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(54) Title: RECOMBINANT ASPARTYL PROTEASE ANTIGEN OR ANTIBODY THERETO FOR USE IN IMMUNOTHERAPY OF FUNGAL DISEASES

(57) Abstract: The invention related to the use of a recombinant aspartyl protease antigen, in particular a recombinant *Cryptococcus* spp. aspartyl protease Pep1 protein or an antibody thereto recognizing specific protective epitopes for the immunotherapy of fungal diseases.



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Recombinant aspartyl protease antigen or antibody thereto for use in immunotherapy of fungal diseases

The present invention relates to the use of a recombinant aspartyl protease antigen, in particular a recombinant *Cryptococcus* spp. aspartyl protease Pep1 protein, as vaccine for the treatment of fungal diseases. The invention relates also to protective antibodies recognizing specific epitopes of said recombinant aspartyl protease antigen, in particular anti-Pep1 protective monoclonal antibodies, and their use for the treatment of fungal diseases.

Fungal diseases are becoming a major public health concern and an increasing economic burden. Candidiasis, aspergillosis and cryptococcosis represent the main life-threatening fungal illnesses worldwide. Invasive candida infections occur can be predicted at around 300,000 worldwide per year, with a mortality of 30-55%. Invasive aspergillosis can occur in different patients groups including leukaemic cases, stem cell transplants and chronic obstructive pulmonary disease, with a mortality over 50%- even with treatment. Cryptococcosis is an opportunistic infection in subjects with cellular immune defects like those infected with HIV at a late stage of their disease, or those with solid organ transplantation, chronic lymphoid leukemia, or prolonged corticosteroid treatment. Disseminated meningoencephalitis is the main and most severe presentation and is always fatal unless treated. The Center for Diseases Control estimates that 1 million patients are diagnosed with cryptococcosis worldwide and that more than 600,000 die each year (Park *et al.*, AIDS, 2009, 23, 525-530). The causative agent is *Cryptococcus* spp., an encapsulated yeast present in the environment that is acquired through inhalation. The isolates responsible for infections are serotypes A, B, C or D, haploid or diploid, and mating type alpha (MAT α) or a. Overall, haploid *C. neoformans* MAT α serotype A isolates represent the most prevalent clinical isolates worldwide. Diagnosis relies on culture of viable yeasts from clinical specimens such as cerebrospinal fluid (CSF), blood, urine, bronchoalveolar lavages or on detection of soluble capsular polysaccharide (CPS) in body fluids such as CSF and serum (Perfect *et al.*, Clin. Infect. Dis., 2010, 50, 291-322). Less fatal infections but which affect large numbers of people worldwide include cutaneous fungal infections, nail infections and athletes foot. Hair infection which is common in young children is predicted to affect some 200 million people worldwide.

Despite antifungal treatment, invasive fungal infections such as cryptococcosis, candidiasis, and aspergillosis are still fatal.

Therefore, there is a need for adjuvant therapy for antifungal infections.

5 The inventors have produced a recombinant aspartyl protease of *C. neoformans* variety *neoformans*, named rPep1, from a cDNA previously cloned and sequenced but not expressed (Patent Application CA 2 354 100). Pep1 is a 438 amino acid protein of SEQ ID NO: 1 containing two putative active sites containing consensus sequences common to all aspartyl proteases (Figure 1). The inventors have
10 found that the aspartyl protease Pep1 is relatively conserved in fungi that can be recovered during infection in humans or animals; the C-terminal region is quite conserved whereas the N-terminal region (first 100 amino acids of Pep1) is variable (Figure 2; Table I). All analyzed aspartyl protease sequences have two catalytic motifs, SEQ ID NO: 2 and SEQ ID NO: 3, corresponding to positions 145 to 149 and
15 327 to 331 of SEQ ID NO: 1. The catalytic motifs are included in the consensus sequences SEQ ID NO: 4 and SEQ ID NO: 5 corresponding to positions 142 to 155 and 326 to 333 of SEQ ID NO: 1.

 Using a mouse model of cryptococcosis, the inventors have shown that vaccination with rPep1 resulted in the production of detectable anti-Pep1
20 antibodies in mice. Vaccination with rPep1 prior to inoculation of *C. neoformans* resulted in decreased fungal burden and prolonged survival, with mice surviving the infection 100 days post-inoculation. Vaccination with rPep1 on day 7 post-inoculation in mice with established cryptococcosis provided prolonged survival and led to a drastic decrease in yeasts counts in the target organs in survival mice.

25 Anti-Pep1 monoclonal antibodies (mAbs) were produced by immunization of mice with rPep1 protein and protective monoclonal antibodies recognizing specific peptide epitopes of Pep1 protein have been identified (Figure 1 and Figure 2). The epitopes correspond to the peptides in positions 1 to 15 (peptide 1), 271 to 285 (peptide 2), 296 to 310 (peptide 3) and 346 to 360 (peptide 4) of Pep1
30 (Figure 1 and 2); peptide 1 is specific of *Cryptococcus* spp. while the other peptide epitopes are common to fungi; peptides 3 and 4 are more conserved than peptide 2, with peptide 3 being the most conserved peptide epitope (Figure 2; Table II and III).

Serotherapy with mAbs directed against different epitopes of Pep1 provided prolonged survival in mice treated before infection and even in mice with an already established infection, leading to sterilization of all target organs in some cases.

C. neoformans rPep1 and homologous aspartyl proteases of other pathogenic fungi (named Pep1 aspartyl proteases) are thus useful as antigens in preventive or therapeutic vaccines (active immunotherapy) against fungal diseases. The antibodies recognizing specific epitopes of said antigens which have a protective effect against fungal diseases are thus useful for the prevention and/or treatment of fungal diseases (passive immunotherapy).

Therefore, the invention relates to a recombinant antigen or antibody thereto for use in the treatment of a fungal disease, wherein said antigen is chosen from:

a) a recombinant aspartyl protease protein comprising an amino acid sequence having at least 48 % identity with SEQ ID NO: 1 and comprising two catalytic motifs, SEQ ID NO: 2 and SEQ ID NO: 3, in positions corresponding to positions 145 to 149 and 327 to 331 of SEQ ID NO: 1, and further comprising at least one protective epitope, or a functional variant thereof; and

b) a polypeptide fragment of the protein in a) comprising at least one of said protective epitope(s), and,

wherein said antibody is an antibody recognizing specifically at least one of said protective epitope(s) or a functional fragment thereof comprising at least the antibody binding region.

Definitions

In the following description:

- “a”, “an”, and “the” include plural referents, unless the context clearly indicates otherwise. As such, the term “a” (or “an”), “one or more” or “at least one” can be used interchangeably herein.

- The percent amino acid sequence identity is defined as the percent of amino acid residues in a Compared Sequence that are identical to the Reference Sequence after aligning the sequences and introducing gaps if necessary, to achieve the maximum sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways known to a

person of skill in the art, for instance using publicly available computer software. When using such software, the default parameters, e.g., for gap penalty and extension penalty, are preferably used. Sequence alignments and sequence analyses shown herein were performed using Geneious v6.1.2 (Biomatters, New Zealand), ClustalW
5 Alignment with BLOSUM 62 Matrix, Gap open cost 10, GAP extent cost 0.1.

- As used herein, the term "antibody" refers to a glycoprotein produced by lymphoid cells in response to stimulation with an immunogen. Antibodies possess the ability to react *in vitro* and *in vivo* specifically and selectively with an antigenic determinant or epitope eliciting their production or with an
10 antigenic determinant closely related to the homologous antigen. The term "antibody" is meant to encompass constructions using the binding variable region of such an antibody, and other antibody modifications.

- As used herein, the term "recognizes specifically" refers to antibodies that have a relatively high affinity to one or more epitopes of a protein of
15 the invention, but which do not substantially recognize and bind to peptides other than the one(s) of interest. As used herein, the term "relatively high affinity" means a binding affinity between the antibody and the protein of interest of at least 10^{-6} M, and preferably of at least about 10^{-7} M and even more preferably 10^{-8} M to 10^{-10} M. Determination of such affinity is preferably conducted under standard competitive
20 binding immunoassay conditions which is common knowledge to the person of ordinary skill in the art.

- an "epitope" or antigenic determinant refers to the portion of an antigen that is recognized by an antibody. The portion recognized by the antibody may be a continuous sequence (a single peptide) or a discontinuous sequence (more than
25 one peptide).

- a "protective effect" refers to the reduction of subsequent (preventive treatment) or established (therapeutic treatment) disease symptoms caused by a fungal infection. The protective effect comprises a partial or total inhibition of the infection that causes the disease. A complete inhibition of the infection
30 corresponds to the eradication or sterilization of the infection and the resulting disease.

- a "protective antigen or antibody" refers to an antigen or

antibody having a protective effect. The protective effect of the antigen is mediated by the induction of a protective immune response directed against the antigen. The immune response includes a protective humoral response comprising the induction of specific protective antibodies recognizing the protective epitopes of the antigen. The use of the antigen for treating the fungal disease thus refers to active immunotherapy as opposed to passive immunotherapy when the protective antibody is used for the treatment. The protective effect of the antigen or antibody according to the invention can be verified in appropriate animal models of fungal diseases which are well-known in the art and illustrated in the examples of the present application. The protective effect of the antigen/antibody can be determined by measuring various parameters such as the reduction of objective clinical signs of the disease, the increase of survival, and/or the decrease of fungal load in target organ(s).

- a "protective epitope" refers to the portion of an antigen that is recognized by a protective antibody.
- "individual" or "subject" refers to an individual susceptible to infection with a pathogenic fungus.
- "fungal disease" refers to a disease caused by infection with a pathogenic fungus.
- unless specified otherwise, "treatment" refers to a preventive or prophylactic treatment that is administered prior to infection and/or a therapeutic treatment that is administered after infection with a pathogenic fungus.

The present invention relates to a recombinant antigen for use in the treatment of a fungal disease, wherein said antigen is chosen from:

- a) a recombinant aspartyl protease protein comprising an amino acid sequence having at least 48 % identity with SEQ ID NO: 1 and comprising two catalytic motifs, SEQ ID NO: 2 and SEQ ID NO: 3, in positions corresponding to positions 145 to 149 and 327 to 331 of SEQ ID NO: 1 and further comprising at least one protective epitope, said epitope(s) corresponding to one or more of the peptides in positions 1 to 15, 271 to 285, 296 to 310 and 346 to 360 of SEQ ID NO: 1, or a functional variant thereof; and

b) a polypeptide fragment of the protein in a) comprising at least one of said protective epitope(s).

The recombinant antigen for the use of the invention is derived from an aspartyl protease encoded by a fungal gene homologous to *C. neoformans* *PEP1* gene.

5 Therefore, the recombinant antigen or protein for the use of the invention is named Pep1 aspartyl protease, recombinant aspartyl protease Pep1, recombinant Pep1 aspartyl protease or rPep1 aspartyl protease antigen or protein.

Fungal aspartyl protease genes are well-known in the art and available in public data bases. Fungal genes homologous to *C. neoformans* *PEP1* can
10 be easily identified using standard sequence analysis software. The recombinant protein for the use of the invention is a Pep1 aspartyl protease encoded by a fungal gene or a functional variant thereof. It is antigenic, e.g. it is recognized by antibodies specific for the aspartyl protease protein from which it is derived, in particular it is recognized by (protective) antibodies directed to the specific protective epitopes of
15 said protein. It is also immunogenic, e.g., capable of inducing a specific protective immune response in an individual resulting in the production of detectable specific protective antibodies. A functional variant is derived from a Pep1 aspartyl protease by the introduction of mutations (deletion(s), insertion(s) and/or substitutions(s)) at specific positions in the sequence of the Pep1 aspartyl protease. A functional variant is
20 antigenic and immunogenic.

In some embodiments, the recombinant protein is a recombinant aspartyl protease of a pathogenic fungus or a functional variant thereof.

The pathogenic fungus may be any fungus causing a disease in humans or animals.

25 Preferably the pathogenic fungus is selected from the group consisting of: *Aspergillus* spp., *Candida* spp., *Clavispora* spp., *Coccidioides* spp., *Cryptococcus* spp., *Exophiala* spp., *Fusarium* spp., *Histoplasma* spp., *Kluyveromyces* spp., *Mucorales*, such as *Lichtheimia* spp., *Mucor* spp. and *Rhizopus* spp., *Meyerozyma* spp., *Microsporum* spp., *Neosartorya* spp., *Paracoccidioides* spp.,
30 *Pneumocystis* spp., *Talaromyces* spp., *Trichophyton* spp., *Trichosporon* spp, and other related pathogenic fungi.

Aspergillus spp. is Aspergillus clavatus (XP_001271141), Aspergillus flavus (KOC16935, XP_002381878), Aspergillus fumigatus (XP_754479), Aspergillus nidulans (XP_660507), Aspergillus niger (XP_001399855, AAA20876), Aspergillus oryzae (XP_001819842, XP_001825179, EIT76268),
5 Aspergillus parasiticus (KJK63739), Aspergillus terreus (XP_001213854), and other species.

Candida spp. is Candida albicans (P10977, XP_713194, EEQ47270, KHC45840, KGQ89270, KHC38341, CAA31962), Candida dubliniensis (XP_002418645), Candida glabrata (XP_449442), Candida parapsilosis (CCE40477),
10 Candida tropicalis (XP_002547417), and other species.

Clavispora spp. is Clavispora lusitaniae (XP_002615242) and other species.

Coccidioides spp. is Coccidioides immitis (XP_001244246), Coccidioides posadasii (AAZ92540), and other species.

15 Cryptococcus spp. is Cryptococcus gattii (serotypes B and C: KIR48411, KIR53730, XP_003191823, KIR86636, KGB75517), Cryptococcus neoformans variety grubii (serotype A; XP_012046817), Cryptococcus neoformans variety neoformans (serotype D, XP_566887), and other species.

Exophiala spp. is Exophiala dermatitidis (XP_009158792),
20 Exophiala oligosperma (KIW43340), Exophiala spinifera (KIW14941), Exophiala xenobiotica (XP_013321677), and other species.

Fusarium spp. is Fusarium fujikuroi (CCT71500), Fusarium oxysporum (EWY86405, EGU83511, KNB13049), Fusarium sp. (CEG04259), and other species.

25 Histoplasma spp. is Histoplasma capsulatum (XP_001542991, EGC40857, EEH04825) and other species.

Kluyveromyces spp. is Kluyveromyces lactis (XP_453326), Kluyveromyces marxianus (BAO41873) and other species.

Lichtheimia spp. is Lichtheimia corymbifera (CDH51907, CDH52330, CDH52400), Lichtheimia ramosa (CDS08210, CDS03074, CDS05348)
30 and other species.

Meyerozyma spp. is Meyerozyma guilliermondii (XP_001483416) and other species.

Microsporum spp. is Microsporum gypseum (XP_003175087) and other species.

5 Mucor spp. is Mucor circinelloides (EPB89553, PB83353) and other species.

Neosartorya spp. is Neosartorya fischeri (XP_001263323) and other species.

10 Paracoccidioides spp. is Paracoccidioides brasiliensis (XP_010756433, AAP32823, EEH19972) and other species.

Pneumocystis spp. is Pneumocystis jirovecii (CCJ31368) and other species.

