 5% fish gelatin or

1% BSA in PBS and incubated for 30 min at RT on a rotation incubator. Cell pellets were collected by centrifugation (4000-5000 x g, 5 min) and resuspended in 250-400 µL of pre-incubated phage mix. After incubation for 1-2 h on a rotation incubator at RT cell samples were washed 5 x with 1mL PBS-T (0,05% Tween 20) and 5 x with 1 mL PBS. After these washes, selected samples (extended wash samples) were incubated in 1 mL PBS-T on a rotation incubator overnight at 4°C or RT followed by 5 washes with 1 mL PBS. For phage elution selection samples were resuspended in 250-400 µL 0,1 M TEA in ddH2O and incubated for 10-20 min on a rotation incubator RT. Then cells were sedimented by centrifugation (>15 000 x g, 5 min) and the output phage containing supernatants were transferred to 125-200 µL 1M Tris/HCl in ddH2O (pH 7.5) for neutralisation. Infections of *E. coli* TG1 with the output phages were conducted as described in Example 1. From the selection outputs either phages were produced for sequential rounds of selection or single clones were screened for antigen binding by ELISA.

Results

Screening of clones derived from two sequential rounds of selection on supernatant from zymolyase treated, formaldehyde inactivated *C. albicans* hyphae with *C. albicans* SC5314 immune VHH phage libraries led to isolation of hyphae specific VHH CAW3A8. CAW3A8 does not show any affinity for viable or formaldehyde inactivated *C. albicans* yeast cells in ELISA or immunofluorescence microscopy experiments. Binding to viable or formaldehyde inactivated *C. albicans* hyphae was confirmed by ELISA (with an apparent low nanomolar affinity, see Figure 11) and immunofluorescence microscopy experiments. Immunofluorescence microscopy experiments with a set of deletion mutants (see Figure 12 and Tables 3- 5) demonstrated that the antigen is abundant in hyphal elongations and that no binding for an Als3 double deletion mutant could be detected. Whole cell ELISAs were performed with VHH CAW3A8 on viable hyphae of the *C. albicans* mutant strains listed in Tables 3-5. VHH CAW3A8 showed binding in ELISA to all of the hyphae forming mutants with exception of the Als3 double deletion mutant (1843).

Sequence analyses of the selected clones resulted in the amino acid sequences provided in the Table 3. In between brackets behind an amino acid residue are the amino acid residues that differ from the originally identified sequence. These substitutions are also able to bind to Als3.

The method described in Example 2 to select VHH against cell wall protein Als3 was and can be followed for other cell wall proteins of *C. albicans* for which deletion mutants are available. The results depicted in Figure 13 show that VHH were successfully isolated against a number of proteins from of *C. albicans*.

Example 3 Selection of functional VHH using direct screening using the in vivo Galleria test

Whereas the above described example is based on binding of VHH to cell wall proteins of *C. albicans*, binding is not the only route to obtain VHH that have the potential to block infection caused by *C. albicans*. A direct screening on the functionality of the selected VHH is also possible. One such route is based on the moth *Galleria mellonella* [Segal and Frenkel, 2018. J Fungi 4: 21 10.3390/jof4010021; Pereira et al., 2018. J Fungi 4: 128 10.3390/jof4040128). For testing of VHHs in a *G. mellonella C. albicans* infection model for protective features (*G. mellonella*) larvae were maintained according to standard methods (see Fuchs et al., 2010. Virulence 1: 475-482). *C. albicans* cells were derived from fresh overnight cultures and washed in PBS before use. 10^4 to 10^9 *C. albicans* cells suspended in PBS were injected into a foreleg of *G. mellonella*. At different time points endotoxin free VHH in PBS is injected into the foreleg to reach final concentrations of 10 µM VHH or less with regards to the volume of fungal suspension already injected. Alternatively, the fungal suspensions are pre-incubated with 10 µM or less endotoxin free VHH for 15-60 min at 37°C before injection. Untreated or PBS-only, *C. albicans* cells-only, VHH-only and irrelevant-VHH control injected larvae are taken along. The injected larvae were kept in the dark at 37°C. Survival of larvae is documented and analyzed. Results of a single VHH and of a mixture of VHHs on the survival of *G. mellonella* are provided in Figures 14A and 14B.

Table 2. *Candida albicans* strains

mutant ID	name	parent	genotype	reference
parental strain for mutants	BWP17		*ura3::imm434/ura3::imm434 iro1::imm434/iro1::imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*	Wilson et al. 1999
M1477	BWP17 + Clp30	BWP17	wild type, RPS1/rps1::(HIS1 ARG4 URA3)	Zakikhany et al. 2007
M2067	ece1Δ/Δ	BWP17	ece1::HIS1/ece1::ARG4 RPS1/rps1::URA3	Moyes et al. 2016

Table 3. Amino acid sequences of VHH that bind to the adhesion protein Als3 or to Als4.

Table 3A. Amino acid sequences of Als3 binders

EVQLVESGGGLVQAGGSLRLSCSYSGSAIFDVAT[A,S]MA[G]WYRQAPGKQRELVAT[A,S]ITVGGTTNYADSVKGRFTISRDNP[A]R[K,H]NTL[V]YLQMNSLKPEDTAVYYCNS[A]DPRWGNTWGQGTQVTVSS

Table 3B. Amino acid sequences of Als4 binders

E[D]VQLVESGGGLVQPGGSLR[K]LSCAASGRTLSNYG[A]GGWFRQAPGKEREI[F]VAY[A]ISASGKTTHYT[P]DSVKGRFTISRDNAKNTMTLQMNSLKPEDTAVYYCAV[A,L]RRFYGSNWYSSNAYDYWGQGTQVTVSS

Note: In between brackets behind an amino acid residue are the amino acid residues that differ from the originally identified sequence. VHH sequences with these mono- or multiple substitutions are also able to bind to their target: Als3 or Als4. For example: S94 in Table 3A can be substituted by A and this VHH still binds Als3.

Table 4. *Candida albicans* strains and [double] mutations thereof used for selection of VHH against cell wall proteins

Table 4A

***C. albicans* strain**	**Genotype**	**Reference**
SC5314	wild type – whole genome sequence (WGS) data available	WGS-data available at www.candidagenome.org
ATCC2091	clinical isolate /not sequenced	www.atcc.org/products/all/2091.aspx
NGY205	ura3Δ::imm434/ura3Δ::imm434, och1Δ::hisG/och1Δ::hisG, RPS1/rps1Δ::Clp10	Bates et al. (2005), DOI: 10.1074/jbc.M510360200
NGY111	ura3Δ::imm434/ura3Δ::imm434 mnt1-mnt2Δ::hisG/mnt1-mnt2Δ::hisG-URA3-hisG	Munro et al. (2005), DOI: 10.1074/jbc.M411413200
1843	iro1-ura3Δ : : λimm434/iro1-ura3Δ : : λimm434 als3laΔ/als3saΔ-URA3	Zhao et al. (2004), DOI: 10.1099/mic.0.26943-0
C1637	Hyr1 reintegrant, Δura3::imm434/Δura3::imm434, HYR1/Δhyr1::hisG, Δhyr1::hisG::URA3::hisG + Clp10-HYR1	Belmonte et al. (2014), unpublished, University of Aberdeen
C1H7-1A1E2	hwp1Δ::hisG/hwp1Δ::hisG eno1::URA3 URA3	Sundstrom et al . (2002), DOI: 10.1128/IAI.70.6.3281-3283.2002
DSY1768	cht2Δ : : hisG-URA3-hisG/cht2Δ : : hisG	Selvaggini et al. (2004), DOI: 10.1099/mic.0.26661-0
GPY102	utr2Δ::hisG/utr2Δ::hisG crh12Δ::hisG/crh12Δ::hisG crh11Δ::hisG/crh11Δ::hisG-URA3-hisG	Pardini et al. (2006), DOI: 10.1074/jbc.M606361200
Pga29,30,31Δ	pga29/pga30/pga31 Δ:: ARG4 / pga29/pga30/pga31 Δ:: HIS1 , rps1 Δ /RPS1 ::Clp10 (parent strain = BWP17)	Ibe C. et al. (2018), unpublished, University of Aberdeen
M2008	ura3:: imm434/ura3:: imm434 his1::hisG/his1::hisG *pra1::HIS1/pra1::ARG4* +Clp10	Cit ulo et al. (2012), DOI: 10.1371/journal.ppat.1002777
584	ywp1Δ : : ARG4/ywp1Δ : : URA3-dpl200 his1 : : hisG/his1 : : hisG	Granger et al. (2005), DOI: 10.1099/mic.0.27663-0
CAS5	Δphr1::hisG-URA3-hisG/PHR1 Δura3::imm434/Δura3::imm434	Saporito-Irwin et al. (1995), DOI: 10.1128/MCB.15.2.601
CFM-2	Δphr2::hisG/Δphr2::hisG-URA3-hisG Δura3::imm434/Δura3::imm434	Mühlschlegel et al. (1997),DOI: 10.1128/MCB.17.10.5960
RML2	Caecm33Δ : : hisG/Caecm33Δ : : hisG-CaURA3-hisG	Raquel Mart ínez-Lopez et al.(2004), DOI: 10.1099/mic.0.27320-0
CaLC700	mkc1Δ::FRT/mkc1Δ::FRT	LaFayet e et al., (2010), DOI: 10.1371/journal.ppat.1001069
Hyr1Δ	Δhyr1::hisG/Δhyr1::hisG::URA3::hisG	Bailey et al. 1996, DOI: 10.1128/jb.178.18.5353-5360.1996

Table 4B

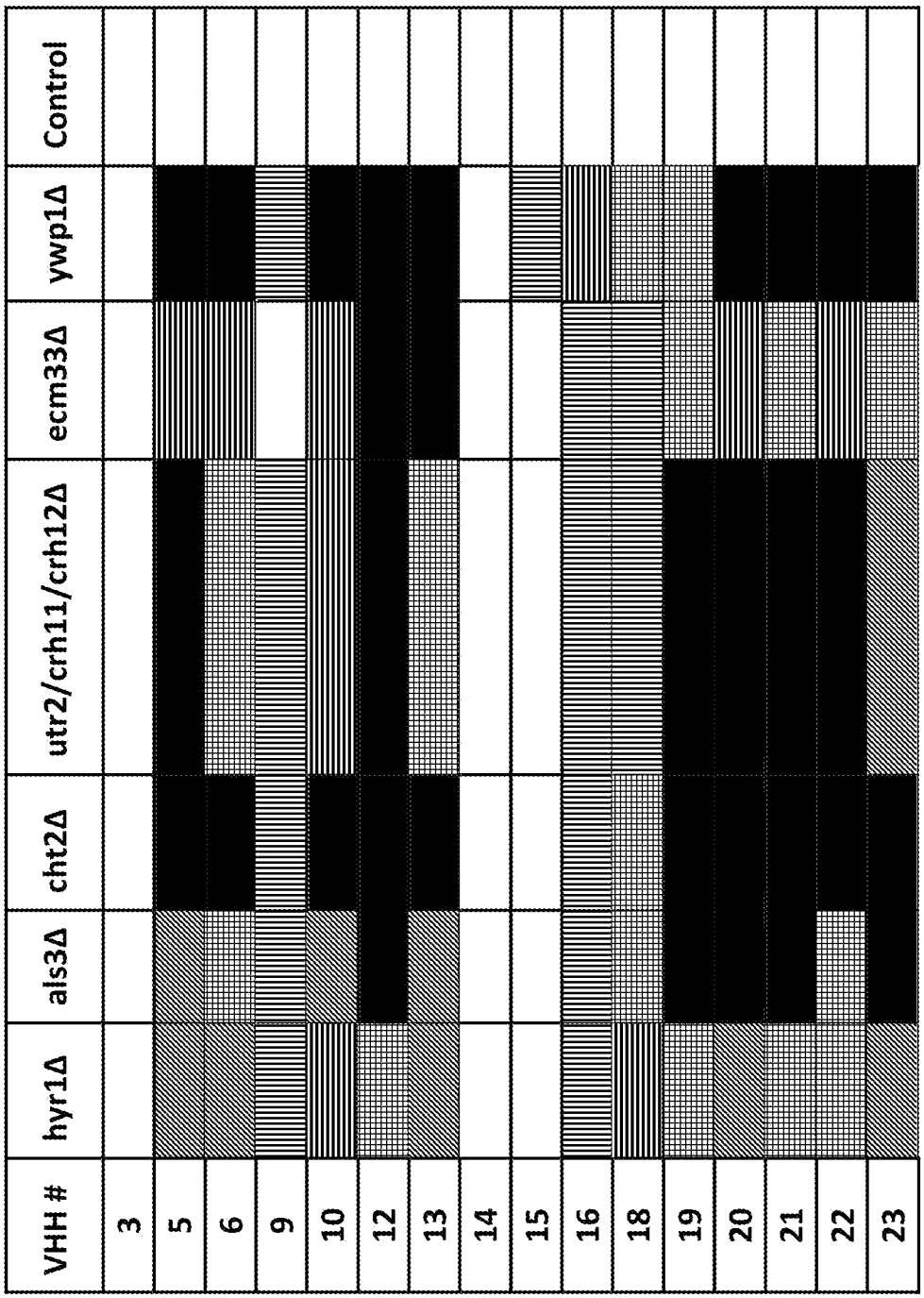
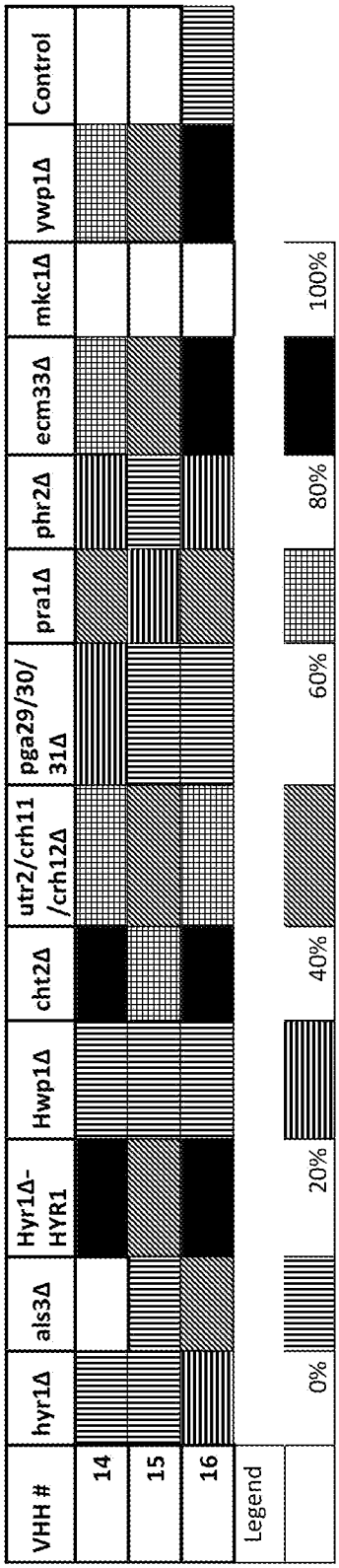

VHH #
hyr1Δ
als3Δ
cht2Δ
utr2/crh11/crh12Δ
ecm33Δ
ywp1Δ
Control
3
5
6
9
10
12
13
14
15
16
18
19
20
21
22
23

Table 4B (continued)



Example 4 Creation of family specific sub-libraries

Subfamily libraries contain VHH related to the parent VHH in terms of partial sequence identity with specificity towards Ece1p and Ece1p-derived peptides such as candidalysin. In comparison to the parent VHH, the family member VHH may have higher affinity and/or neutralising activity towards Ece1p and Ece1p-derived peptides such as candidalysin, as well as improved physico-chemical stability or/and a higher yield during production. Subfamily libraries for the parent VHH antibody CAL-F1 or H1 is created by PCR amplification of VHH antibody DNA sequences as described herein.

By applying PCR primers that anneal to the CDR3 region of the VHH antibodies and employing flanking primers for conserved regions of the plasmid/phagemid (e.g. pUR8100) or the VHH framework regions, amplicons containing related sequences to the parent VHH are created. Up- and/or downstream PCR amplifications using specific CDR3 up- or downstream primers in combination with flanking primers for conserved plasmid or VHH framework regions on whole library plasmid preparations or cDNA samples, will create one or two overlapping amplicons. These amplicons are purified and, in the case of two amplicons, fused by overlap extension PCR. In overlap extension PCR the two overlapping amplicons serve as template and amplification using the flanking primers of the constant plasmid or VHH framework regions yields a fusion product containing the complete VHH sequence due to hybridisation of the overlapping region. The PCR or overlap extension PCR products are digested with restriction endonucleases, purified and ligated into a vector/phagemid (e. g pUR8100). Highly efficient transformation of an appropriate bacterial or fungal strain, such as *Escherichia coli* TG1 by electroporation, yields the subfamily library for application in VHH display selections, such as phage display. These selection will result in isolation of family specific VHH with improved properties over the parent VHH.

Primer pair examples for CAL1-F1 subfamily library amplification from a library containing vector pUR8100 are:

M13rev-for 5'-GAGCGGATAACAATTTCACACAGG-3'

plus F1-CDR3-rev: 5'- TGACCCCACGAAGCATACATCG-3
F1-CDR3-for: 5'-CGATGTATGCTTCGTGGGGTCA-3'
plus MPE25-rev: 5'-TTTCTGTATGGGGTTTTGCTA-3'.

Examples of primer concentrations are 0,05 -5 µM. DNA template concentrations are e. g. 0.1-2000 ng. PCR and overlap extension PCR conditions depend on the primer pair and polymerase applied and can be e.g. 5 min 92-99°C/25-40 cycles of 30 s 92-99°C - 30 s 48-74°C - 30-90 s 74°C/ 1-10 min 68-74°C.

A similar procedure can be used to obtain family members of H1 and of the other VHH antibodies that bind non-candidalysin Ece1p-derived peptides.

Example 5 Construction of vectors that can transform lactic acid bacteria

Transformation of the described vectors results in either secretion of VHH by these lactic acid bacteria in the gastro intestinal tract or in expression of the VHH on their cell walls, thereby creating cells that bind to adhesin molecules on the surface of hyphae of *C. albicans*.

To achieve a long residence time in the intestine lactic acid bacteria have been used that bind to the mucosae of the intestine. Other types of lactic acid bacteria have a long relative residence in the vagina as they bind to the mucosae of the vagina.
Lactic acid bacteria that secrete VHH carry one of the following genetic constructs:

QLF1 -Pamy-SSamy-VHHF1-E tag-TAA-
QLH1 -Pamy-SSamy-VHHH1-E-tag-TAA
QLA1 -Pamy-SSamy-VHHA1-E-tag-TAA

Coding sequences for VHHF1-E tag, VHHH1-E tag and VHHA1 E tag were codon-optimised for expression in lactic acid bacteria and synthesized (see Table 6). These synthesized genes (QLF1 or QLH1 or QLA1) have on their 5' end recognition sequences for the restriction enzyme Cfa1 and on their 3' end,

after the E-tag and a stop codon TAA, for the restriction enzyme Xho1. These restriction sites enable integration of these constructs in pLP401 (Hultberg et al., 2007. BMC Biotechnol 7:58 10.1186/1472-6750-7-58), which plasmid was treated with Cfa1 and Xho1 and the largest fragment was obtained by separation on molecular weight. These fragments of the vector and the VHH inserts were subsequently ligated resulting in pLPQ01 secreted, pLPQ02 secreted and pLPQ03 secreted and transformed to *E. coli* according to standard procedures. After growth of *E. coli* these plasmids were isolated according to standard procedures and checked on integrity by Sanger sequencing.

LAB were transformed with one of the plasmids pLPQ01 or pLPQ02 or pLPQ03 and transformants were selected on MRS plates [Difco] with 3 µg/l erythromycin after cultivating anaerobically at 37 °C for 48 hours.

Table 6. Nucleotide sequences of Pamy [underlined] – SSamy [italic] – [VHH] - E- tag [normal] (A) and the amino acid sequences of SSamy [italic] and E-tag [normal] (B)

Table 6a. Nucleotide sequences

AAGCTTCCAGATCCTGGCGGATCAAATCGAATTGTTGCCGAGAAATATCGCTTTGGTAAT
GATGACTAAAATGTTGCATATGATCACCAACTTTTTTGACAGTAGTTTATCAAATTACTT
GGTCAAAATCAAAGATACTAAACAGCTTCTAAAAAAGCCAATAACCACACGCCTTTGGCG
TGATTATCAGCTTTCAAGTTTCAGTTACTAAAACTAATACTGACTATAAAACAGAAGCAA
AAAAATTTTCGATTTTTATGAAAACGGTTGCAAAGAAGTTAGCAAAAATATATAATTTCT
TTTGAAATTGTTCACTTGGCCAAGCTGCAGTTTCAATATTTTAATAAAGGGGGCAGTAAA
AAGTGAAAAAAAAAGAAAAGTTTCTGGCTTGTTTCTTTTTTAGTTATAGTAGCTAGTGTTT
TCTTTATATCTTTTGGATTTAGCAATCATTCTAAACAAGTTGCTCAAGCGGCTAGTGATA
CGACATCAACTGATCACTCAAGCAATGATACAGCTGATTCTGTTAGCGACGGTGTTATTT
TGCATGCG[.....]GGTGCGCCGGTGCCGTATCCGGATCCGCTGGAACC...