15 Rhizopus spp. is Rhizopus delemar (EIE89256, EIE77417, EIE82161), Rhizopus microsporus (CEG73869, CEG74799, CEI95549, CEG76436, CEG73870) and other species.

Talaromyces spp. is Talaromyces marneffei (KFX49218, XP_002143541) and other species.

20 Trichophyton sp. is Trichophyton interdigitale (EZF32786), Trichophyton rubrum (XP_003232712, EZF25931), Trichophyton tonsurans (EGD99457), Trichophyton verrucosum (XP_003020295) and other species.

Trichosporon spp. is Trichosporon asahii (EKD04292, XP_014177670, EKD04292) and other species.

25 More preferably, the pathogenic fungus is selected from the group consisting of: Aspergillus spp., Candida spp., Cryptococcus spp., Mucorales, Trichosporon spp., Trichophyton spp., Microsporum spp., Exophiala spp., Fusarium spp., Pneumocystis spp. and the species responsible for severe endemic mycoses, namely Coccidioides spp., Histoplasma spp., Paracoccidioides spp., and Talaromyces spp.

30 In some embodiments, the recombinant protein comprises an amino acid sequence having at least 60 % identity with SEQ ID NO: 1. Pep1 aspartyl proteases of some Aspergillus spp., Talaromyces spp., Kluyveromyces spp., Paracoccidioides spp., Coccidioides spp., Trichosporon spp., Candida spp., Exophiala.

spp., *Clavispora* spp., *Rhizopus* spp., *Meyerozyma* spp., *Histoplasma* spp., *Lichtheimia* spp., and *Microsporum* spp. have at least 60 % sequence identity with SEQ ID NO: 1 (Table I).

In a preferred embodiment the recombinant protein comprises an amino acid sequence having at least 70 % identity with SEQ ID NO: 1. Pep1 aspartyl proteases of some *Trichosporon* spp. in particular *Trichosporon asahii* (EKD04292) have at least 70 % sequence identity with SEQ ID NO: 1 (Table I).

In another preferred embodiment, the recombinant protein comprises an amino acid sequence having at least 80 % identity with SEQ ID NO: 1. Pep1 aspartyl proteases of *Cryptococcus* spp. including *Cryptococcus neoformans* variety *grubii* (serotype A), *Cryptococcus neoformans* variety *neoformans* (serotype D) and *Cryptococcus gattii* (serotypes B and C) Pep1 aspartyl proteases have at least 80 % sequence identity with SEQ ID NO: 1 (Table I). Preferably, the recombinant protein is a *Cryptococcus* spp. protein; more preferably a *Cryptococcus* spp. protein comprising or consisting of any one of SEQ ID NO: 1 (XP_566887), SEQ ID NO: 6 (XP_012046817), SEQ ID NO: 7 (KGB75517), SEQ ID NO: 8 (KIR48411), SEQ ID NO: 9 (KIR53730), SEQ ID NO: 10 (KIR86636) or SEQ ID NO: 11 (XP_003191823).

In another preferred embodiment, the recombinant protein comprises an amino acid sequence having at least 90 % identity with SEQ ID NO: 1. Pep1 aspartyl proteases of *Cryptococcus neoformans* including *Cryptococcus neoformans* variety *grubii* (serotype A) and *Cryptococcus neoformans* variety *neoformans* (serotype D) Pep1 aspartyl proteases have at least 90 % sequence identity with SEQ ID NO: 1 (Table I). Preferably, the recombinant protein is a *Cryptococcus neoformans* protein; more preferably a *Cryptococcus neoformans* protein comprising or consisting of any one of SEQ ID NO: 1 (XP_566887) and SEQ ID NO: 6 (XP_012046817); even more preferably, a recombinant protein comprising or consisting of SEQ ID NO: 1.

In some embodiments, the catalytic motif of SEQ ID NO: 2 is included in a consensus sequence of SEQ ID NO: 4 and/or the catalytic motif of SEQ ID NO: 3 is included in a consensus sequence of SEQ ID NO: 5.

In some embodiments, the protective epitope(s) correspond to one or more of the peptides in positions 1 to 15 (peptide 1), 271 to 285 (peptide 2), 296 to 310 (peptide 3) and 346 to 360 (peptide 4) of SEQ ID NO: 1.

In a preferred embodiment, the peptide in positions 1 to 15 (peptide 1) consists of SEQ ID NO: 12; this peptide is specific of *Cryptococcus* spp. and strictly conserved in *Cryptococcus* spp..

In another preferred embodiment, the peptide in positions 271 to 285 (peptide 2) consists of a sequence having at least 60 %, preferably at least 70 %, 80 % or 90 % identity with SEQ ID NO: 13. Preferably, the peptide 2 is selected from the group consisting of: SEQ ID NO: 13 and SEQ ID NO: 14.

In another preferred embodiment, the peptide in positions 296 to 310 (peptide 3) consists of a sequence having at least 60 %, preferably at least 70 %, 80 % or 90 % identity with SEQ ID NO: 15. Preferably, the peptide 3 consists of SEQ ID NO: 15

In another preferred embodiment, the peptide in positions 346 to 360 (peptide 4) consists of a sequence having at least 60 %, preferably at least 70 %, 80 % or 90 % identity with SEQ ID NO: 16. Preferably, the peptide 4 consists of SEQ ID NO: 16.

The polypeptide fragment, which is different from a full-length aspartyl protease Pep1 protein consists of up to 400 amino acids, preferably up to 300, 250, 200, 150, 100, 90, 80, 70, 60, 50, 40, 30 or 20 amino acids of a recombinant protein as defined above. The polypeptide fragment is an antigenic and immunogenic fragment of the recombinant protein. The polypeptide fragment comprises advantageously at least one of the peptides 1 to 4 as defined above.

The present invention also relates to an antibody for use in the treatment of a fungal disease,

wherein said antibody binds to an antigen being chosen from:

a) a recombinant aspartyl protease protein comprising an amino acid sequence having at least 48 % identity with SEQ ID NO: 1 and comprising two catalytic motifs, SEQ ID NO: 2 and SEQ ID NO: 3, in positions corresponding to positions 145 to 149 and 327 to 331 of SEQ ID NO: 1 and further comprising at least one protective epitope, said epitope(s) corresponding to one or more of the peptides

in positions 1 to 15, 271 to 285, 296 to 310 and 346 to 360 of SEQ ID NO: 1, or a functional variant thereof; and

b) a polypeptide fragment of the protein in a) comprising at least one of said protective epitope(s), and,

5 wherein said antibody is an antibody recognizing specifically at least one of said protective epitope(s) or a functional fragment thereof comprising at least the antibody binding region.

In particular, the antibody binds to a recombinant antigen as defined above.

The antibody is a polyclonal or monoclonal antibody, preferably a
10 monoclonal antibody.

An antibody for the use of the invention may comprise a whole antibody, an antibody fragment, a polyfunctional antibody aggregate, or in general a substance comprising one or more specific binding sites from an antibody. The antibody fragment may be a fragment such as an Fv, Fab or F(ab')₂ fragment or a
15 derivative thereof, such as a single chain Fv fragment (ScFv). The Fv fragment may advantageously be from a *Camelidae* antibody (nanobodies® or V_{HH}). The antibody or antibody fragment may be non-recombinant, recombinant or humanized. The antibody may be of an immunoglobulin isotype, e.g., IgG, IgM, and so forth. In addition, an aggregate, polymer, derivative and conjugate of an immunoglobulin or a
20 fragment thereof can be used where appropriate.

In some embodiments, the antibody or functional fragment recognizes specifically at least one of the peptides 1 to 4 as defined above.

In a preferred embodiment, the antibody recognizes specifically the peptides of SEQ ID NO: 12 and SEQ ID NO: 15, the peptide of SEQ ID NO:13, or the
25 peptides of SEQ ID NO:13 and SEQ ID NO:16; said antibody is preferably a monoclonal antibody.

In a more preferred embodiment, the antibody is a monoclonal antibody produced by an hybridoma selected from the hybridomas deposited by the Applicant, according to the Budapest Treaty, at CNCM (Collection Nationale de
30 Cultures de Microorganismes, 25 rue du Docteur Roux, Paris) on November 17, 2014, under the accession number CNCM I-4914, I-4915 and I-4916 or a Fv, Fab or Fab'2 fragment thereof. These hybridomas are also referred to as B4-1 (CNCM I-4914), J1-

26 (CNCM I-4915) and J17-14 (CNCM I-4916). More preferably, the monoclonal antibody is produced by the hybridoma deposited at CNCM under accession number I-4914.

5 The invention encompasses the use of several rPep1 aspartyl protease antigens and/or antibodies as defined above, either as a mixture wherein the different rPep1 aspartyl protease antigens and/or antibodies are used simultaneously, or as a combination wherein the different rPep1 aspartyl protease antigens and/or antibodies are used separately or sequentially.

10 In some embodiments, the rPep1 aspartyl protease antigens and/or antibodies are used simultaneously, separately or sequentially with at least one immunotherapeutic agent and/or antifungal agent.

The immunotherapeutic agent is for example another antigen, another antibody or an immunomodulatory drug. Other antigens and antibodies include in particular fungal antigens and antigens from pathogenic microorganisms (viruses, bacteria, parasites) and antibodies thereto.

15 Antifungal agents include standard antifungal drugs which are used for treating fungal diseases such as Polyenes (Amphotericin B, Candicidin, Filipin, Hancin, Natamycin, Nystatine, Rimocidin and the like), Azoles (Imidazoles, triazoles, thiazoles), Allyamines (Amorolfine, Butenafine, Naftifine, Terbinafine and the like), Echinocandins (Anidulafungin, Caspofungin, Micafungin and the like) and others.

In some embodiments, the rPep1 aspartyl protease antigen and/or antibody are used in the form of a composition comprising a pharmaceutically acceptable vehicle, carrier and/or adjuvant.

25 Carriers and adjuvants are usually used in vaccine compositions comprising the antigen according to the invention.

The composition may comprise a mixture of aspartyl protease antigens and/or antibodies as defined above, and eventually also at least one immunotherapeutic agent and/or antifungal agent, as defined above.

30 The pharmaceutical vehicles and carriers are those appropriate to the planned route of administration, which are well known in the art.

Non-limitative examples of carriers suitable for use in the composition of the invention include uni- or multi-lamellar liposomes, ISCOMS, virosomes, viral pseudoparticules, saponin micelles, saccharid (poly(lactide-co-glycolide)) or gold microspheres, and nanoparticules.

5 Non-limitative examples of adjuvants suitable for use in the composition of the invention include: CpG oligodeoxynucleotide, Freund adjuvant, polyI:C (polyinosine-polycytidylic acid), oil emulsion, mineral substances, bacterial extracts, saponin, aluminium salts, monophosphoryl-lipid A and squalene.

A preferred composition for the use according to the invention is a
10 vaccine composition comprising at least one rPep 1 aspartyl protease antigen of the invention, and an aluminium salt and a CpG oligodeoxynucleotide, as adjuvants. In some more preferred embodiments, the composition comprises a recombinant Pep1 aspartyl protease protein having at least 80 % identity with SEQ ID NO: 1, preferably a recombinant *Cryptococcus* spp. aspartyl protease Pep1 protein (encoded by a
15 *Cryptococcus* spp. aspartyl protease Pep1 gene). The composition may further comprises at least one immunotherapeutic agent and/or antifungal agent, as defined above.

Aluminium salt includes any insoluble aluminium salt capable of enhancing and/or directing the immune response to a vaccine antigen.

20 Non-limitative examples of aluminium salts suitable for use in the composition of the invention include aluminium potassium sulfate, aluminium hydroxide, aluminium phosphate and mixtures thereof.

In some preferred embodiment, the aluminium salt is aluminium hydroxide, more preferably aluminium hydroxide gel.

25 CpG oligodeoxynucleotide (CpG ODN) includes any short single-stranded synthetic DNA molecules containing unmethylated CpG motif(s) which are recognized by Toll-like receptor 9 (TLR9) leading to strong immunostimulatory effects.

The invention encompasses the use of the various types of CpG
30 immunostimulants including Class A (Type D), Class B (Type K), Class C, Class P, and Class S CpGs.

In some preferred embodiment, the CpG ODN is a class B CpG.

CpG ODNs of the B-Class stimulate strong B cell and NK cell activation and cytokine production. In some more preferred embodiment, said CpG is a phosphorothioate (nuclease resistant) CpG of SEQ ID NO: 17.

Another preferred composition for the use according to the invention
5 is a pharmaceutical composition comprising at least one monoclonal antibody recognizing specifically at least one of the peptides 1 to 4 according to the invention, and a pharmaceutically acceptable vehicle.

In a preferred embodiment of the pharmaceutical composition, the antibody recognizes specifically the peptides of SEQ ID NO: 12 and SEQ ID NO: 15,
10 the peptide of SEQ ID NO:13, or the peptides of SEQ ID NO:13 and SEQ ID NO:16.

In a more preferred embodiment of the pharmaceutical composition, the antibody is a monoclonal antibody produced by an hybridoma selected from the hybridomas deposited at CNCM under accession number I-4914, I-4915 and I-4916 or a Fv, Fab or Fab'2 fragment thereof. More preferably, the monoclonal antibody is
15 produced by the hybridoma deposited at CNCM under accession number I-4914.

In some preferred embodiments, the pharmaceutical composition comprises a mixture of monoclonal antibodies recognizing specifically at least one of the peptides 1 to 4 according to the invention, as defined above.

The composition of the invention comprises a therapeutically
20 effective dose of aspartyl protease antigen, and eventually CpG and aluminium salts (vaccine composition) or of antibody (pharmaceutical composition), sufficient to induce a protective effect against a fungal disease in the individual to whom it is administered.

The protective effect of the composition according to the invention
25 can be readily verified by various assays, using appropriate animal models of fungal diseases which are known to the person of ordinary skill in the art such as those described in the examples of the present Application. In particular, the administration of the composition according to the invention to an individual, before or after infection with a pathogenic fungus results in decreased fungal burden and prolonged
30 survival compared to non-vaccinated individual, as shown in the examples of the present invention.

The effective dose is determined and adjusted depending on factors

such as the composition used, the route of administration, the physical characteristics of the individual under consideration such as sex, age and weight, concurrent medication, and other factors, that those skilled in the medical arts will recognize.

The invention provides also a method for treating a fungal infection,
5 comprising: administering to an individual a therapeutically effective amount of the composition as described above.

The composition of the present invention is generally administered according to known procedures used for vaccination and serotherapy, at dosages and for periods of time effective to induce a protective effect against the fungal disease in
10 the individual. The administration may be by injection or by oral, sublingual, intranasal, rectal or vaginal administration, inhalation, or transdermal application. The injection may be subcutaneous, intramuscular, intraveinuous, intraperitoneal, intradermal or else. Preferably, the administration of the vaccine composition is subcutaneous, intramuscular or intradermal. Preferably, the administration of the
15 pharmaceutical composition is intraveinuous.

The protective effect of the vaccine or pharmaceutical composition is obtained when the composition is administered before (preventive effect) or after (therapeutic effect) the fungal infection is diagnosed.

Therefore, the composition may be administered before or after the
20 individual has been diagnosed with fungal infection.

In some embodiments, the antigen and/or antibody composition of the invention is used for the therapeutic treatment of a fungal disease (after diagnosis of fungal infection).