Table 6b. Amino acid sequences

MKKKKSFWLVSFLVIVASVFFISFGFSNHSKQVAQAASDTTSTDHSSNDTADSVSDGVIL
HA.....GAPVPYPDPLE

Lactic acid bacteria that express on their surface VHH have one of the following genetic chemically synthesized constructs:

QLF1 -Pamy-SSamy-VHHF1-E tag-anchor sequence

QLH1 -Pamy-SSamy-VHHH1-E-tag-anchor sequence

QLA1 -Pamy-SSamy-VHHA1-E-tag-anchor sequence

having on their 5' end a sequence recognized by Cfa1 and on their 3'end sequence recognized by Xho1. These constructs were treated with the appropriate restriction enzymes and cloned in pLP401 as described by Hultberg et al., 2007 (Hultberg et al., 2007. BMC Biotechnol 7:58 10.1186/1472-6750-7-58), resulting in pLPQ01 anchored, pLPQ02 anchored and pLPQ03 anchored. After transformation into *E. coli* and isolation, the plasmids were transformed into lactic acid bacteria and transformants were obtained by plating on selection media as described above.

Example 6. Construction of vectors that enable production of single VHH by *S. cerevisiae.*

The anti-candidalysin VHH genes Cal-F1- and Cal-H1 were ordered via Twist Bioscience. The construct was ordered as a non cloned gene with flanking cloning sites for restriction enzymes SacI and BstEII. The F1 or H1 VHH gene fragment was restricted with these enzymes and after cleaning of the restricted DNA the gene was ligated in pYQVQ11.

The pYQVQ11 plasmid containing the F1 or H1 gene was transformed through heat-shock into the *Escherichia coli* strain DH5α. Colonies were allowed to grow overnight at 37 °C on LB agar plates supplemented with 2% glucose and 100 µg/ml ampicillin.

Six transformants were collected from the plate and grown again overnight at 37 °C with a rotation speed of 140 RPM in LB medium supplemented with 2% glucose and 1:2000 ampicillin for DNA isolation. Prior to DNA isolation the transformants plasmid were controlled by multiplying the VHH gene with PCR using yeast forward and reverse primers annealing on pYQVQ11. The PCR product was analysed on 1% agarose gel to check for the presence of the F1 or H1 gene.

After assuring that the transformants indeed contained the desired gene, DNA was isolated from two of the transformants using the nucleospin plasmid easypure kit (Machery Nagel). The isolated DNA was send for sequencing to verify the correct insert was ligated into the vector. The sequencing results were aligned against the initial F1 or H1 genes sequence and analysed and only when sequence was correct the next step was performed.

The isolated plasmid DNA was transformed into *Saccharomyces cerevisiae* strain VWK18 as described in Example 1.

DNA was isolated out of the two yeast transformant clones using the nucleospin plasmid easypure kit. The two transformant clones were analysed by sequencing using yeast forward and reverse primers. The sequencing results were aligned against the initial F1 or H1 gene sequences and analysed.

The VHH was purified using the tag containing the EPEA sequence and affinity chromatography, on the CaptureSelect™ C-tag column. The purified VHH was buffer exchanged to PBS by desalting membrane. The functionality of the individual clones was tested using ELISA similar as described in Example 1.

Example 7 Site specific neutralisation of candidalysin

It is well known that secretion of proteins by hyphae forming yeasts -a process that starts with bud formation- is mainly a process that occurs at the tip of the hyphae. At that site the cell wall is remodelled and new building blocks for the cell wall and extracellular enzymes are transported as Golgi vesicles. These vesicles fuse with the cell membrane at the tip and unload their cargo there. There is sufficient evidence that this route is also followed by *C. albicans*. There is good experimental evidence indicating that the hyphae formation and the expression of certain cell surface proteins such as Als3, Als4, Hwp1 and Ssa1 are essential for *C. albicans* to infect epithelial cells. Moreover Moyes et al. (Moyes et al., 2016. Nature 532: 64-68) provided evidence that ECE1 or processed forms thereof use the same route, which means that ECE1 is mainly secreted at the tip of the hyphae. After secretion

the ECE1 or processed forms thereof diffuse through the relative thin cell wall and enter in a relative thin water-like layer above the extra cellular matrix of epithelial cells. Subsequently, candidalysin, one of the processed forms of ECE1, passes this layer and enters the cell membrane of the epithelial cells and subsequently ensures lysis of these cells (see Figures 3 and 4).

We reasoned that because of the relative high concentration of candidalysin in the cell wall of the tip of the hyphae, this is the most effective place to capture candidalysin by anti candidalysin VHH. Therefore we screened a large number of VHH to find out which VHH, isolated from the libraries #7 and # 11, will bind to molecules characteristic for the hyphae using immunofluorescent techniques and a combination of whole cell ELISA's and *C. albicans* mutants that carry mutations in several genes encoding cell wall proteins (see Table 4).

This resulted in a selection of a number of VHH that bind to Als3, a protein characteristic for the tip of the hyphae and involved in the adhesion of the hyphae to epithelial cells. These VHH are coded VHH 3, VHH 14 [also described as clone 3A8 or VHH CAW3A8), VHH 15 and VHH 16 (see Figure 4B).

Moreover using the same libraries and selection methods clones that bind to Als4 have been obtained (see Figure 4C).

Moreover 2 clones, VHH 1 and 4, recognize biomolecules that are specific for hyphae, but the molecular nature of these antigens has not yet been determined. Anti Als3, anti-Als4 and the 2 unknown hyphae specific biomolecules can serve as anchor for bi-specific VHH that capture Ece1 or processed products thereof, like candidalysin, when passing the cell wall of the hyphae.

Theoretically such bihead should lower the number of candidalysin molecules that passes the ECM of epithelial cells. Even if not all candidalysin molecules are captured by these biheads, their effects can be very large, because only when the concentration of candidalysin at the surface of the target cell is high enough, multimeric forms of candidalysin will be formed and these multimeric forms can lyse the target cell

The next step was testing the hypothesis that capturing of candidalysin by anti-candidalysin VHH was more effective when these VHH's were present in a bi-specific bihead, comprising a VHH binding to Als3, notably VHH 14, 15 and 16 and a VHH binding the candidalysin. Figures 15 and 16 show the protection provided by bihead, when compared to individual VHH's.

Table 7. Whole-cell ELISAs with cell wall mutants and selected VHHs.

The antibodies were tested against formaldehyde-inactivated cells and the absorbance measured at 490nm (subtracting the background 655nm) for yeast cells (a) and hyphae (b). The grey scale for the absorbance was set on the background signal given by the control (no cells). R indicates the signal intensity of the negative controls (0-15%), O (15-30%), Y (30-50%), C (50-70%), L (70-85%), and G the highest signal in the plate (85-100%). The measurements were the average of two technical replicates.

Table 7A

VHH#	*hyr1Δ*	*als3Δ*	*cht2Δ*	*utr2/crh11/ crh12Δ*	*ecm33Δ*	*yup1Δ*	Control
3	R	R	R	R	R	R	R
5	C	C	L	G	Y	G	R
6	C	L	G	L	Y	G	R
9	O	O	O	O	R	O	R
10	Y	C	G	Y	Y	G	R
12	LG	G	G	G	G	G	R
13	L	C	G	L	LG	G	R
14	R	R	R	R	R	R	R
15	R	R	R	R	R	OR	R
16	O	O	YO	O	OR	Y	R
18	Y	L	LG	O	O	G	R
19	LG	G	G	G	LG	G	R
20	LG	G	G	LG	Y	G	R
21	LG	G	G	G	LG	G	R
22	LG	LG	G	G	Y	G	R
23	C	G	G	LG	LG	G	R

Table 7B

VHH#	*hyr1Δ*	*als3Δ*	*hyr1Δ –HYR1*	*hup1Δ*	*cht2Δ*	*utr2/crh11/ crh12Δ*	*pga29/30 /31Δ*	*pra1Δ*	*phr2Δ*	*ecm33Δ*	*mkc1Δ*	*ywp1Δ*	*Control*
14	O	R	G	O	G	L	Y	C	Y	G	R	G	R
15	O	OR	L	OR	LG	C	O	Y	OR	L	R	L	R
16	Y	C	G	O	G	L	O	C	YC	G	R	G	R

Table 8. Cell-based ELISA's of hyphae-selected VHH

Dose response ELISA of selected VHHs against filamentous forms of wild type *C. albicans* SC5314. Serial dilutions of the VHHs (1 μ M, 100, 1nM) were tested. In this table values of 1 nM VHH concentration are presented

		ELISA
VHH #	Specificity	Abs 490nm (AU)
18		0,5
15	ALS3	0,7
22		0,9
16	ALS3	1
5	ALS4	1,2
9	Bud	1,2
3	ALS3	1,4
19		1,55
10	ALS4	1,55
13	ALS4	1,8
23		2
14	ALS3	2,1
2	cell pole	2,1
12	ALS4	2,15
20		2,4
21		2,9
6	ALS4	3,2

Claims

1. A single heavy chain variable domain antibody (VHH) that binds a cell wall component and/or secreted protein, such as Ece1p and/or a peptide derived therefrom, of *Candida* spp., preferably of *C. albicans*.

2. The VHH antibody according to claim 1, which binds and neutralizes a cytolytic pore-forming peptide toxin, termed candidalysin.

3. The VHH antibody of claim 1 or claim 2, comprising CDR1, CDR2 and CDR3 amino acid sequences as depicted in Figure 4a.

4. The VHH antibody of any one of claims 1-3, comprising amino acid sequences as depicted in Figure 4b.

5. The VHH antibody of claim 1 that binds to a non-candidalysin peptide and which antibody reduces or eliminates mammalian cell lysis by Ece1p derived peptides such as candidalysin.

6. The VHH antibody of claim 1, that binds an adhesion protein of *Candida* spp., preferably of *C. albicans*.

7. The VHH antibody according to claim 5 or claim 6, that binds a protein of the Als-, Hpw- and Yff/Hyr family of adhesin molecules of *Candida* spp. preferably *C. albicans,* most preferably to adhesin Als3.

8. The VHH antibody of any one of claims 1-7, that is fused to an immunoglobulin Fc region or functional part thereof, preferably derived from IgGl, IgG2, IgG3, IgG4), more preferably derived from IgG1.

9. The VHH antibody of claim 8, wherein the Fc region or functional part thereof is human or a humanized lama Fc or functional part thereof.

10. A bi- or multi-specific antibody comprising a VHH of any one of claims 1-9.

11. The bi- or multi-specific antibody according to claim 10, wherein the at least two VHHs are non-competing and non-interfering VHHs.

12. The bi- or multi-specific antibody of claim 10 or 11, comprising at least a VHH that recognize a hyphae specific cell wall protein, and a VHH that recognizes ECE1 and an ECE1 derived peptide such as candidalysin.

13. The bi- or multi-specific antibody of claim 10 or 11, which protects *Galleria mellonella* against death after infection with *C. albicans*.