In a preferred embodiment, the antigen and/or antibody composition
25 of the invention used for the therapeutic treatment of individuals which have been previously diagnosed with a fungal infection using standard diagnosis assays which are well-known in the art.

In addition, the diagnosed patients may be classified in different groups depending upon the severity of the fungal disease; the patients diagnosed with
30 a mild fungal disease based on clinical signs and/or biological marker levels will be administered a standard antifungal treatment; whereas the patients diagnosed with a severe fungal disease will be administered the antigen and/or antibody composition of

the invention alone or in combination with a standard antifungal treatment. The use of the antigen and/or antibody composition of the invention increases the efficiency of standard antifungal treatments.

In some embodiments, the pharmaceutical composition or the vaccine composition according to the invention are administered simultaneously, separately or sequentially, with at least one immunotherapeutic and/or antifungal agent as defined above.

In some other embodiments, the pharmaceutical composition and the vaccine composition according to the invention are administered simultaneously, separately or sequentially, possibly in combination with at least one immunotherapeutic and/or antifungal agent as defined above.

In some embodiments, the composition of the invention is used for the treatment of animals such as pets (cats, dogs and others), livestock and the like.

In other embodiments, the composition of the invention is used for the treatment of humans.

The composition of the invention is used for the treatment of a fungal disease (mycosis) caused by a pathogenic fungus such as those listed above. The antigen and antibody thereto are chosen so as to induce a protective effect against the fungal disease that is treated. The antigen is chosen in a fungal genus close to that of the fungus causing the disease that is treated based on aspartyl protease Pep1 sequence identity. For example a *Cryptococcus* spp. aspartyl protease Pep1 antigen is used for treating cryptococcosis as well as other fungal diseases.

The composition may be used for the treatment of superficial mycoses, cutaneous mycoses, subcutaneous mycoses and systemic mycoses due to primary or opportunistic pathogens. Systemic mycoses are in particular candidiasis, aspergillosis, cryptococcosis, mucormycosis, fusariosis, pneumocystosis, dermatophytomycosis, trichosporoniosis, and endemic mycosis such as penicilliosis marneffei, coccidioidomycosis, histoplasmosis or paracoccidioidomycosis. Dermatophytomycosis are caused by pathogenic fungus such as *Trichophyton* spp. and *Microsporum* spp., for example.

In some embodiments, the fungal disease is a systemic mycoses as defined above. In some preferred embodiments, the fungal disease is candidiasis,

aspergillosis, cryptococcosis, trichosporonosis, mucormycosis, fusariosis, pneumocystosis, penicilliosis marneffei, coccidioidomycosis, histoplasmosis or paracoccidioidomycosis; preferably candidiasis, aspergillosis, cryptococcosis, trichosporonosis, mucormycosis, fusariosis, or pneumocystosis; more preferably
5 cryptococcosis.

A preferred use of the invention is the use of a recombinant *Cryptococcus* spp aspartyl protease Pep1 protein having at least at least 80 % identity with SEQ ID NO: 1 or a monoclonal antibody thereto recognizing specifically the peptides of SEQ ID NO: 12 and SEQ ID NO: 15, the peptide of SEQ ID NO:13, or the
10 peptides of SEQ ID NO:13 and SEQ ID NO:16 for the treatment of a systemic mycoses as defined above, preferably candidiasis, aspergillosis, cryptococcosis, trichosporonosis, mucormycosis, fusariosis, pneumocystosis, penicilliosis marneffei, coccidioidomycosis, histoplasmosis or paracoccidioidomycosis; more preferably candidiasis, aspergillosis, cryptococcosis, trichosporonosis, mucormycosis, fusariosis,
15 or pneumocystosis; still more preferably cryptococcosis. The recombinant *Cryptococcus* spp aspartyl protease Pep1 protein comprises advantageously any one of SEQ ID NO: 1 and 6 to 11, preferably SEQ ID NO: 1. Preferably, the monoclonal antibody is produced by an hybridoma selected from the hybridomas deposited at CNCM under accession number I-4914, I-4915 and I-4916 or a Fv, Fab or Fab'2
20 fragment thereof; even more preferably, the monoclonal antibody is produced by the hybridoma deposited at CNCM under accession number I-4914.

Another aspect of the invention is a vaccine composition comprising at least one rPep 1 aspartyl protease antigen of the invention, and an aluminium salt and a CpG oligodeoxynucleotide, as adjuvants. In some more preferred embodiments,
25 the composition comprises a recombinant Pep1 aspartyl protease protein having at least at least 80 % identity with SEQ ID NO: 1, preferably a recombinant *Cryptococcus* spp. aspartyl protease Pep1 protein (encoded by a *Cryptococcus* spp. aspartyl protease Pep1 gene). The composition may further comprises at least one at least one immunotherapeutic agent and/or antifungal agent, as defined above.

30 Another aspect of the invention is protective antibody of the invention, in particular an antibody recognizing specifically at least one of the peptides 1 to 4 as defined above, preferably the peptides of SEQ ID NO: 12 and SEQ

ID NO: 15, the peptide of SEQ ID NO:13, or the peptides of SEQ ID NO:13 and SEQ ID NO:16; preferably a monoclonal antibody, more preferably a monoclonal antibody produced by an hybridoma selected from the hybridomas deposited at CNCM under accession number I-4914, I-4915 and I-4916 or a Fv, Fab or Fab'2 fragment thereof; even more preferably, the monoclonal antibody is produced by the hybridoma deposited at CNCM under accession number I-4914.

Another aspect of the invention is a pharmaceutical composition comprising at least one protective antibody of the invention or a functional fragment thereof, as defined above; in particular a composition comprising a mixture of said antibodies; preferably a composition further comprising at least one immunotherapeutic and/or antifungal agent as defined above.

Another aspect of the invention is an antigenic polypeptide fragment as defined above. The antigenic polypeptide fragment of the invention is used to generate new antibodies specific for the recombinant aspartyl protease antigen of the invention, in particular protective monoclonal antibodies.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques which are within the skill of the art. Such techniques are explained fully in the literature.

In addition to the above arrangements, the invention also comprises other arrangements, which will emerge from the description which follows, which refers to exemplary embodiments of the subject of the present invention, with reference to the attached drawings in which:

- **Figure 1** represents *Cryptococcus neoformans* v. *neoformans* Aspartyl protease Pep1 amino acid sequence (SEQ ID NO: 1). The two putative active sites common to all aspartyl proteases are in bold; the consensus sequences of the active sites are in italics. The sequence of the epitopes recognized by the therapeutic monoclonal antibodies J1-26, J17-14 and B4-1 is underlined: SEQ ID NO: 12 (J1-26); SEQ ID NO: 13 (J17-14 and B4-1); SEQ ID NO: 15 (J1-26); SEQ ID NO: 16 (B4-1).

- **Figure 2** represents the alignment of *Cryptococcus neoformans* v. *neoformans* aspartyl protease Pep1 amino acid sequence (SEQ ID NO: 1) with that of homologous aspartyl proteases from human pathogenic fungi (SEQ ID NO: 6, 9, 11, 8, 7, 10 and 21 to 91). The two putative active sites common to all aspartyl

proteases and the sequences of the epitopes recognized by the therapeutic monoclonal antibodies are shown. The consensus sequence is SEQ ID NO: 92.

- **Figure 3** shows the effect of prior immunization with recombinant Pep1 protein (rPep1) in various adjuvants on the survival of BALB/c mice inoculated with 10^5 H99. Mice (4-5 mice/groups) received an initial administration followed by 2 boosts with rPep1 (day 0, 14 and 35), and were inoculated on day 60 and survival was recorded daily ($p = 0.0009$, log-rank test).

- **Figure 4** illustrates the effect of prior vaccination with rPep1 on survival (**A**) and fungal burden (**B**) of BALB/c male mice inoculated with *C. neoformans* var. *neoformans* (NIH52D). Mice were treated with PBS (Control), adjuvant (Alum+CpG) or rPep1/Alum+CpG (day 0, 14 and 35) prior to inoculation (day 60) with 10^5 yeasts (14 mice in each group). Survival (7 mice/group) was recorded daily up to 100 dpi ($p = 0.0019$, log-rank test). CFU/g of brain, lungs or spleen were determined in mice sacrificed 7 dpi (3 mice) and 14dpi (4 mice) ($p < 0.0083$ for brains at 14dpi, Kruskal Wallis test). Each mouse is represented by one symbol. The 4 mice surviving at 100dpi had no viable yeasts in brain, spleen and lungs.

- **Figure 5** illustrates the effect of vaccination with rPep1 on survival (**A**) and fungal burden (**B and C**) of BALB/c male mice inoculated with *C. neoformans* var. *neoformans* (NIH52D). Mice were treated with PBS (Control), rPep1/Alum+CpG 15 days prior to (rPep1 D-15) or 7 days (rPep1 D+7) after inoculation with 10^5 yeasts (14 mice in each group). Survival (7 mice/group) was recorded daily up to 100 dpi (panel A). CFU/g of brain, lungs or spleen were determined in the surviving mice (2 in each group, panel B) and in mice sacrificed 14 dpi (3 mice) and 21dpi (4 mice) (panel C). Each mouse is represented by one symbol.

- **Figure 6** illustrates the effect of vaccination with rPep1 on fungal burden (**A**) and severity of meningitis (**B**) of BALB/c male mice inoculated with *C. neoformans* var. *neoformans* (NIH52D). BALB/c male mice that have been inoculated with 10^5 *C. neoformans* var. *neoformans* (NIH52D)) and treated 7 days later with either adjuvant or rPep1 in adjuvant (5 mice/group) were sacrificed 14 days after inoculation. The fungal load (CFU/g of organ) was determined in spleen, brain

and lung (A) and the score of severity of meningitis was determined blindly on sagittal section of one hemisphere (B).

- **Figure 7** illustrates the effect of vaccination with rPep1 on immune mediators after inoculation of *C. neoformans* var. *neoformans* (NIH52D). BALB/c male mice that have been inoculated with 10^5 *C. neoformans* var. *neoformans* (NIH52D)) and treated 7 days later with either adjuvant or rPep1 in adjuvant (5 mice/group) were sacrificed 14 days after inoculation. The mediators were measured in the supernatants of brain homogenates or in serum using the Mouse-23-Plex Panel (Bio-Rad). Only the statistically significant and biologically relevant results are presented (Kruskall Wallis test). * $p < 0.05$ ** $p < 0.01$

- **Figure 8** shows the effect on survival (A) and fungal burden (B, C) of vaccination with rPep1 in BALB/c male mice 7 days after inoculation with *C. neoformans* var. *grubii* (H99). Mice were inoculated with 10^3 yeasts and treated at 7 dpi with 1 injection of PBS (Control without vaccine; wo Vacc) or rPep1/Alum+CpG (w rPep1 vacc.; 14 mice in each group). (A) Survival was recorded daily up to 63 dpi (7 mice/group, $p = 0.010$ log-rang test), and (B) fungal burden in the target organs determined for the 4 mice surviving at day 70dpi. (C) CFU/g of brain, lungs or spleen were determined at 14 (4 mice/group) and 21 dpi (3 mice/group) (non statistically different except for spleens on day 21, $p = 0.0495$). Each mouse is represented by one symbol.

- **Figure 9** illustrates the effect of anti-Pep1 mAb B4-1 on the course of experimental cryptococcosis in BALB/c mice inoculated with 10^5 NIH52D. Mice were injected ip with 20 μ g (A) or 100 μ g (B) of B4-1, 1 day before (D-1) or 1 or 7 days after (D+1 or D+7) inoculation of the yeasts. Survival of mice was significantly prolonged by B4-1 at 20 μ g (D-1 or D+7) and at 100 μ g. Moreover, fungal load was decreased in all mice surviving 100 dpi (C).

- **Figure 10** illustrates the effect of passive serotherapy with the monoclonal anti-pep1 antibody B4-1 7 days after inoculation of *C. neoformans* var. *neoformans* (NIH52D) on survival of BALB/c male mice.

Mice were injected ip with PBS (Control), or Mab B4-1 at different doses 7 days after inoculation with 10^5 yeasts (7 mice in each group). Survival was recorded daily up to 100 dpi (A). Survival of mice was significantly prolonged when mice were injected with the lowest dosages of the antibody. Residual fungal load was determined at 100dpi in the target organs of surviving mice (1 each in the 70 and 200 μ g groups, and 4 each in the 7 and 20 μ g groups) (B). Of note, 2/4 mice in the 7 μ g group and 3/4 mice in the 20 μ g group had no viable yeast detectable.

- **Figure 11** shows the effect of the various anti-Pep1 mAbs on the course of experimental cryptococcosis in BALB/c mice inoculated with 10^5 NIH52D. Mice were injected ip with 20 μ g of B4-1, J1-26 and J17-17 at 7dpi. Survival was recorded up to 63 days post-inoculation (A) and fungal burden was assessed in surviving mice (B).

- **Figure 12** shows the effect of the various anti-Pep1 mAbs on the course of experimental cryptococcosis in BALB/c mice inoculated with 10^5 H99. Mice were injected ip with 20 μ g of B4-1, J1-26 and J17-17 at 7dpi. Survival was recorded up to 54dpi.

- **Figure 13** shows the effect of anti-*C. neoformans* mAbs (10 μ g/ml) on *C. neoformans* growth. Yeasts (H99 or 52D) at 10^4 /ml in YPD were distributed in the presence of various mAbs (at 10 μ g/ml): anti-glucuronoxylomannan mAb E1, anti-Pep1 mAbs (B4-1, J1-26 or J17-14), unrelated IgG1 mAb or no mAb. Growth was recorded using the Bioscreen C Automated Microbiology Growth Curve Analysis System (Thermofisher). The Richards non-linear regression model was used to determine lag times (Latency), slope (Slope) and maximal growth (Maximal Growth) for each condition. Each dot represents one experiment (result of triplicate wells).

- **Figure 14** shows the comparison of *C. neoformans* H99 phagocytosis by the murine macrophages cell line J774 in the presence of anti-Pep1 mAbs (B4-1 or J17-14) using anti-GXM mAb (E1) as the opsonin. The condition tested were the antibody B4-1 or J17-14 (each at 0.5 μ g/ml) alone or added together with E1 (+E1) or 30 min before E1 (then E1) at 1 μ g/ml. The phagocytic index obtained in the presence of anti-Pep1 mAbs was normalized (ratio of the experimental

condition to the phagocytic index obtained with E1 at 1 µg/ml in the same experiment).

- **Figure 15** shows the correlation between expression of PEP1 and phagocytic index relative to those of the reference strain H99 for 9 clinical isolates. Compared to H99, the phagocytic index determined at 2 hrs inversely correlated with the intracellular expression of the *PEP1* genes. Bars represent means standard deviations (SD) of duplicates from 2 independent experiments for each of the 9 clinical isolates of *C. neoformans* var. *grubii*. Phagocytosis and gene expression determination were performed as explained above, with H99 as a reference strain and E1 as opsonin. The clinical isolates have been selected in a previous study (Alanio et al. mBio 2011).

There will now be described by way of example a specific mode contemplated by the Inventors. In the following description numerous specific details are set forth in order to provide a thorough understanding. It will be apparent however, to one skilled in the art, that the present invention may be practiced without limitation to these specific details. In other instances, well known methods and structures have not been described so as not to unnecessarily obscure the description.

Example 1: Material and Methods

Experimental examples described below are obtained using following materials and methods.

Strains

Reference strains of *Cryptococcus. neoformans* variety *grubii* H99 (serotype A, MATalpha) and variety *neoformans* NIH52D (serotype D, MATalpha) were stored in 40% glycerol at -80°C.. Yeasts were cultured on Sabouraud agar (SA) medium and subcultured in liquid yeast extract-peptone-glucose medium (YPD, DIFCO) or in yeast nitrogen base (YNB, DIFCO) broth supplemented with 2% glucose (30°C, 150 rpm) for 22 h. Yeasts were collected by centrifugation, washed in phosphate buffered saline (PBS), enumerated with a hemacytometer and suspended at the desire concentration in the appropriate buffer or medium.

Reagents

All reagents were purchased from SIGMA unless otherwise specified. Alhydrogel® 2%, (referred to as Alum), is an aluminium hydroxide wet gel suspension (INVIVOGEN), CpG ODN 1826 (SEQ ID NO: 17; VacciGrade™, CAYLA). Conjugated antibodies were purchased from BIO-RAD.

5 ***Production of anti-rPep1 antibodies***

Monoclonal antibodies were produced after fusion of spleen cells from BALB/c mice immunized with rPep1 and murine myeloma cells (P3X63-Ag8653) at a 1:4 ratio in 45% polyethylene glycol 1000. Four clones were selected and mAbs purified from ascites by ammonium sulfate precipitation. In all cases,
10 production of anti-Pep1 Abs was tested by ELISA and westernblots.

Detection of anti-C. neoformans antibodies and characterization of the epitopes

Antibodies to rPep1 were analyzed by ELISA with rPep1 coated overnight at 4°C (1 µg/ml of rPep1 in carbonate buffer of a 96-well plates) (MaxiSorp®, NUNC). Saturation was done with 1% gelatin in PBS, for 1 h at 37°C
15 and washes with 0.1% Tween® 20 in PBS (PBST). Supernatants, serum samples or peptides preincubated with mAbs (100 µl/well) were incubated for 2 h at 37°C or overnight at 4°C. Dilutions were made in PBST containing 0.25% gelatin (PBSTG). Specific antibodies were detected after incubation with the HRP conjugate (BIO-RAD) followed by addition of the substrate solution (SIGMAFAST™ OPD table set,
20 SIGMA-ALDRICH).

Non-overlapping and overlapping peptides (15-mer) were synthesized (> 90% purity, PROTEOGENIX) to analyze the epitopes recognized by the various mAbs. Peptides were suspended at 5 mg/mL in PBS (with 50 µL DMSO if necessary) and stored at -20°C in aliquots until use. Peptides at various concentrations
25 (1250, 750, 250, 50 or 25 µM) were incubated for 1 hr at 37°C vol:vol with the various mAbs at 5 ng/mL all in PBSTG. Controls consisted in bovine serum albumin (25 µg/ml) or buffer (negative controls) and rPep1 (50 µg/mL, positive control). Optical densities (OD) were measured at 492 nm in a microplate reader (LABSYSTEMS MULTISKAN RC). Results were expressed as percent inhibition.

30 ***Experimental infections***

Six week-old outbred OF1 (CHARLES RIVER) or BALB/cJRj (JANVIER) male mice were housed 7/cage in our animal facilities and received food

and water ad libitum. Mice were identified individually. Infections were performed by intravenous (iv) inoculation (10^3 to 10^6 yeasts/mouse). Yeasts viability was assessed by colony forming units (CFU) enumeration onto Sabouraud agar.

In the OF1 model, mice exhibit individual patterns of susceptibility to *C. neoformans* infection, independently of the inoculum size. Some of the mice develop acute, disseminated and rapidly lethal infections, whereas others survive for several weeks with limited chronic infection. In the BALB/c model, all mice die with a mean survival time after inoculation depending on the inoculum size. BALB/c were thus used to assess the efficacy of passive and active immunizations.

Outcome was evaluated either by survival or by fungal burden. Survival was recorded daily and weight recorded weekly up to 100 day post-inoculation (dpi). In selected experiments, blood was also withdrawn weekly from the lateral tail vein and immediately used for antibody (Ab) determination (20 μ l). For fungal burden evaluation, mice were euthanized at selected time after inoculation, blood was withdrawn by cardiac puncture (1 ml) and serum stored at -20°C until assayed. Brain, lungs and spleens were aseptically removed for CFU determination. Tenfold dilutions of the homogenates were plated in duplicate on Sabouraud-chloramphenicol agar Petri dishes and incubated at 30°C for 48 hrs. Results were expressed as log₁₀ CFU/g of organ. All experiments were approved by the institutional committee (CETEA 02196.01).

Efficacy of the recombinant protein rPep1 prior to or after challenge with C. neoformans

Preliminary experiments were done to determine the best adjuvant for rPep1 immunization in 4 groups of 5 BALB/c mice receiving subcutaneously (sc) a vol:vol mixture of rPep1 (10 μ g) and one of the following (i) Freund's complete adjuvant; (ii) Alum; (iii) 10 μ g CpG ; (iv) a mixture of 5 μ g CpG/Alum (Alum+CPG) with boosts 14 and 35 days later. Controls were injected with saline mixed with each adjuvant. Anti-rPep1 Abs titers were determined initially and 7 days after the 2nd and 3rd challenge.

Subsequent experiments testing the prophylactic efficacy of rPep1 used for adjuvant Alum d0 and d14, and 5 μ g Alum+CpG on d35. Experiments were also performed to test the efficacy of rPep1 as therapeutic vaccine. In that case,

immunization was done at 7dpi with 10µg rPep1 or saline in 5µg Alum+CpG. Details (strain, inoculum size, day of sacrifice, number of animals) are specified in the results section. All injections were performed at the same time when endpoints included assessment of survival and fungal burden at specific time points.

5 *Efficacy of anti-Pep1 mAbs prior to or after challenge with C. neoformans*

The effect of passive serotherapy with anti-rPep1 mAbs was first assessed with mAb A9-4 at 20 or 100 µg in PBS injected intraperitoneally (ip) before (d-1) or after (1dpi or 7dpi) inoculation. Subsequent experiments compared the efficacy of 20 µg of A9-4, B4-1, J1-26, and J17-14 injected 7 dpi with either strain.

10 *Growth curves*

Yeasts (H99 or 52D) in stationary phase were suspended at 10^4 /ml in YPD. Growth in the presence of Mab (final concentration 0.1, 1 or 10 µg/ml) was measured as increased in turbidity using the Bioscreen C Automated Microbiology Growth Curve Analysis System (Thermofisher) set up to record optical density every 15 min for 32
15 hrs. Mabs were either E1 anti-glucuronoxylomannan mAb (Dromer et al. Infect. Immun. 1987) or anti-Pep1 mAbs (B4-1, J1-26 or J17-14) or an unrelated mAb IgG1. Each condition was tested in triplicate wells. Lag times, slope of the curve and maximal growth were calculated using the Richards non-linear regression model. Each parameter was normalized using the corresponding parameter obtained on H99
20 alone during the same run. Experiments were repeated twice except for mabs J1-26 and J17-14 that were only tested once.

Phagocytosis of C. neoformans by J774 cells

The experiments were performed as described before (Alanio et al. mBio 2011). Briefly, J774 cell suspensions (10^5 in fresh medium per well of a 24-wells culture
25 plate) were incubated at 37°C in 5% CO₂ for 48 h. The day of the experiment, calcofluor-stained *C. neoformans* suspension and mabs were added in fresh medium at the desired concentrations to the J774 cell monolayer, and incubated at 37°C and 5% CO₂ for 2 h (phagocytosis assay, *C. neoformans*/J774 ratio, 5:1). The anti-glucuronoxylomannan antibody E1 (Dromer et al. Infect. Immun. 1987) was used at 1
30 µg/ml as opsonin in all experiments except when testing the opsonic activity of the anti-Pep1 mabs alone. Several experiments were performed in order to test: (i) the

effect of the various anti-Pep1 mabs alone at 50, 5 and 0.5µg/ml; (ii) the effect of the various anti-Pep1 mabs at 50, 5 and 0.5µg/ml on the opsonic activity of E1 added concomitantly (+E1); or (iii) 30 min after the addition of the anti-Pep1 mabs (then E1).

- 5 At the end of the incubation, nonadherent extracellular yeast cells were then removed by PBS washings, and incubation was stopped to assess phagocytosis. Phagocytosis was determined after staining residual extracellular yeasts using anti-IgG-FITC, additional PBS washings, and macrophage lysis with distilled water. The samples were then centrifuged, resuspended in 1% paraformaldehyde in PBS (PFA-PBS),
10 vortexed, and sonicated for 3 min before analysis. Flow cytometry analyses were performed using MacsQuant analyzer and MacsQuantify software 2.0 (Milteniy BioTeC) to provide absolute quantification. Samples were analyzed using FlowJo 8.7 software (Tree Star, Inc.). Aggregates were excluded by gating relevant events in the forward scatter/side scatter (FSC/SSC) contour plot. The phagocytic index (PI) was
15 the number of events in the Calco^{high} FITC^{neg} gate after 2 hours. Results were expressed as the ratio of PI for the clinical strains compared to the H99 parameter determined in the same run. We assessed that results obtained during the two independent experiments were reproducible. Means of replicates were then used for analyses and shown on the graphs.

20 Expression of *PEPI*

- RNA extraction was performed on the clinical isolates and H99 cells coincubated with J774 cells for 2 hrs. J774 cells were washed twice with PBS, scraped, lysed in 2 ml 0.05% SDS-ice-cold water, and vortexed, and the pellet was collected after 3 min of centrifugation at 2,000 relative centrifugal force (RCF). RTL lysis buffer (500 µl;
25 Qiagen) and 1:100 β-mercaptoethanol (Sigma) were added to the *C. neoformans* pellets. The suspensions were transferred to Ceramique Magna Lyser green bead tubes (Roche Diagnostics), homogenized three times with the Magna Lyser instrument (30 s at 7,000 rpm), and centrifuged (3 min at 10,000 RCF). RNA extraction was performed on 350 µl supernatant using the RNeasy minikit (Qiagen). RNAs were quantified and
30 qualified using the Nanodrop spectrometer (ThermoFisher Scientific, Inc.). cDNA was generated from Turbo DNase (Ambion)-treated RNA using the Transcriptor first-

strand cDNA synthesis kit (Roche Diagnostics). Quantitative reverse transcription-PCR (RT-PCR) using 10 µl of Light-Cycler 480 SYBR green I master, 2 µl of cDNA, and specific primers for *PEP1* in a LightCycler 480 (Roche Diagnostics) consisted of a denaturation step at 95°C, 45 cycles of amplification (95°C for 5 s, 60°C for 5s, and 72°C for 5 s). Each cDNA was analyzed in duplicate and normalized with the corresponding *GAPDH* gene expression. Fold changes for each isolate were assessed compared to H99 under the same conditions. Two independent RNA extractions for each condition were analyzed blindly, and an internal calibrator consisting of the cDNA of H99 was used in each RT-PCR run as recommended.

10 **Example 2: Production of recombinant aspartyl protease Pep1 (rPep1) from *C. neoformans* var *neoformans***

A 1590 bp amplicon containing the cDNA of *PEP1* (SEQ ID NO: 18) was obtained with the primers PEP1BamHI (SEQ ID NO: 19) and PEP1XhoI (SEQ ID NO: 20), and subcloned into the pGEM-TE cloning vector (PROMEGA).
15 The 1.6-kb insert was then digested by *BamHI* and *XhoI*, and subcloned into the pHAT10/11/12 expression vector (ClonTech) and used to transform *E. coli* strain BL1. Purification of the recombinant protein (rPep1) was conducted according to the manufacturer's instructions. Preservation of the immunoreactive epitope of the rPep1 was assessed by immunoblotting with the pooled immune sera from survivors.

20 **Example 3: Use of rPep1 as vaccine against *C. neoformans* infection**

Preliminary experiments were done to determine the best adjuvant for rPep1 immunization. All rPep1-immunized mice had strong antibody response against rPep1. Vaccination with rPep1/CpG+Alum prior to inoculation with 10⁵ yeasts H99 was associated with the best survival compared to other adjuvants and controls
25 (Figure 3, p=0.0009; log-rank test).

Preimmunisation with rPep1/CpG+Alum provided prolonged survival in mice inoculated with 52D (Figure 4A, p=0.0019 log-rank test) with no detectable CFU in the target organs of the 4 mice surviving 100 dpi. CFU burden between the 3 groups did not differ at 7 dpi but was significantly decreased in the
30 vaccinated mice compared to controls at 14 dpi in brains and spleens (Figure 4B).

One sc injection of rPep1/Alum+CpG at 7dpi provided prolonged survival of mice inoculated with 10⁵ NIH52D comparable to that afforded by 1

immunization 15 day prior to inoculation (Figure 5A, $p = 0.042$, log-rank test) with drastic decreased in the fungal load in the surviving mice (Figure 5B) and a trend towards decreased fungal load at 21 dpi significant only in the brain (Figure 5C, $p = 0.0231$, Kruskal Wallis test).

5 Fungal load especially in the brain (Figure 5C and Figure 6A), and severity of meningitis (Figure 6B) were significantly decreased in mice inoculated with NIH52D and treated with the therapeutic vaccine. Analysis of the inflammation mediators showed a global decrease in mice treated by the vaccine (Figure 7). Whether the decreased fungal burden is per se responsible for this observation or
10 whether the neutralization of Pep1 by anti-Pep1 antibody induced by the vaccination is responsible for the decreased inflammation remains to be determined.

The effect of one sc injection of rPep1/Alum+CpG after inoculation was confirmed on mice inoculated with 10^3 H99 ($p=0.0004$, log-rank test, Figure 8A). The 4 mice surviving at 63 dpi had no viable yeasts in their brains and 2/4 had sterile
15 spleen and lungs (Figure 8B). There was a non-significant trends towards less CFU in the target organs of vaccinated compared to control mice sacrificed at 14 and 21 dpi (Figure 8C).

Example 4: Protection of mice by passive immunization with anti-Pep1 antibodies

20 Several mAbs were produced and deposited at the CNCM on Nov 17, 2014: CNCM I-4914 (B4-1), CNCM I-4915 (J1-26) and CNCM I-4916 (J17-14). Determination of the epitopes recognized by the various mAbs produced against rPep1 was done by competitive ELISA and showed that three Mabs recognized different epitopes: SEQ ID NO: 12 and SEQ ID NO: 15 are recognized by J1-26; SEQ
25 ID NO: 13 is recognized by J17-14 and B4-1 and SEQ ID NO: 16 is recognized by B4-1.

Anti-Pep1 mabs, especially B4-1 seem to affect the normal growth of *C. neoformans* H99 and 52D by altering the growth curves (increasing the latency), decreasing the maximal growth and altering the slope which is not seen or not as
30 much with E1 and an unrelated mAb (Figures 13). The effect seems more pronounced for 52D.

mAbs B4-1 did not modify the phagocytosis by murine macrophages in the presence of E1, a monoclonal antibody specific for *Cryptococcus neoformans* capsular polysaccharide (Dromer et al., Infect. Immun., 1987, 55, 742-748). None of the anti-Pep1 mabs promoted the phagocytosis of H99 by J774 cells in comparison with E1 (Figure 14). The combination of E1+ B4-1 did not alter phagocytosis compared to E1 alone. The addition of E1 after preincubation with the anti-Pep1 mAbs resulted in an increased phagocytosis compared to the reference condition and to concomitant incubation. This suggests the neutralization of potential anti-phagocytic activity of Pep1.