14. The antibody of any one of claims 1-13, for use as a medicament.

15. The antibody of any one of claims 1-13, for use in a method of prophylactic treatment of an immune compromised person that may become infected with *C. albicans*.

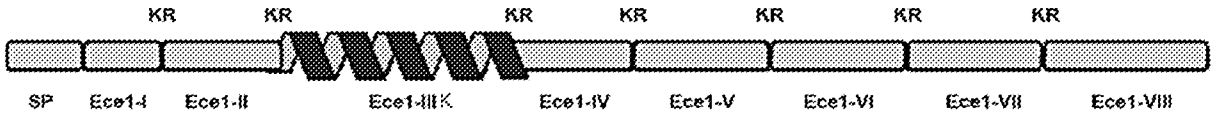
16. A microbicide or apheresis device comprising an antibody of any one of claims 1-7, 10-13.

17. A pharmaceutical composition comprising an antibody of any one of claims 1-13.

18. A host cell, preferably a lactic acid bacterium, expressing an antibody of any one of claims 1-7, 10-13, either as secreted antibody, or as an antibody on its surface.

19. The antibody according to any one of claims 1-13, having a C-terminal extension containing at least the amino acid sequence A-C-A–A. for use in a diagnostic application.

Figure 1




```
Ece1-I      1~ MKFSKIACATVFALSSQAAIIHHAPEFNMKR
Ece1-II    32~ DVAPAAPAAPADQAPTVPAPQEFNTAITKR
               DVAPAAPAAPADQAPTVPAPQEFNTAITKRSIIGIIMGILGNIPQVIQIIMSIVKAFKGNK (57)
Ece1-III   62~ SIIGIIMGILGNIPQVIQIIMSIVKAFKGNKR
               SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK (699) ---------- Candidalysin
               SIIGIIMGILGNIPQVIQIIMSIVKAFKGN (36)
Ece1-IV    94~ EDIDSVVAGIIADMPFVVRAVDTAMTSVASTKR
Ece1-V    127~ DGANDDVANAVVRLPEIVARVATGVQQSIENAKR
               DGANDDVANAVVRLPEIVA (258)
Ece1-VI   161~ DGVPDVGLNLVANAPRLISNVFDGVSETVQQAKR
               DGVPDVGLNLVANAPR (80)
Ece1-VII  195~ DGLEDFLDELLQRLPQLITRSAESALKDSQFVKR
                                  SAESALKDSQFV (250)
               DGLEDFLDELLQRLPQLIT (306)
Ece1-VIII 229~ DAGSVALSNLIKKSIETVGIENAAQIVSERDISSLIEEYFGKA
                                            DISSLIEEYFGKA (16)
```

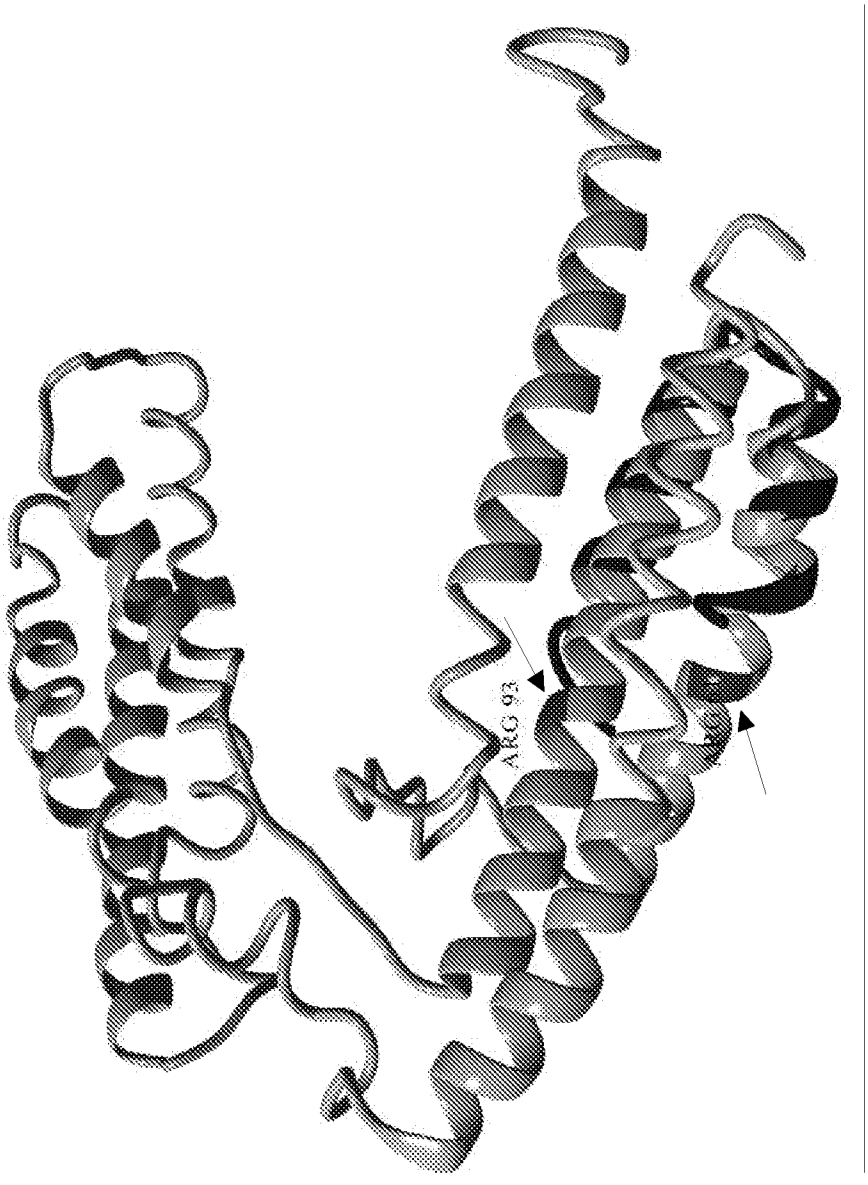


Figure 2

Figure 3

1. SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK
2. SIIGIIMGILGNIPQVIQIIMSIVKAFKGNKR
3. SIIGIIMGILGNIPQVIQIIMSIVKAFKGNKDYKDDDDK
4. DYKDDDDKSIIGIIMGILGNIPQVIQIIMSIVKAFKGNK

Figure 4
4A

F1	CDR1	CDR2	CDR3
	GTIFSLN	TITETTG	YNDYSMYAS
	GSIFSLN	TLTETTG	YNDYSLYAS
	GTIFSIN	TITESTG	YNDYSLYSS
	GSLFSIN	TITETSG	YNEFSMYAS
	GMIFSLN	TITDTTG	YNEYSMFAS
		TLTDTTG	YNEYSLYAS
		TLTDTSG	

H 1	CDR1	CDR2	CDR3
	SGFTLDY	SGFTLDYY	DMVYLCLGPSFYDY
	SGYTLDY	SGFSLDYY	DMVLCGIAPSFYDY
	SGFTLDY		DMVLCGLGPSEYDY
	SGYTLNY		DMVLCGAAPSEYDY
	SGFSLDY		DMVICGLGPSFYDY
	SGFSLNY		DMVICGAGPSFYDY
			DMVYLCLGPSYYDY

4B
Family F1

1. E[D]VQLVESGGGLVQAGGSLRLSCAT[A]SGT[M]IFSLND[A]MGWYRQA
41. PGKQRELVAS[A]ITITE[G]TTGYADS[A]VKGRFTISRDNAKGTVF[Y]L
81. QMNSLT[K,N]PEDTAVYYCNYYNDYSM[L]YASWGQGTQVTVSS

Family H1
1. E[D]VQLVESGGD[G]LVQPGGSLRLSCAV[A]SGFTLDYYT[A]IGWFRQA
41. PGKEREGVSC[W]SGFTLDYYADSVKGRFTISRDNAKN[S]TVY
81. LQMNSLTPEDTAVYYCAA[T]DMVLCGLGPSFYDYWGQGTQVT
121. VSS

4C
Anti-ALS3

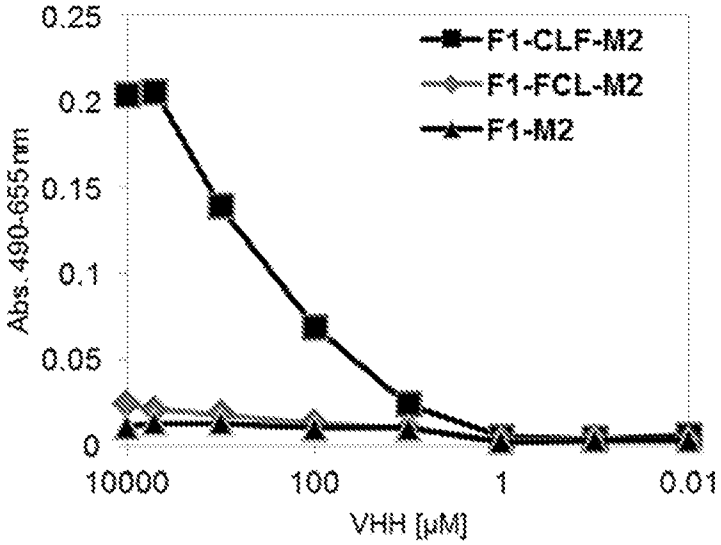
E[D]VQLVESGGGLVQAGGSLRLSCSYSGSAIFDVTMAWYRQAPGKQRELVAT[A]ITVGGTTNYADSVKGRFTISRDNP[A]R[K
]NTLYLQMNSLKPEDTAVYYCNS[A]DPRGNTWGQGTQVTVSS

Anti ALS4

E[D]VQLVESGGGLVQPGGSLR[K]LSCAASGRTLSNYG[A]GGWFRQAPGKEREI[F]VAY[A]ISASGKTTHYT[P]DSVKGRFTIS
RDNAKNTMTLQMNSLKPEDTAVYYCAV[A,L]RRFYGSNWYSSNAYDYWGQGTQVTVSS

Figure 5

A


0.25
0.2
0.15
0.1
0.05
0
Abs. 490-655 nm
10000
100
1
0.01
VHH [µM]
F1-CLF-M2
F1-FCL-M2
F1-M2

B

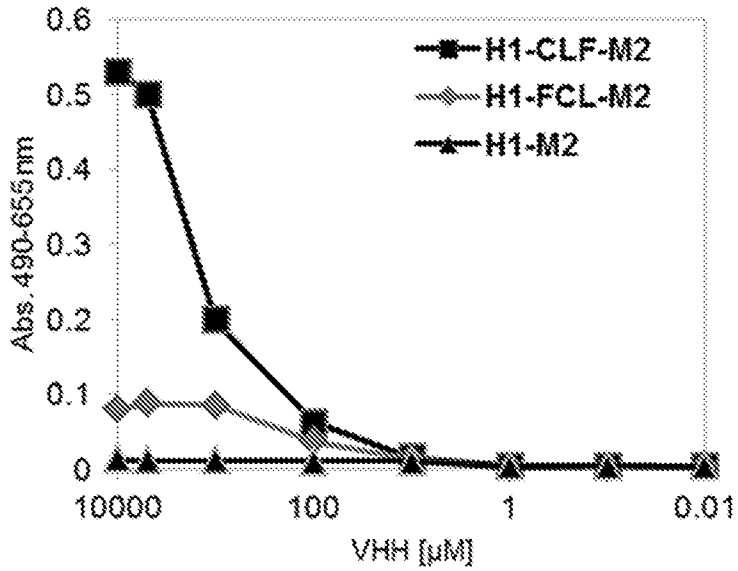
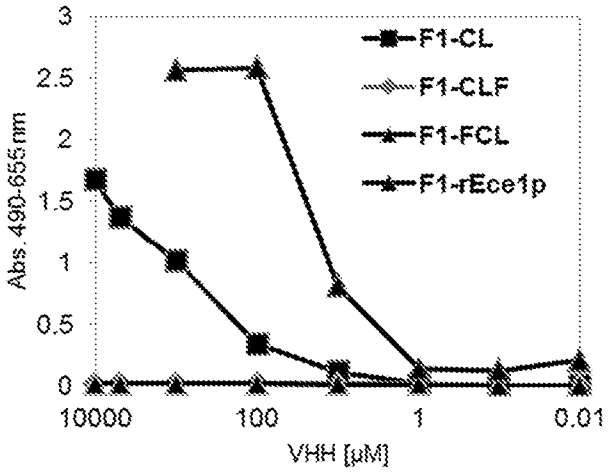

0.6
0.5
0.4
0.3
0.2
0.1
0
Abs. 490-655 nm
10000
100
1
0.01
VHH [µM]
H1-CLF-M2
H1-FCL-M2
H1-M2

Figure 6

A


F1-CL
F1-CLF
F1-FCL
F1-rEce1p
Abs. 490-655 nm
VHH [µM]
3
2.5
2
1.5
1
0.5
0
10000
100
1
0.01

B

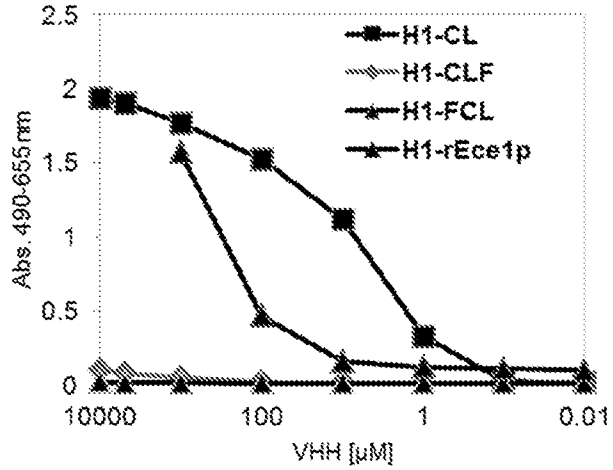
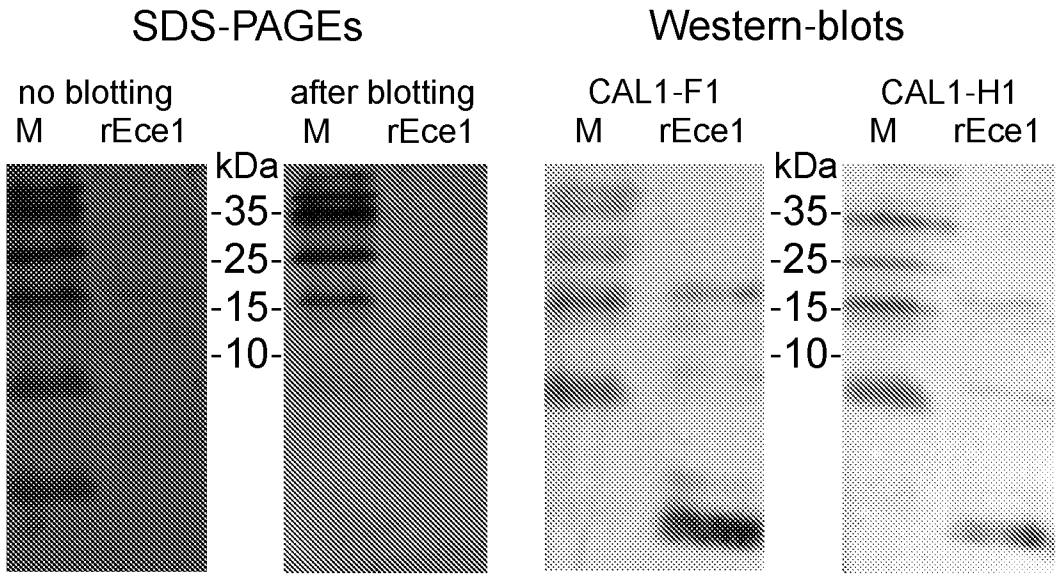

H1-CL
H1-CLF
H1-FCL
H1-rEce1p
Abs. 490-655 nm
VHH [µM]
2.5
2
1.5
1
0.5
0
10000
100
1
0.01

Figure 7


SDS-PAGEs
Western-blots
no blotting
after blotting
CAL1-F1
CAL1-H1
M
rEce1
M
rEce1
M
rEce1
M
rEce1
kDa
-35-
-25-
-15-
-10-
kDa
-35-
-25-
-15-
-10-