These results are confirmed by the correlation between phagocytic index and expression of *PEP1* in clinical isolates (Figure 15) which suggests that Pep1 may have anti-phagocytic properties that the monoclonal antibodies may be able to revert as suggested by the experiments using a combination of E1 and anti-Pep1 mAbs.

Injection of mAb B4-1 to mice provided prolonged survival when injected at 20µg (Figure 9A, $p=0.028$, log-rank test), or 100µg 24 h prior to or 1 day or 7 days (Fig 9B, $p=0.004$) after inoculation with NIH52D. The dose-response experiment (Figure 10) suggests that increasing the dose (70 and 200 µg) of mab B4-1 injected does not provide better survival and may even be less effective than smaller doses (20 and 7 µg) in terms of survival and even residual fungal load since the majority of the surviving mice were even completely cured of the infection.

A comparison of the 3 mAbs after inoculation with NIH52D, showed no decrease in fungal burden at 21dpi. Prolongation of survival after inoculation with NIH52D was seen mostly with B4-1 and J17-14 (Figure 11A, $p=0.0662$), whereas fungal burden in all surviving mice was decreased at 63 dpi except in the brain of mice inoculated with J17-14 (Figure 11B).

Injection of mAb B4-1, J17.14 or J1.26 to mice infected with H99 (10^5 /mouse) provided prolonged survival (Figure 12, $p=0.0182$ log-rank test). Prolongation of survival was seen mostly with B4-1 (Figure 12, $p=0.0118$). Fungal burden was decreased at 54 dpi in 2 of the 3 surviving mice (1 treated with B4.1 and 1 with J1.26).

Table I: Sequence identity of Pep1 with other fungal aspartyl proteases

Organism	GenBank ID	% Pairwise Identity
<i>Cryptococcus neoformans</i>	XP_566887	100.00%
<i>Cryptococcus neoformans</i>	XP_012046817	94.10%
<i>Cryptococcus gattii</i>	XP_003191823	86.80%
<i>Cryptococcus gattii</i>	KIR53730	86.80%
<i>Cryptococcus gattii</i>	KIR48411	86.20%
<i>Cryptococcus gattii</i>	KGB75517	84.40%
<i>Cryptococcus gattii</i>	KIR86636	83.20%
<i>Trichosporon asahii</i>	EKD04292	71.20%
<i>Talaromyces marneffeii</i>	XP_002143541	67.50%
<i>Talaromyces marneffeii</i>	KFX49218	66.00%
<i>Trichosporon asahii</i>	XP_014177670	65.30%
<i>Kluyveromyces marxianus</i>	BAO41873	65.00%
<i>Rhinocladiella mackenziei</i>	XP_013268148	65.00%
<i>Paracoccidioides brasiliensis</i>	AAP32823	64.90%
<i>Coccidioides immitis</i>	XP_001244246	64.80%
<i>Saccharomyces cerevisiae</i>	EGA76604	64.80%
<i>Trichosporon asahii</i>	EKD04292	64.80%
<i>Candida auris</i>	KND96412	64.70%
<i>Coccidioides posadasii</i>	AAZ92540	64.70%
<i>Saccharomyces cerevisiae</i>	AJV96513	64.70%
<i>Saccharomyces cerevisiae</i>	AJW09566	64.70%
<i>Saccharomyces cerevisiae</i>	AJW12026	64.70%
<i>Saccharomyces cerevisiae</i>	NP_015171	64.70%
<i>Saccharomyces cerevisiae</i>	AJW20271	64.70%
<i>Saccharomyces cerevisiae</i>	AJW18989	64.70%
<i>Saccharomyces cerevisiae</i>	AJU25730	64.70%
<i>Saccharomyces cerevisiae</i>	AJW16371	64.70%
<i>Saccharomyces cerevisiae</i>	GAA26821	64.70%
<i>Paracoccidioides brasiliensis</i>	XP_010756433	64.60%
<i>Exophiala dermatitidis</i>	XP_009158792	64.40%
<i>Saccharomyces cerevisiae</i>	AJV92838	64.40%
<i>Saccharomyces cerevisiae</i>	AJV94247	64.40%
<i>Saccharomyces cerevisiae</i>	EEU09100	64.40%
<i>Paracoccidioides brasiliensis</i>	EEH19972	64.30%
<i>Clavispora lusitaniae</i>	XP_002615242	64.20%
<i>Rhizopus delemar</i>	EIE89256	64.20%
<i>Saccharomyces cerevisiae</i>	EDZ68928	64.20%
<i>Saccharomyces cerevisiae</i>	EHM99929	64.10%
<i>Sporothrix schenckii</i>	ERT02753	64.10%

<i>Aspergillus oryzae</i>	XP_001819842	63.70%
<i>Geotrichum candidum</i>	CDO51223	63.50%
<i>Meyerozyma guilliermondii</i>	XP_001483416	63.40%
<i>Rhizopus microsporus</i>	CEG74799	63.30%
<i>Candida albicans</i>	CAA31962	63.20%
<i>Debaryomyces hansenii</i>	XP_458031	62.20%
<i>Histoplasma capsulatum</i>	EGC40857	62.10%
<i>Histoplasma capsulatum</i>	XP_001542991	61.80%
<i>Histoplasma capsulatum</i>	EEH04825	61.80%
<i>Lichtheimia ramosa</i>	CDS05348	61.70%
<i>Kluyveromyces lactis</i>	XP_453326	61.30%
<i>Microsporum gypseum</i>	XP_003175087	61.20%
<i>Malassezia pachydermatis</i>	KOS12980	60.90%
<i>Aspergillus nidulans</i>	XP_660507	60.70%
<i>Pneumocystis jirovecii</i>	CCJ31368	58.90%
<i>Trichoderma harzianum</i>	KKP05288	58.90%
<i>Candida glabrata</i>	XP_449442	58.60%
<i>Malassezia globosa</i>	XP_001729651	58.50%
<i>Rhizopus delemar</i>	EIE82161	58.20%
<i>Trichophyton interdigitale</i>	EZF32786	58.10%
<i>Trichophyton tonsurans</i>	EGD99457	58.10%
<i>Trichophyton rubrum</i>	EZF25931	57.90%
<i>Trichophyton rubrum</i>	XP_003232712	57.90%
<i>Trichoderma atroviride</i>	XP_013942144	57.50%
<i>Trichophyton verrucosum</i>	XP_003020295	57.30%
<i>Fusarium</i> sp.	CEG04259	57.20%
<i>Fusarium oxysporum</i>	EWY86405	57.10%
<i>Fusarium oxysporum</i>	EGU83511	57.10%
<i>Fusarium oxysporum</i>	KNB13049	57.10%
<i>Fusarium fujikuroi</i>	CCT71500	57.00%
<i>Lichtheimia corymbifera</i>	CDH52400	56.60%
<i>Candida orthopsilosis</i>	XP_003867779	56.50%
<i>Rhizopus microsporus</i>	CEG73869	56.50%
<i>Rhizopus microsporus</i>	CEI95549	56.50%
<i>Candida parapsilosis</i>	CCE40477	56.10%
<i>Emmonsia crescens</i>	KKZ62546	56.10%
<i>Rhizopus microsporus</i>	CEG76436	55.90%
<i>Lichtheimia ramosa</i>	CDS08210	55.80%
<i>Rhizopus microsporus</i>	CEG73870	55.80%
<i>Candida dubliniensis</i>	XP_002418645	55.10%
<i>Exophiala xenobiotica</i>	XP_013321677	55.10%
<i>Fonsecaea pedrosoi</i>	XP_013278715	55.00%

<i>Cladophialophora carrionii</i>	XP_008724614	54.80%
<i>Candida tropicalis</i>	XP_002547417	54.70%
<i>Candida albicans</i>	KHC38341	54.60%
<i>Candida albicans</i>	XP_713194	54.60%
<i>Candida albicans</i>	KHC45840	54.60%
<i>Candida albicans</i>	KGQ89270	54.60%
<i>Candida albicans</i>	P10977	54.40%
<i>Candida albicans</i>	EEQ47270	54.40%
<i>Exophiala oligosperma</i>	KIW43340	54.40%
<i>Cladophialophora bantiana</i>	KIW87422	54.30%
<i>Exophiala spinifera</i>	KIW14941	54.30%
<i>Aspergillus clavatus</i>	XP_001271141	54.20%
<i>Aspergillus niger</i>	XP_001399855	54.20%
<i>Aspergillus niger</i>	AAA20876	54.00%
<i>Aureobasidium pullulans</i>	KEQ89895	54.00%
<i>Aspergillus fumigatus</i>	XP_754479	53.90%
<i>Neosartorya fischeri</i>	XP_001263323	53.60%
<i>Chaetomium globosum</i>	XP_001227563	52.90%
<i>Madurella mycetomatis</i>	KOP43023	52.70%
<i>Myceliophthora thermophila</i>	XP_003665229	52.60%
<i>Mucor circinelloides</i>	EPB83353	52.10%
<i>Aspergillus terreus</i>	XP_001213854	51.90%
<i>Lichtheimia corymbifera</i>	CDH51907	51.80%
<i>Mucor circinelloides</i>	EPB89553	51.60%
<i>Lichtheimia ramosa</i>	CDS03074	51.20%
<i>Rhizopus delemar</i>	EIE77417	51.10%
<i>Aspergillus parasiticus</i>	KJK63739	50.10%
<i>Lichtheimia corymbifera</i>	CDH52330	49.90%
<i>Aspergillus flavus</i>	XP_002381878	49.60%
<i>Aspergillus oryzae</i>	EIT76268	49.60%
<i>Aspergillus flavus</i>	KOC16935	49.30%
<i>Aspergillus oryzae</i>	XP_001825179	49.30%

Table II: Sequence identity of peptide epitope 3 of *Cryptococcus* spp. with that of other fungi

[illegible]

Table III: Sequence identity of peptide epitope 4 of *Cryptococcus* spp. with that of other fungi

[illegible]

AAA20876	<i>Aspergillus niger</i>	73.3	73.3	73.3	73.3	73.3	73.3	73.3
XP_001819842	<i>Aspergillus oryzae</i>	66.7	66.7	66.7	66.7	66.7	66.7	66.7
XP_001213854	<i>Aspergillus terreus</i>	60	60	60	60	60	60	60
XP_660507	<i>Aspergillus nidulans</i>	66.7	66.7	66.7	66.7	66.7	66.7	66.7
XP_002143541	<i>Talaromyces marneffei</i>	93.3	93.3	93.3	93.3	93.3	93.3	93.3
KFX49218	<i>Talaromyces marneffei</i>	93.3	93.3	93.3	93.3	93.3	93.3	93.3
EZF32786	<i>Trichophyton interdigitale</i>	80	80	80	80	80	80	80
EGD99457	<i>Trichophyton tonsurans</i>	80	80	80	80	80	80	80
XP_003232712	<i>Trichophyton rubrum</i>	80	80	80	80	80	80	80
EZF25931	<i>Trichophyton rubrum</i>	80	80	80	80	80	80	80
XP_003020295	<i>Trichophyton verrucosum</i>	80	80	80	80	80	80	80
XP_003175087	<i>Microsporum gypseum</i>	80	80	80	80	80	80	80
XP_001244246	<i>Coccidioides immitis</i>	93.3	93.3	93.3	93.3	93.3	93.3	93.3
AAZ92540	<i>Coccidioides posadasii</i>	93.3	93.3	93.3	93.3	93.3	93.3	93.3
EGC40857	<i>Histoplasma capsulatum</i>	80	80	80	80	80	80	80
EEH04825	<i>Histoplasma capsulatum</i>	73.3	73.3	73.3	73.3	73.3	73.3	73.3
XP_001542991	<i>Histoplasma capsulatum</i>	80	80	80	80	80	80	80
EEH19972	<i>Paracoccidioides brasiliensis</i>	80	80	80	80	80	80	80
XP_010756433	<i>Paracoccidioides brasiliensis</i>	80	80	80	80	80	80	80
AAP32823	<i>Paracoccidioides brasiliensis</i>	80	80	80	80	80	80	80
KIW43340	<i>Exophiala oligosperma</i>	66.7	66.7	66.7	66.7	66.7	66.7	66.7
XP_013321677	<i>Exophiala xenobiotica</i>	66.7	66.7	66.7	66.7	66.7	66.7	66.7
KIW14941	<i>Exophiala spinifera</i>	66.7	66.7	66.7	66.7	66.7	66.7	66.7
XP_009158792	<i>Exophiala dermatitidis</i>	73.3	73.3	73.3	73.3	73.3	73.3	73.3
EGU83511	<i>Fusarium oxysporum</i>	66.7	66.7	66.7	66.7	66.7	66.7	66.7
KNB13049	<i>Fusarium oxysporum</i>	66.7	66.7	66.7	66.7	66.7	66.7	66.7
EWY86405	<i>Fusarium oxysporum</i>	66.7	66.7	66.7	66.7	66.7	66.7	66.7
CCT71500	<i>Fusarium fujikuroi</i>	66.7	66.7	66.7	66.7	66.7	66.7	66.7
CEG04259	<i>Fusarium sp.</i>	73.3	73.3	73.3	73.3	73.3	73.3	73.3
CCJ31368	<i>Pneumocystis jirovecii</i>	53.3	53.3	53.3	53.3	53.3	53.3	53.3
CDH51907	<i>Lichtheimia corymbifera</i>	66.7	66.7	66.7	66.7	66.7	66.7	66.7
CDS03074	<i>Lichtheimia ramosa</i>	66.7	66.7	66.7	66.7	66.7	66.7	66.7
CDH52400	<i>Lichtheimia corymbifera</i>	73.3	73.3	73.3	73.3	73.3	73.3	73.3
CDS08210	<i>Lichtheimia ramosa</i>	80	80	80	80	80	80	80

EPB89553	Mucor circinelloides	66.7	66.7	66.7	66.7	66.7	66.7	66.7
EIE89256	Rhizopus delemar	73.3	73.3	73.3	73.3	73.3	73.3	73.3
CEG74799	Rhizopus microsporus	66.7	66.7	66.7	66.7	66.7	66.7	66.7
EPB83353	Mucor circinelloides	73.3	73.3	73.3	73.3	73.3	73.3	73.3
CDH52330	Lichtheimia corymbifera	60	60	60	60	60	60	60
CDS05348	Lichtheimia ramosa	60	60	60	60	60	60	60
CEG73870	Rhizopus microsporus	46.7	46.7	46.7	46.7	46.7	46.7	46.7
CEG73869	Rhizopus microsporus	46.7	46.7	46.7	46.7	46.7	46.7	46.7
CEG76436	Rhizopus microsporus	40	40	40	40	40	40	40
CEI95549	Rhizopus microsporus	46.7	46.7	46.7	46.7	46.7	46.7	46.7
EIE82161	Rhizopus delemar	53.3	53.3	53.3	53.3	53.3	53.3	53.3
EIE77417	Rhizopus delemar	40	40	40	40	40	40	40

CLAIMS

1. An antibody for use in the treatment of a fungal disease,
wherein said antibody binds to an antigen being chosen from:

5 a) a recombinant aspartyl protease protein comprising an amino acid
sequence having at least 48 % identity with SEQ ID NO: 1 and comprising two
catalytic motifs, SEQ ID NO: 2 and SEQ ID NO: 3, in positions corresponding to
positions 145 to 149 and 327 to 331 of SEQ ID NO: 1 and further comprising at least
one protective epitope, said epitope(s) corresponding to one or more of the peptides
in positions 1 to 15, 271 to 285, 296 to 310 and 346 to 360 of SEQ ID NO: 1, or a
10 functional variant thereof; and

b) a polypeptide fragment of the protein in a) comprising at least one
of said protective epitope(s), and,

wherein said antibody is an antibody recognizing specifically at least one of said
protective epitope(s) or a functional fragment thereof comprising at least the antibody
15 binding region.