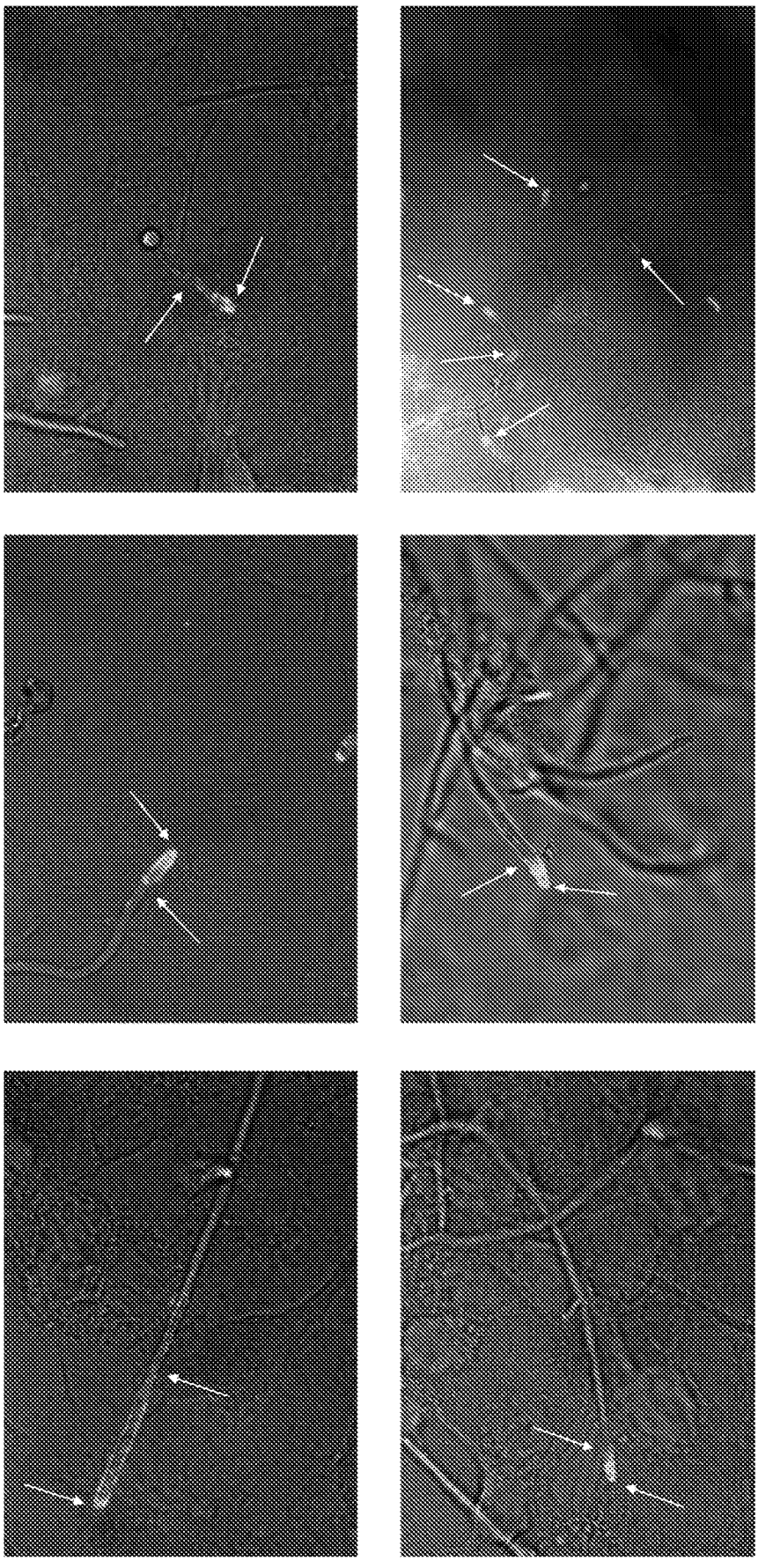


Figure 8

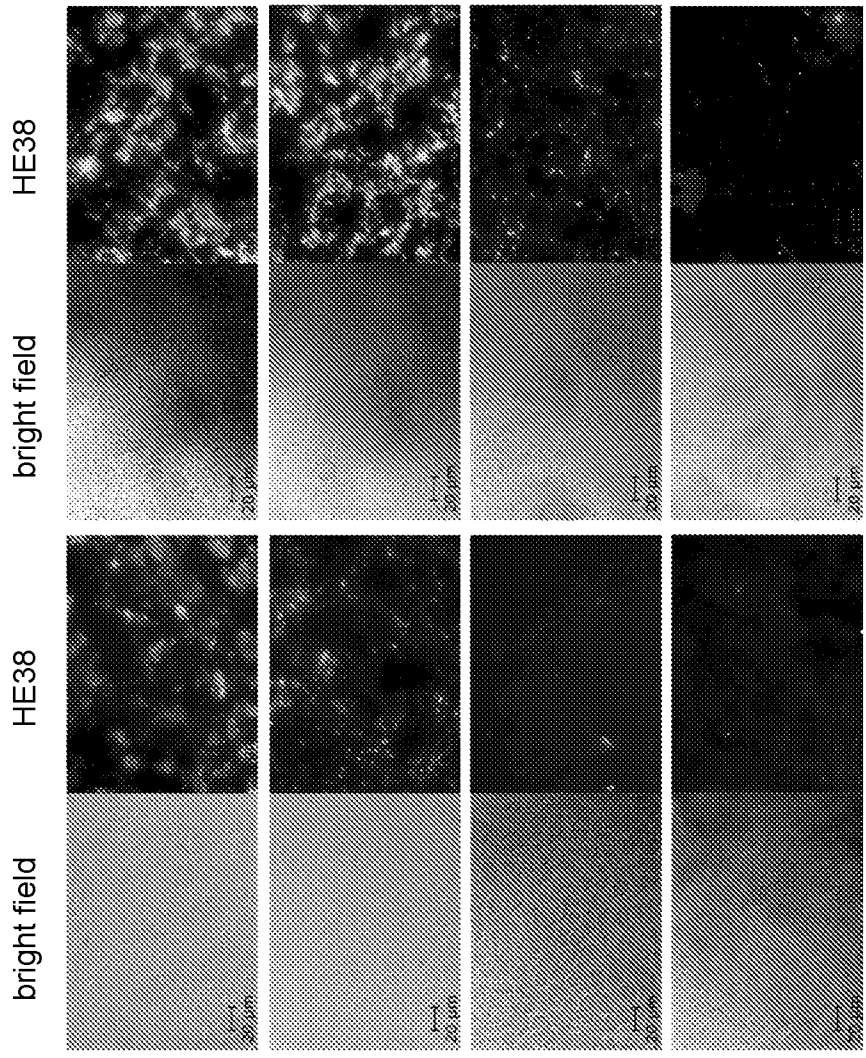
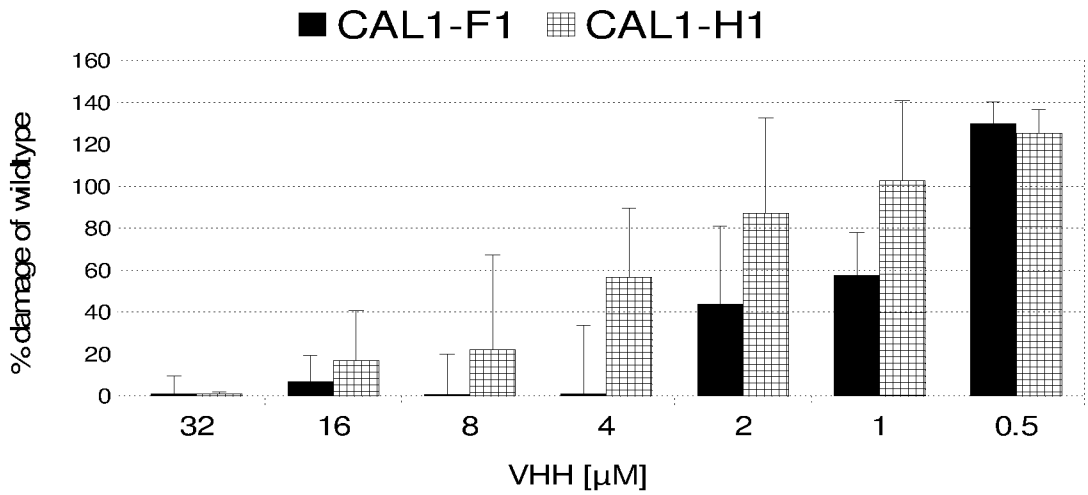

[candida-lysin]
CAL1-F1 [1 µM]
CAL1-H1 [1 µM]
bright field
HE38
bright field
HE38
16 µM
4 µM
1 µM
0.25 µM

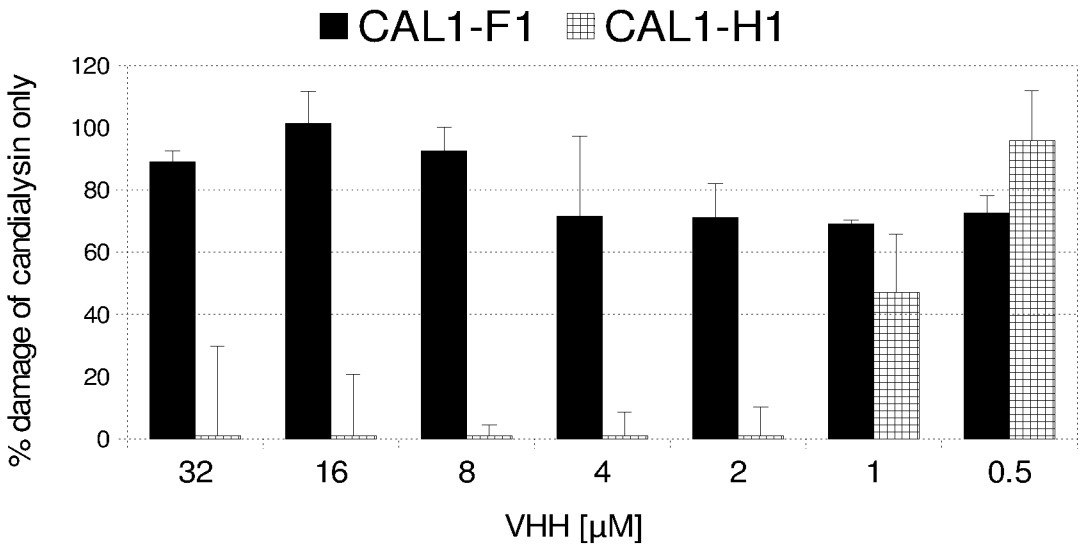
Figure 9

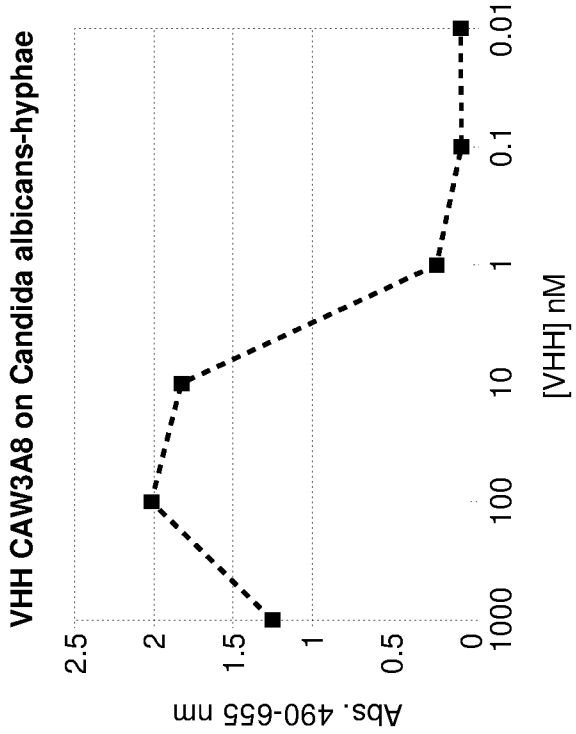
Figure 10

A


CAL1-F1
CAL1-H1
% damage of wildtype
160
140
120
100
80
60
40
20
0
32
16
8
4
2
1
0.5
VHH [µM]

B


CAL1-F1
CAL1-H1
% damage of candialysin only
120
100
80
60
40
20
0
32
16
8
4
2
1
0.5
VHH [µM]


VHH CAW3A8 on Candida albicans-hyphae
Abs. 490-655 nm
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2
1.5
1
0.5
0
1000
100
10
1
0.1
0.01
[VHH] nM

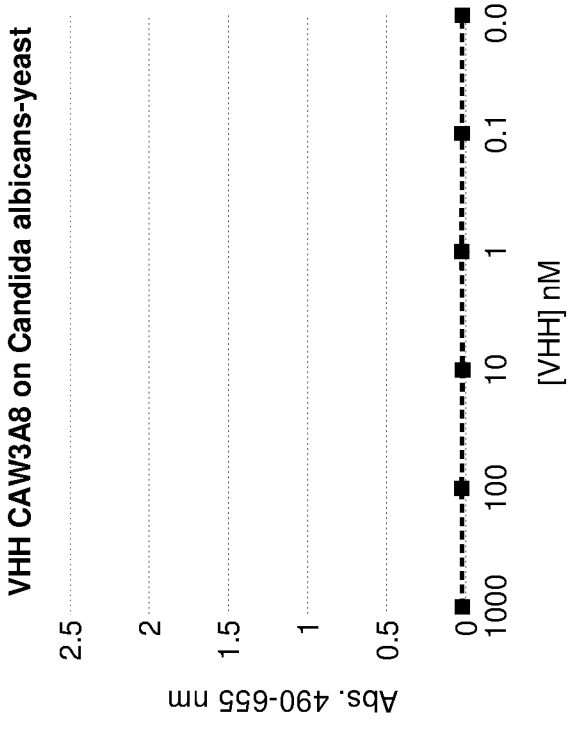

VHH CAW3A8 on Candida albicans-yeast
Abs. 490-655 nm
2.5
2
1.5
1
0.5
0
1000
100
10
1
0.1
0.01
[VHH] nM