2. The antibody for the use according to claim 1, wherein:

- the peptide in positions 1 to 15 consists of SEQ ID NO: 12;
- the peptide in positions 271 to 285 consists of a sequence having at
least 60 % identity with SEQ ID NO: 13;
- 20 - the peptide in positions 296 to 310 consists of a sequence having at
least 60 % identity with SEQ ID NO: 15; and
- the peptide in positions 346 to 360 consists of a sequence having at
least 60 % identity with SEQ ID NO: 16.

3. The antibody for the use according to claim 1 or claim 2, which is a monoclonal
25 antibody recognizing specifically the peptides of SEQ ID NO: 12 and SEQ ID
NO: 15, the peptide of SEQ ID NO: 13, or the peptides of SEQ ID NO:13 and
SEQ ID NO:16, or a functional fragment thereof comprising at least the antigen
binding site.

4. The antibody for the use according to claim 3, which is a monoclonal antibody
30 produced by an hybridoma selected from the hybridomas deposited at CNCM
under accession number I-4914, I-4915 and I-4916 or a Fv, Fab or Fab'2 fragment
thereof.

5. The antibody for the use according to any one of claims 1 to 4, wherein said antibody is used simultaneously, separately or sequentially with at least one immunotherapeutic agent and/or antifungal agent.
6. The antibody for the use according to any one of claims 1 to 5, wherein said treatment of a fungal disease is a therapeutic treatment administered after diagnosis of the fungal infection.
7. The antibody for the use according to any one of claims 1 to 6, wherein said fungal disease is a superficial mycosis, a cutaneous mycosis, a subcutaneous mycosis or a systemic mycosis due to primary or opportunistic pathogenic fungi.
8. The antibody for the use for the treatment of a fungal disease according to claim 7, wherein said fungal disease is selected from the group consisting of: candidiasis, aspergillosis, cryptococcosis, trichosporonosis, mucormycosis, fusariosis, pneumocystosis, dermatophytomycosis, penicilliosis marneffei, coccidioidomycosis, histoplasmosis, and paracoccidioidomycosis.
9. The antibody for the use for the treatment of a fungal disease according to claim 8, wherein said fungal disease is cryptococcosis.
10. A pharmaceutical composition comprising at least an antibody according to any one of claims 1, 2, 3 and 4 or a functional fragment comprising at least the antibody binding region, and a pharmaceutically acceptable vehicle.
11. A recombinant antigen for use in the treatment of a fungal disease, wherein said antigen is chosen from:
 - a) a recombinant aspartyl protease protein comprising an amino acid sequence having at least 48 % identity with SEQ ID NO: 1 and comprising two catalytic motifs, SEQ ID NO: 2 and SEQ ID NO: 3, in positions corresponding to positions 145 to 149 and 327 to 331 of SEQ ID NO: 1 and further comprising at least one protective epitope, said epitope(s) corresponding to one or more of the peptides in positions 1 to 15, 271 to 285, 296 to 310 and 346 to 360 of SEQ ID NO: 1, or a functional variant thereof; and
 - b) a polypeptide fragment of the protein in a) comprising at least one of said protective epitope(s).
12. The recombinant antigen for the use according to claim 11, wherein:
 - the peptide in positions 1 to 15 consists of SEQ ID NO: 12;

- the peptide in positions 271 to 285 consists of a sequence having at least 60 % identity with SEQ ID NO: 13;

- the peptide in positions 296 to 310 consists of a sequence having at least 60 % identity with SEQ ID NO: 15; and

5 - the peptide in positions 346 to 360 consists of a sequence having at least 60 % identity with SEQ ID NO: 16.

13. The recombinant antigen for the use according to claim 11 or claim 12, which is a recombinant aspartyl protease protein of a pathogenic fungus selected from the group consisting of: *Aspergillus* spp., *Candida* spp., *Clavispora* spp., *Coccidioides* spp.,
10 *Cryptococcus* spp., *Exophiala* spp., *Fusarium* spp., *Histoplasma* spp., *Kluyveromyces* spp., *Mucorales* such as *Lichtheimia* spp., *Mucor* spp. and *Rhizopus* spp., *Meyerozyma* spp., *Microsporum* spp., *Neosartorya* spp., *Paracoccidioides* spp., *Pneumocystis* spp., *Talaromyces* spp., *Trichophyton* spp. and *Trichosporon* spp., or a functional variant thereof.

15 14. The recombinant antigen for the use according to any one of claims 11 to 13, which is a recombinant aspartyl protease protein comprising an amino acid sequence having at least 60 % identity with SEQ ID NO: 1.

15. The recombinant antigen for the use according to any one of claims 11 to 14, which is a *Cryptococcus* spp. recombinant aspartyl protease protein comprising an
20 amino acid sequence having at least 80 % identity with SEQ ID NO: 1.

16. The *Cryptococcus* spp. recombinant aspartyl protease protein for the use according to claim 15, which comprises any one of SEQ ID NO: 1 and 6 to 11.

17. The recombinant antigen for the use according to any one of claims 11 to 16, wherein said antigen is used simultaneously, separately or sequentially with at least
25 one immunotherapeutic agent and/or antifungal agent.

18. The recombinant antigen for the use according to any one of claims 11 to 17, wherein said treatment of a fungal disease is a therapeutic treatment administered after diagnosis of the fungal infection.

19. The recombinant antigen for the use according to any one of claims 11 to 18, wherein said fungal disease is a superficial mycosis, a cutaneous mycosis, a
30 subcutaneous mycosis or a systemic mycosis due to primary or opportunistic pathogenic fungi.

20. The recombinant antigen for the use according to any one of claims 11 to 18, wherein said fungal disease is selected from the group consisting of candidiasis, aspergillosis, cryptococcosis, trichosporonosis, mucormycosis, fusariosis, pneumocystosis, dermatophytomycosis, penicilliosis marneffeii, coccidioidomycosis, histoplasmosis, and paracoccidioidomycosis, and is preferably cryptococcosis.

1 / 16

MKTSAILIAALSAAASVEAGIHRMKLEKQTPSSLTGTFFPPSPELEAKWLASKYLGQEYTDQMPLG
(Epitope 1)

GFGGAGKKFKSGNKHTEHPEQNDDEERYWAQMVDQSAHSQMIDVLKGGHGVPLSNYMNAQYF

ATMEIGTPFQTFK**VILD****TGSSNLWVPS**VKCTSIACFLHSKYDSSQSSTYKANGSDFEIHYGSGSLEGF

ISQDTVSIGDLVKKQDFAEATKEPGLAFAFGKFDGILGLGYDTISVNHIIVPPFYNMLNQHLLDEPV

FSFRLGSSDEDEGGEAIFGGIDDSAYS**GELQYVPVRRKGYWEVELESISFGDEELELENTGAAIDTGTS**
(Epitope 2) (Epitope 3)

LIVMPTDVAELLNKE**IGAEKSWNGQYTVDCSTVSSLPVLAFTFGGKDYKLTGDDYILNAGGTCISSF**
(Epitope 4)

TGMDIPAPIGPLWIVGDVFLRKYYTVYDLGKNAVGFASK (SEQ ID NO: 1)

Figure 1



Consensus		Europe 2																Europe 3																Europe 4																Europe 5																Europe 6																Europe 7																Europe 8																Europe 9																Europe 10																Europe 11																Europe 12																Europe 13																Europe 14																Europe 15																Europe 16																Europe 17																Europe 18																Europe 19																Europe 20																Europe 21																Europe 22																Europe 23																Europe 24																Europe 25																Europe 26																Europe 27																Europe 28																Europe 29																Europe 30																Europe 31																Europe 32																Europe 33																Europe 34																Europe 35																Europe 36																Europe 37																Europe 38																Europe 39																Europe 40																Europe 41																Europe 42																Europe 43																Europe 44																Europe 45																Europe 46																Europe 47																Europe 48																Europe 49																Europe 50																Europe 51																Europe 52																Europe 53																Europe 54																Europe 55																Europe 56																Europe 57																Europe 58																Europe 59																Europe 60																Europe 61																Europe 62																Europe 63																Europe 64																Europe 65																Europe 66																Europe 67																Europe 68																Europe 69																Europe 70																Europe 71																Europe 72																Europe 73																Europe 74																Europe 75																Europe 76																Europe 77																Europe 78																Europe 79																Europe 80																Europe 81																Europe 82																Europe 83																Europe 84																Europe 89	
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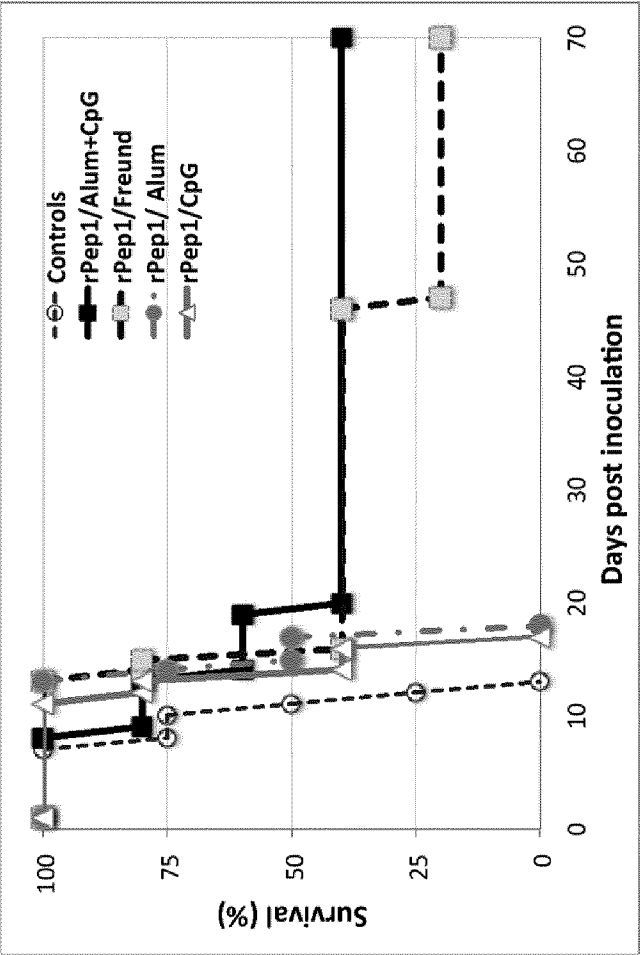


Figure 3

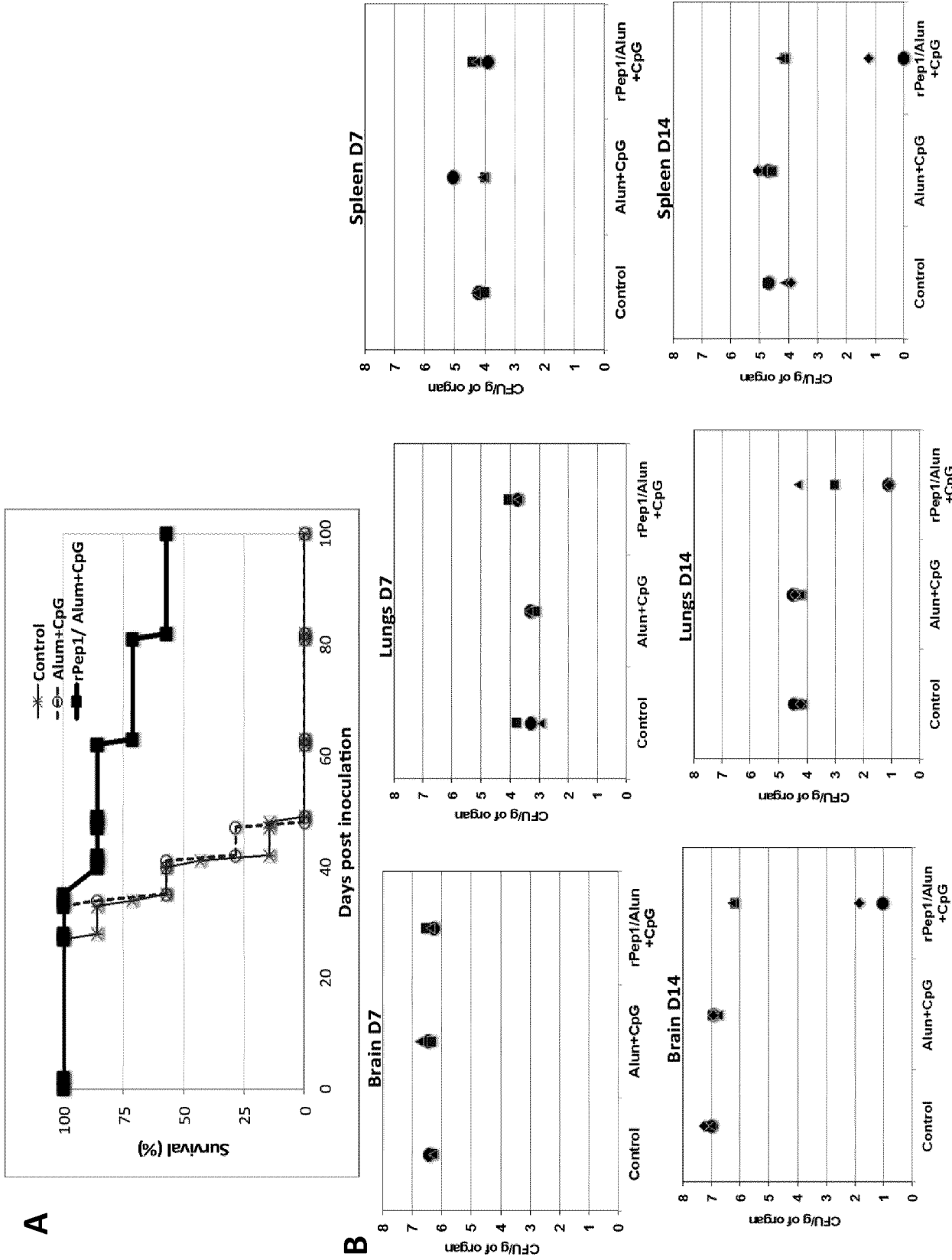


Figure 4

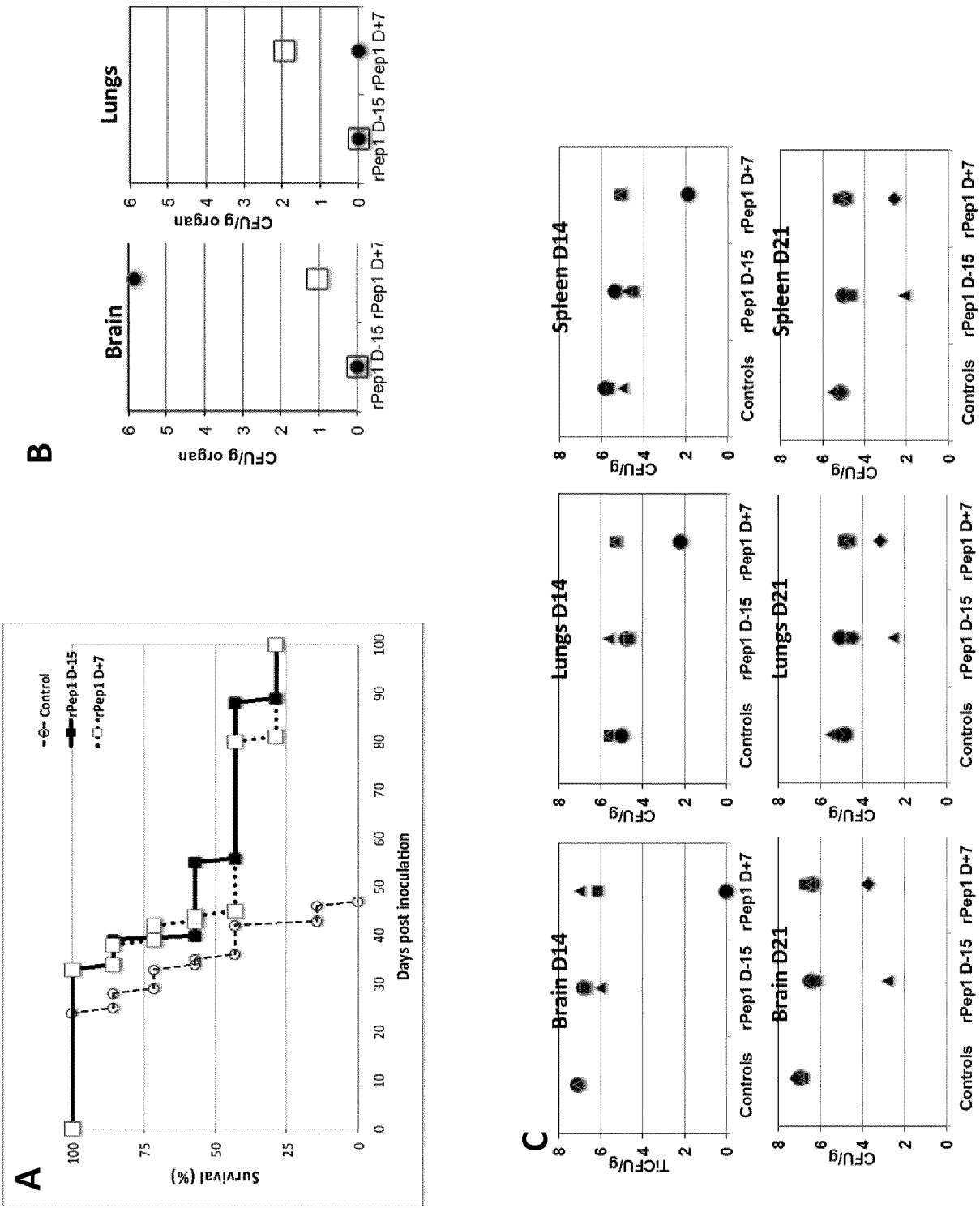


Figure 5

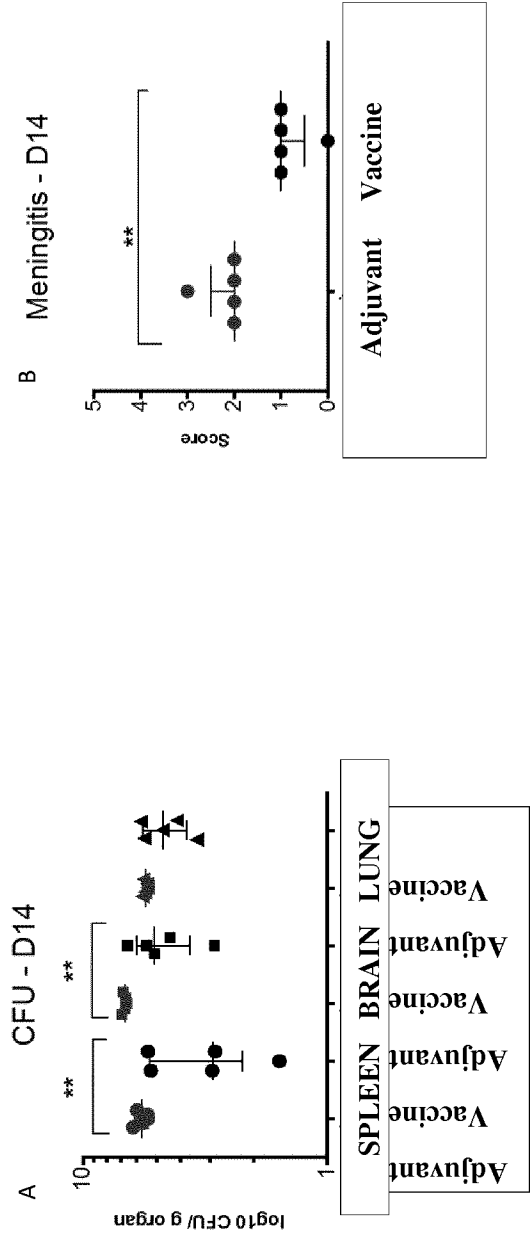


Figure 6

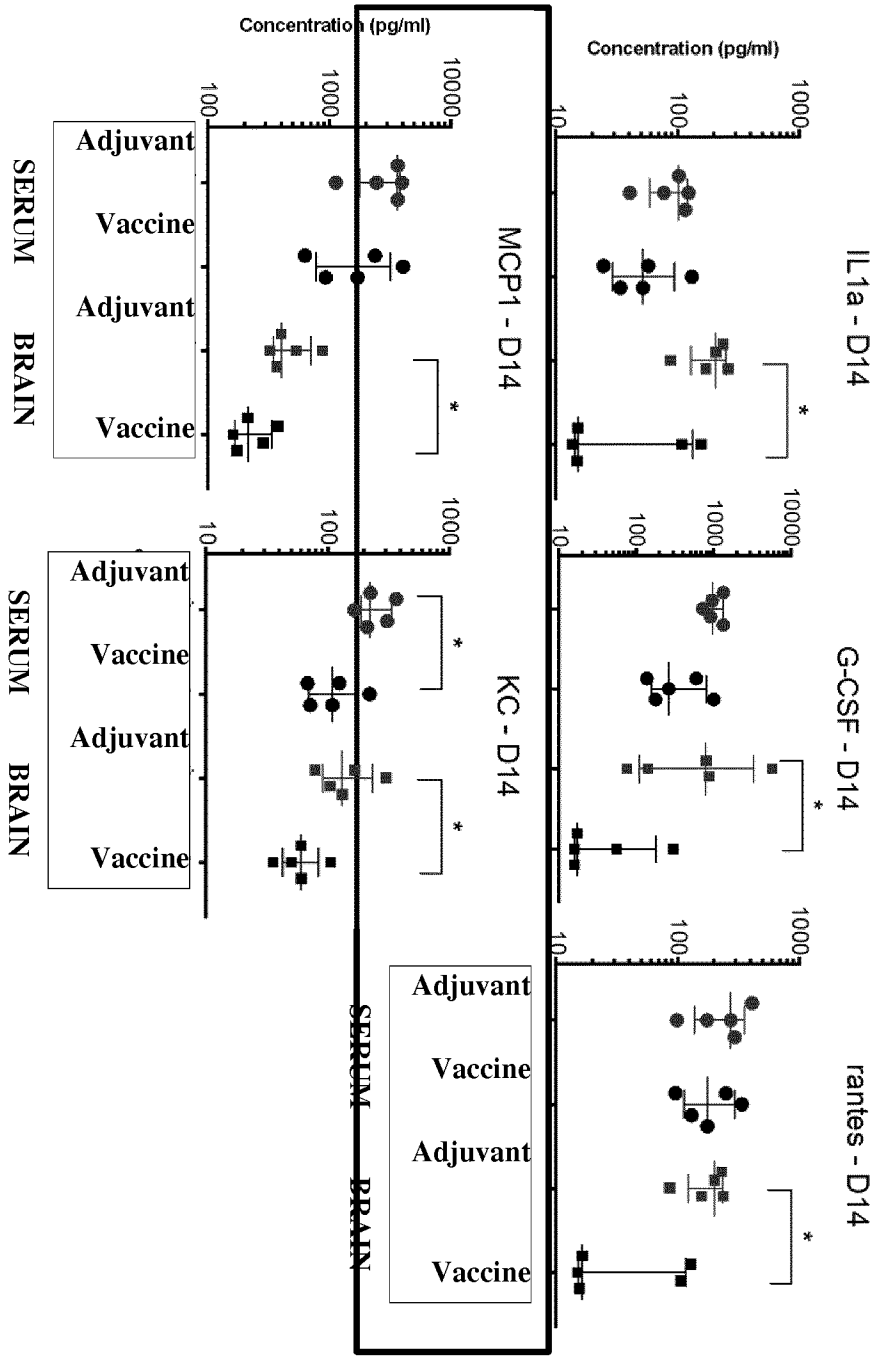
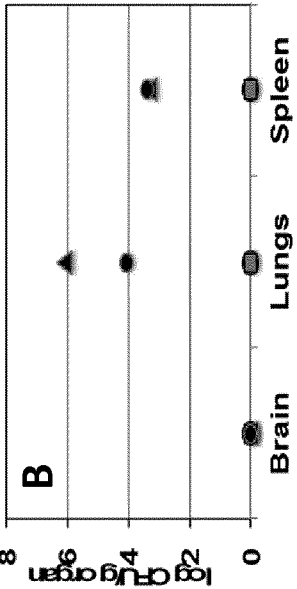
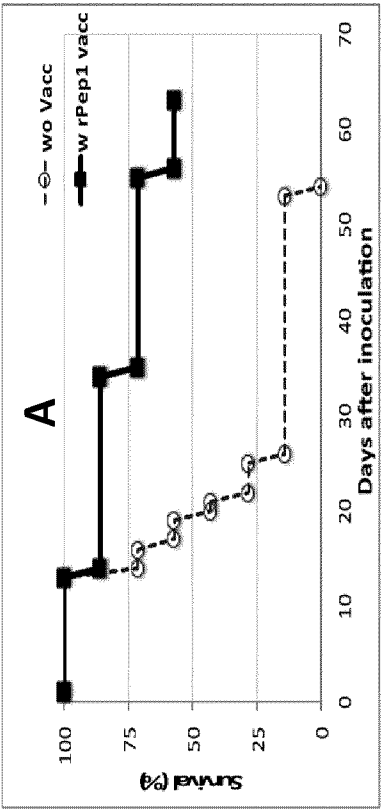


Figure 7

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C

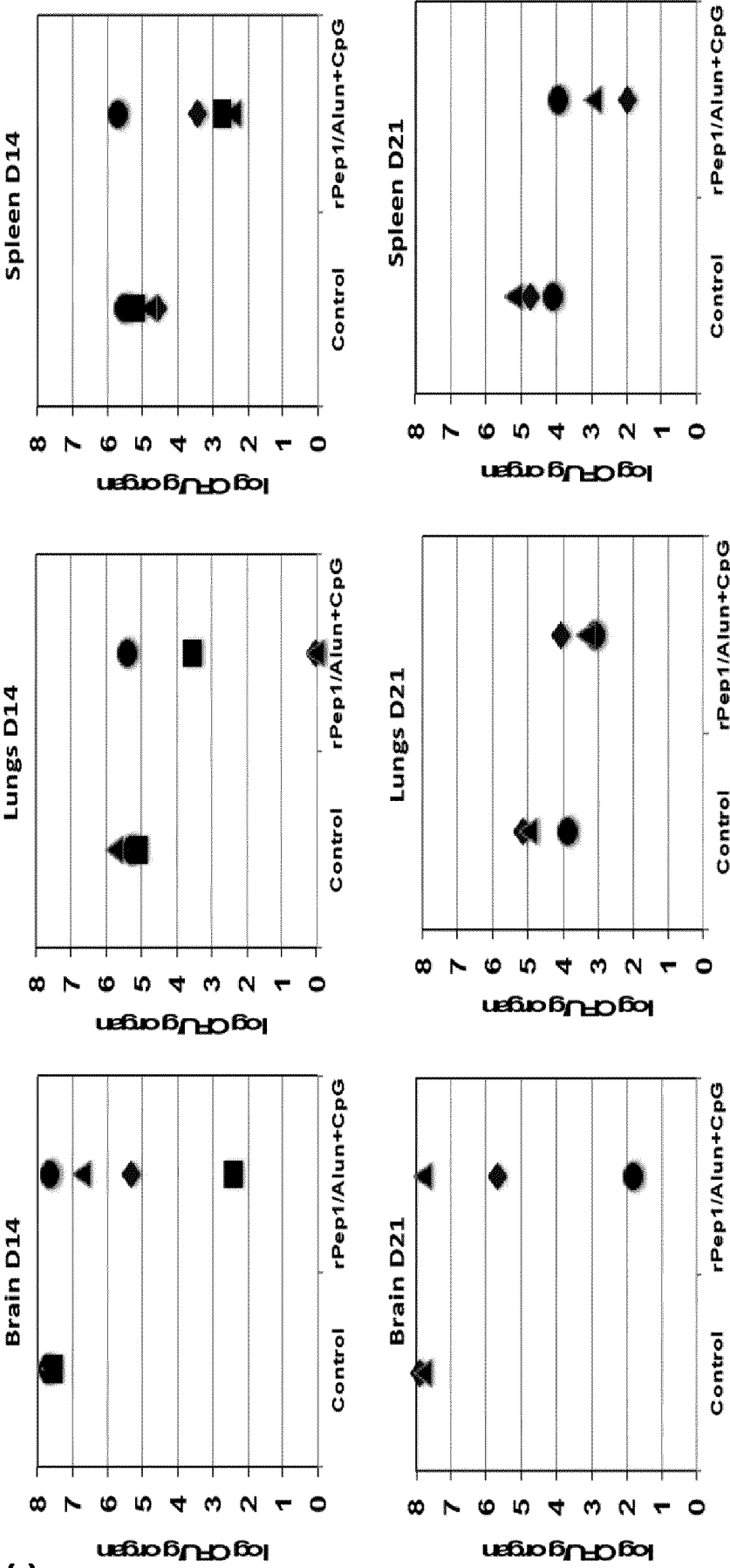
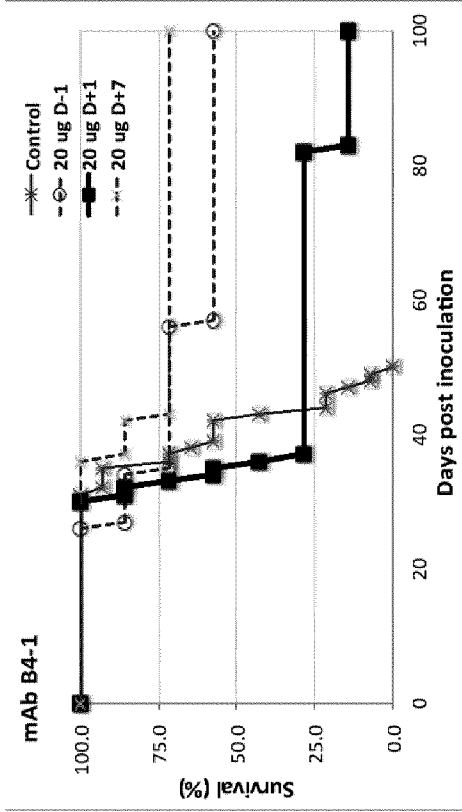