Figure 11

Figure 12

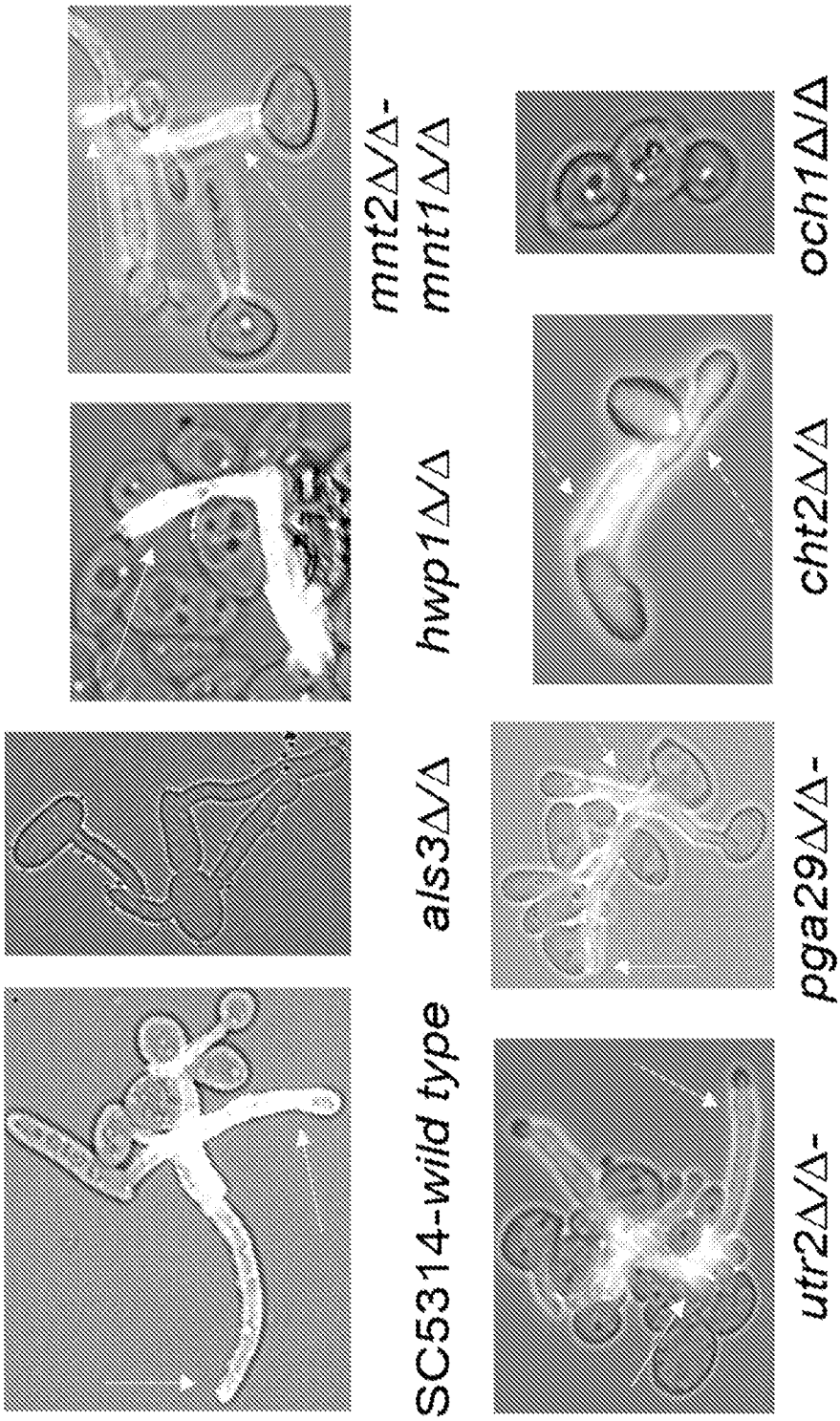
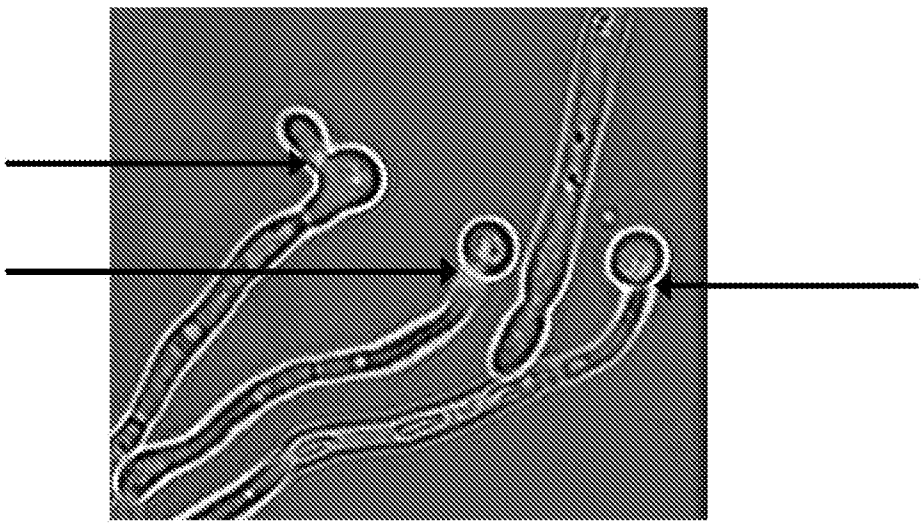

SC5314-wild type
als3Δ/Δ
hwp1Δ/Δ
mnt2Δ/Δ-
mnt1Δ/Δ
utr2Δ/Δ-
chr11Δ/Δ-
chr12-Δ/Δ
pga29Δ/Δ-
pga30Δ/Δ-
pga31Δ/Δ
cht2Δ/Δ
och1Δ/Δ

Figure 13

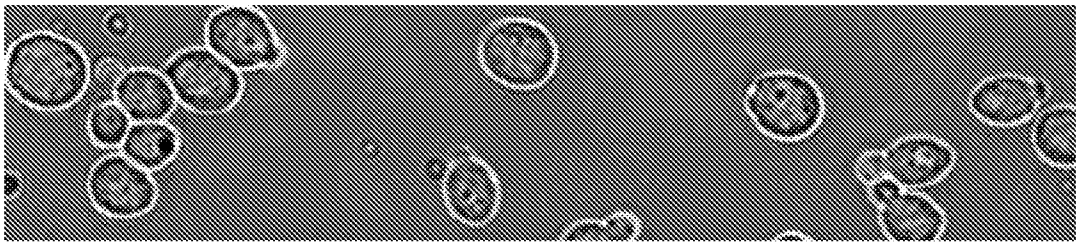
13A

VHH selection			ELISA
Code	VHH #	Specificity	Abs 490nm (AU)
	18		0,5
	15	Als3	0,7
	22		0,9
	16	Als3	1
2 E 9	5	Als4	1,2
2 A 6	9	Bud	1,2
2 C 11	3	Als3	1,4
	19	unknown	1,55
1 E 11	10	Als4	1,55
1 B 11	13	Als4	1,8
	23		2
	14	Als3	2,1
2 D 6	2	cell pole	2,1
1 H 7	12	Als4	2,15
	20		2,4
	21		2,9
2 A 3	6	Als4	3,2

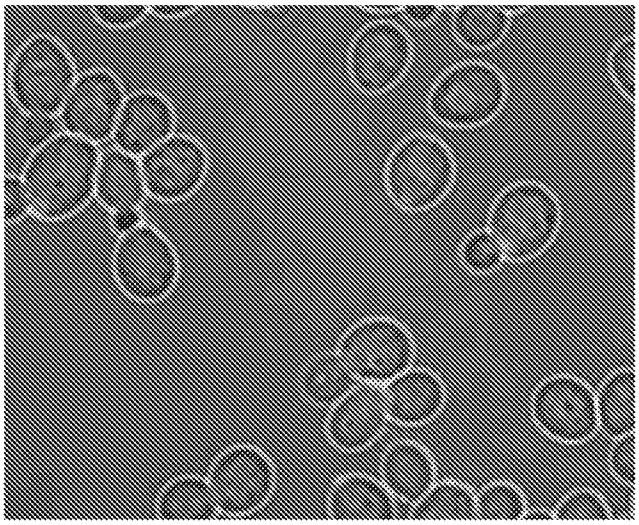
13B



13C



13D



13E

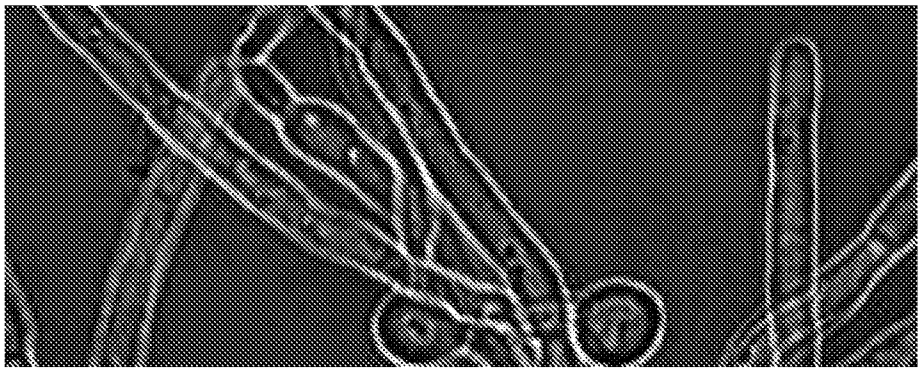
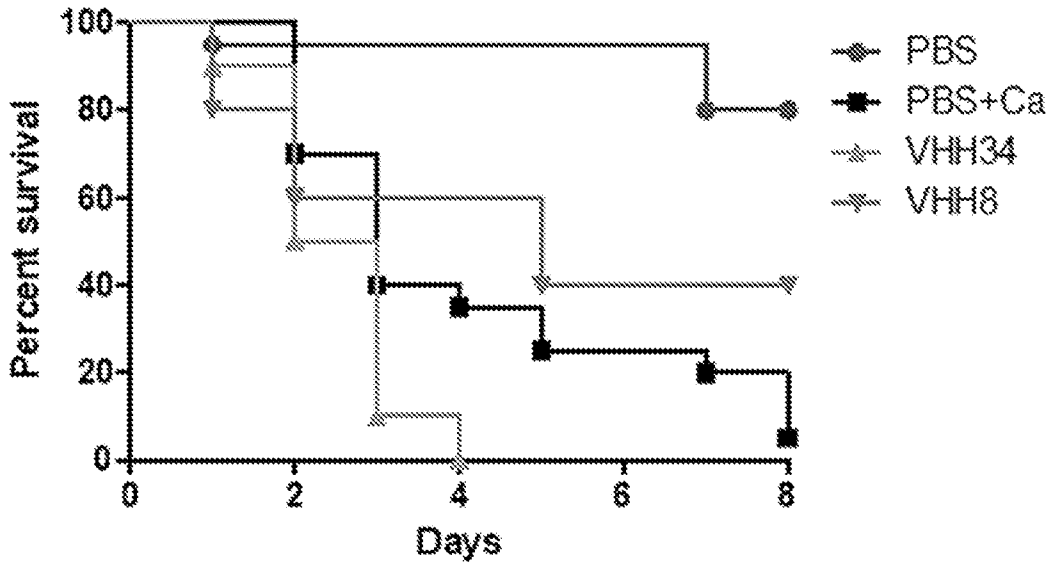


Figure 14

14A



14B

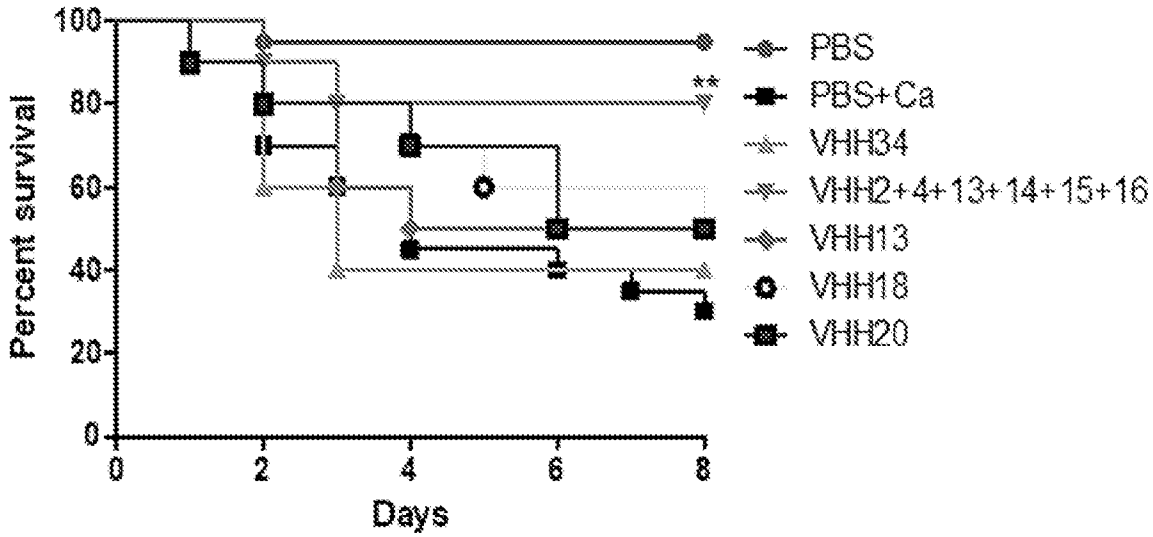


Figure 15

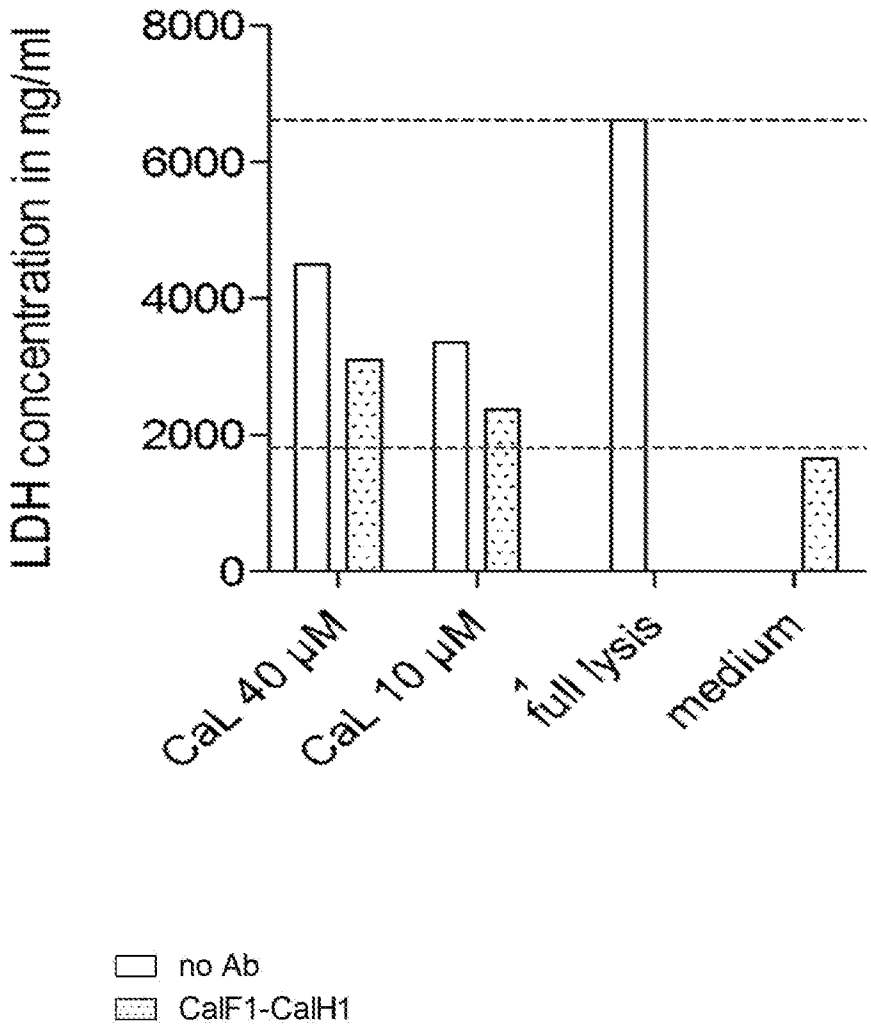
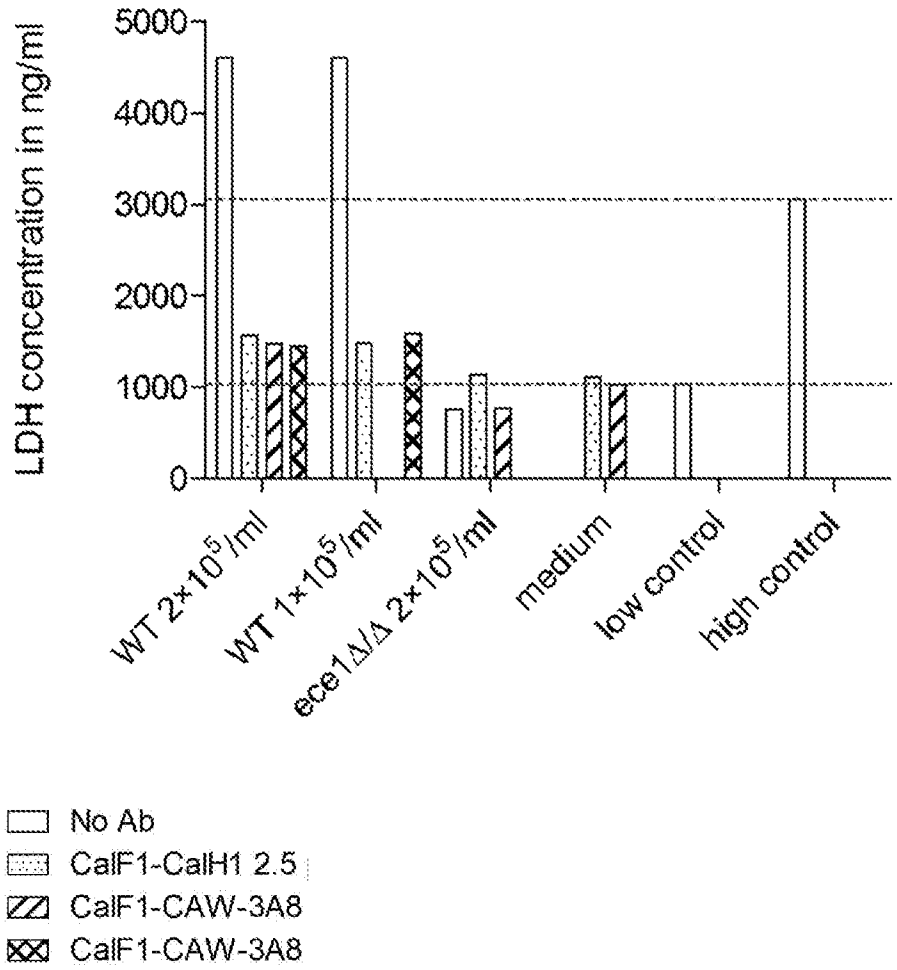

LDH concentration in ng/ml
8000
6000
4000
2000
0
CaL 40 µM
CaL 10 µM
full lysis
medium
no Ab
CalF1-CalH1

Figure 16


LDH concentration in ng/ml
5000
4000
3000
2000
1000
0
WT 2×10^5/ml
WT 1×10^5/ml
ece1Δ/Δ 2×10^5/ml
medium
low control
high control
No Ab
CalF1-CalH1 2.5
CalF1-CAW-3A8
CalF1-CAW-3A8

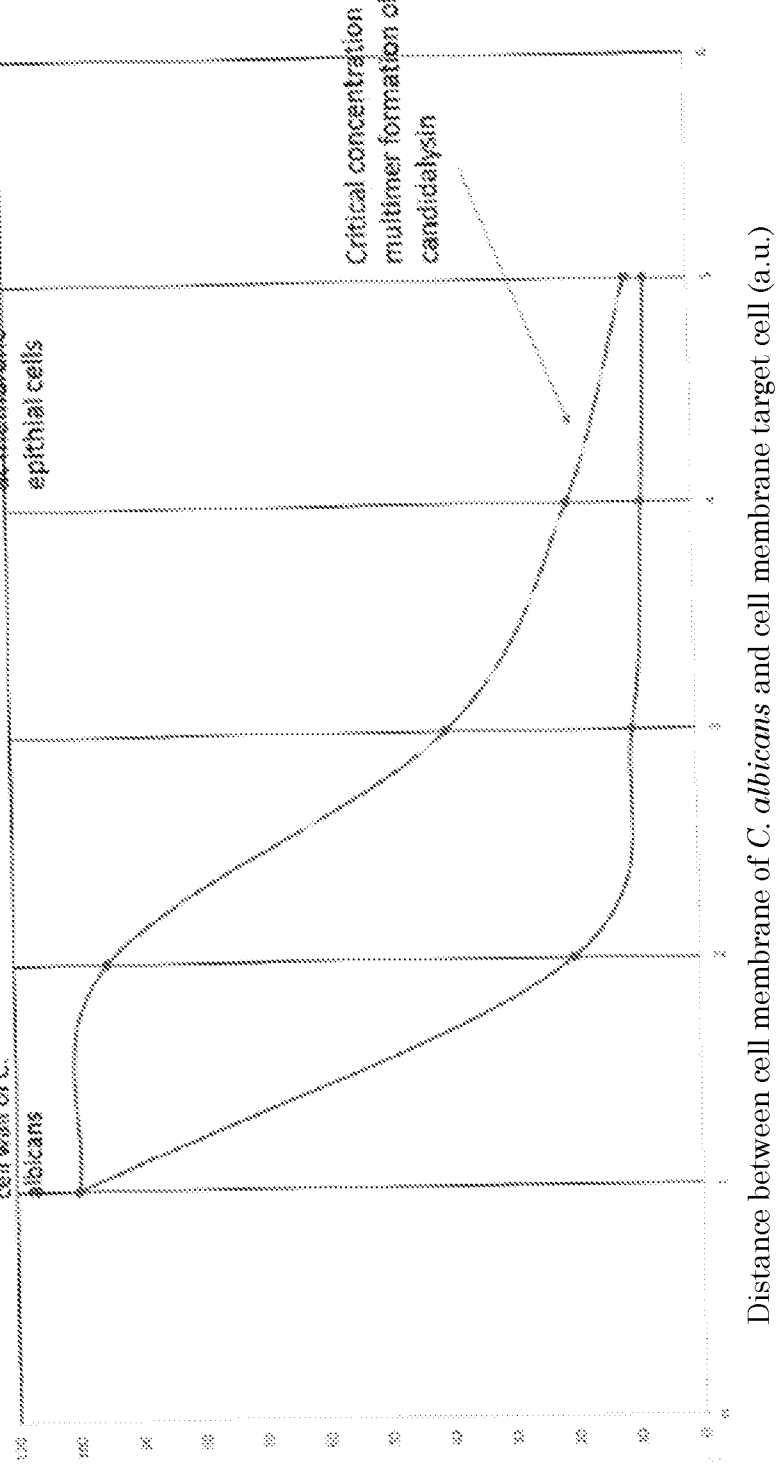

Phase 1
Transport through cell wall of C. albicans
Phase 2
Diffusion
Phase 3
Passing the ECM
Phase 4
Concentration at membrane epithial cells
Critical concentration of multimer formation of candidalysin
Concentration candidalysin (arbitrary units)
Distance between cell membrane of C. albicans and cell membrane target cell (a.u.)

Figure 17