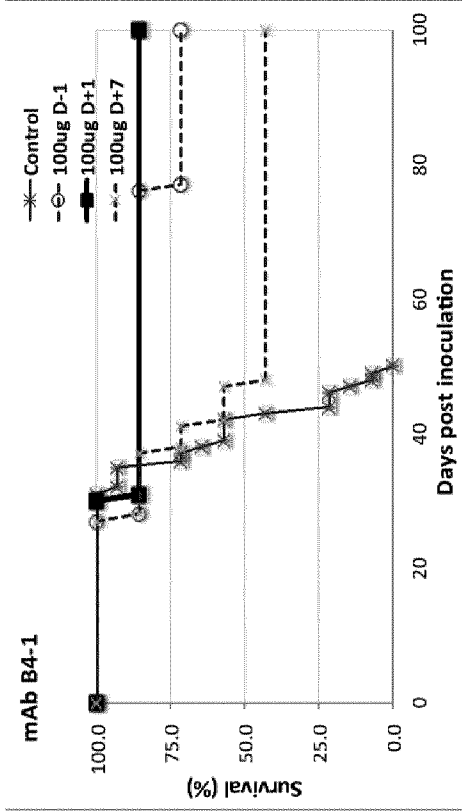
Figure 8

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A



B



CA

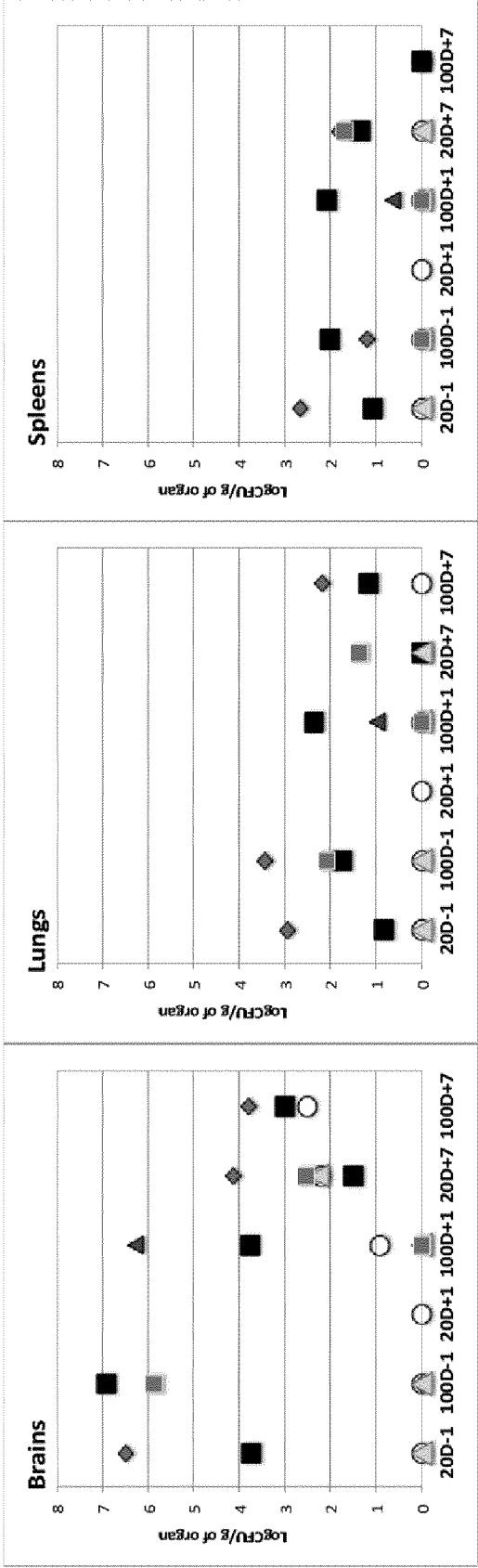


Figure 9

11/16

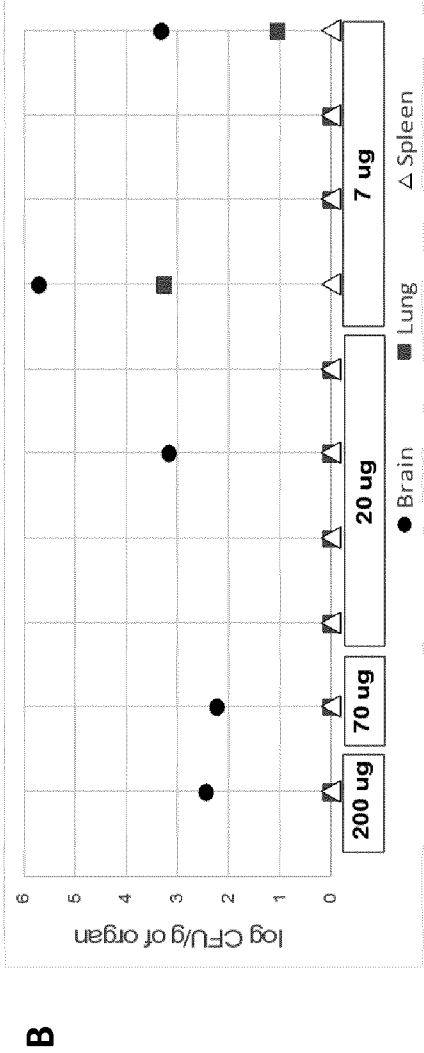
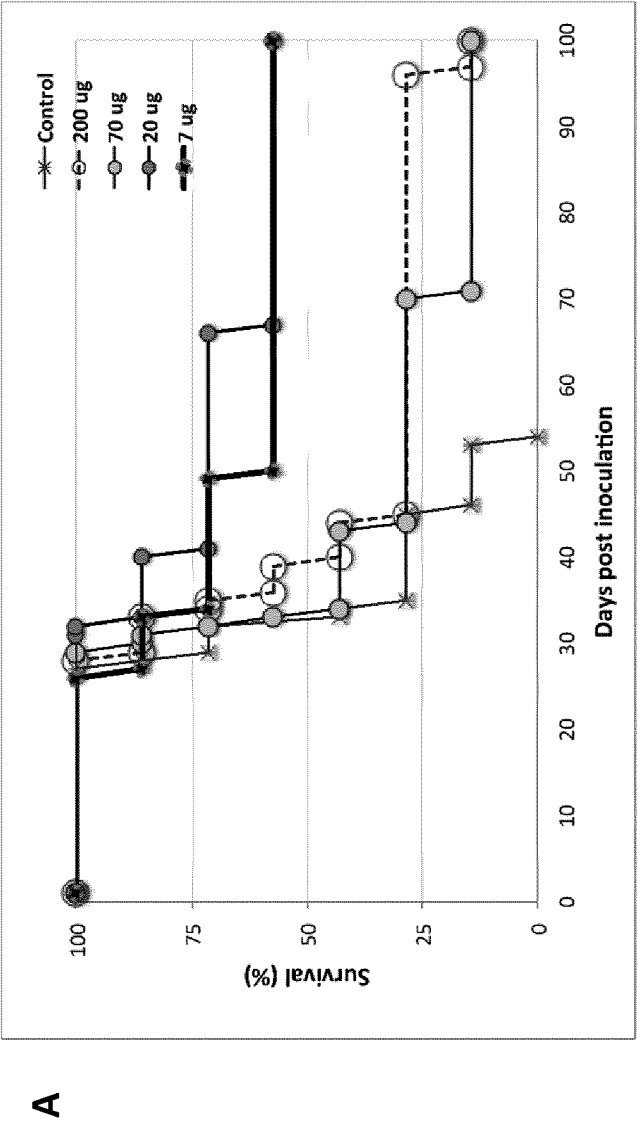


Figure 10

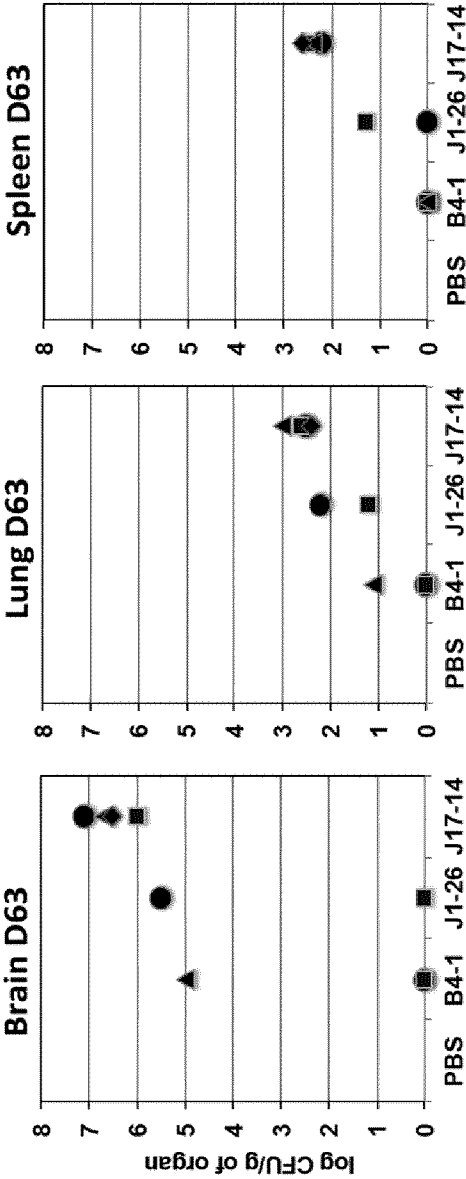
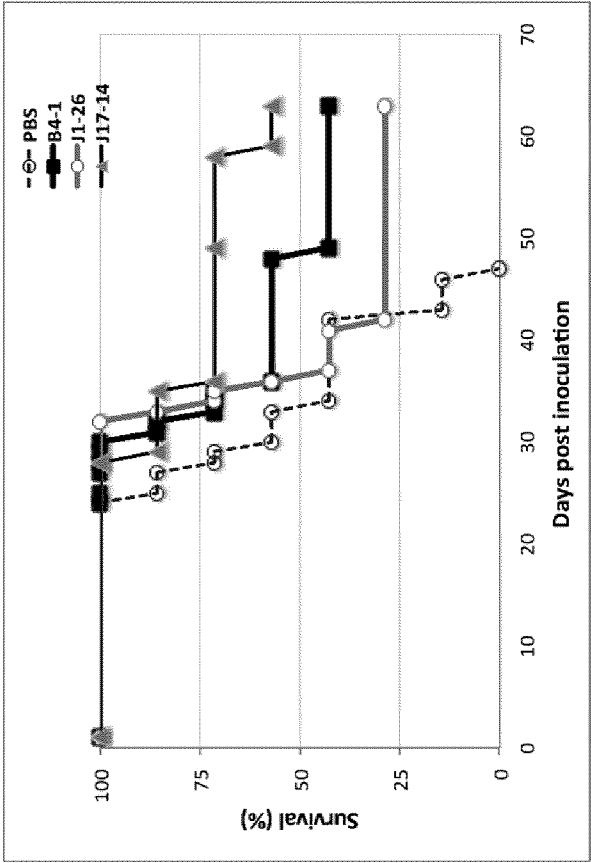


Figure 11

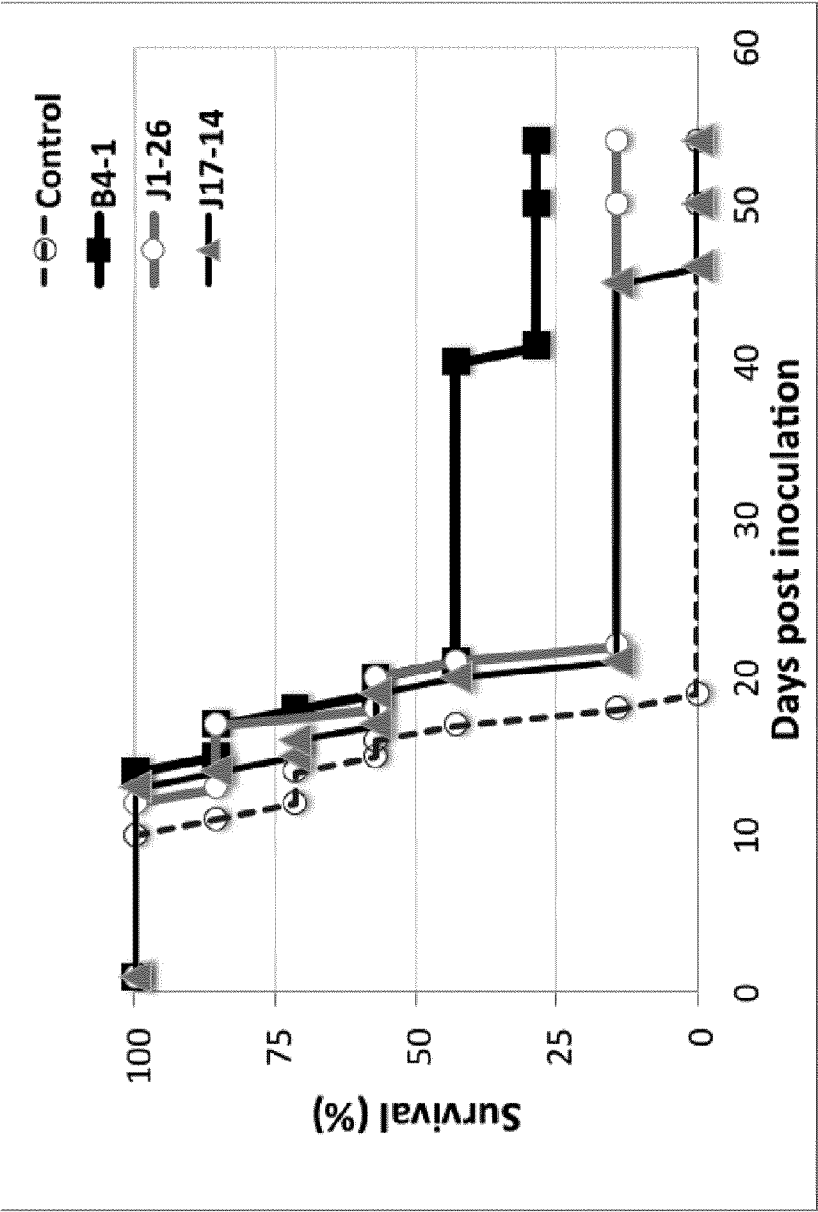


FIGURE 12

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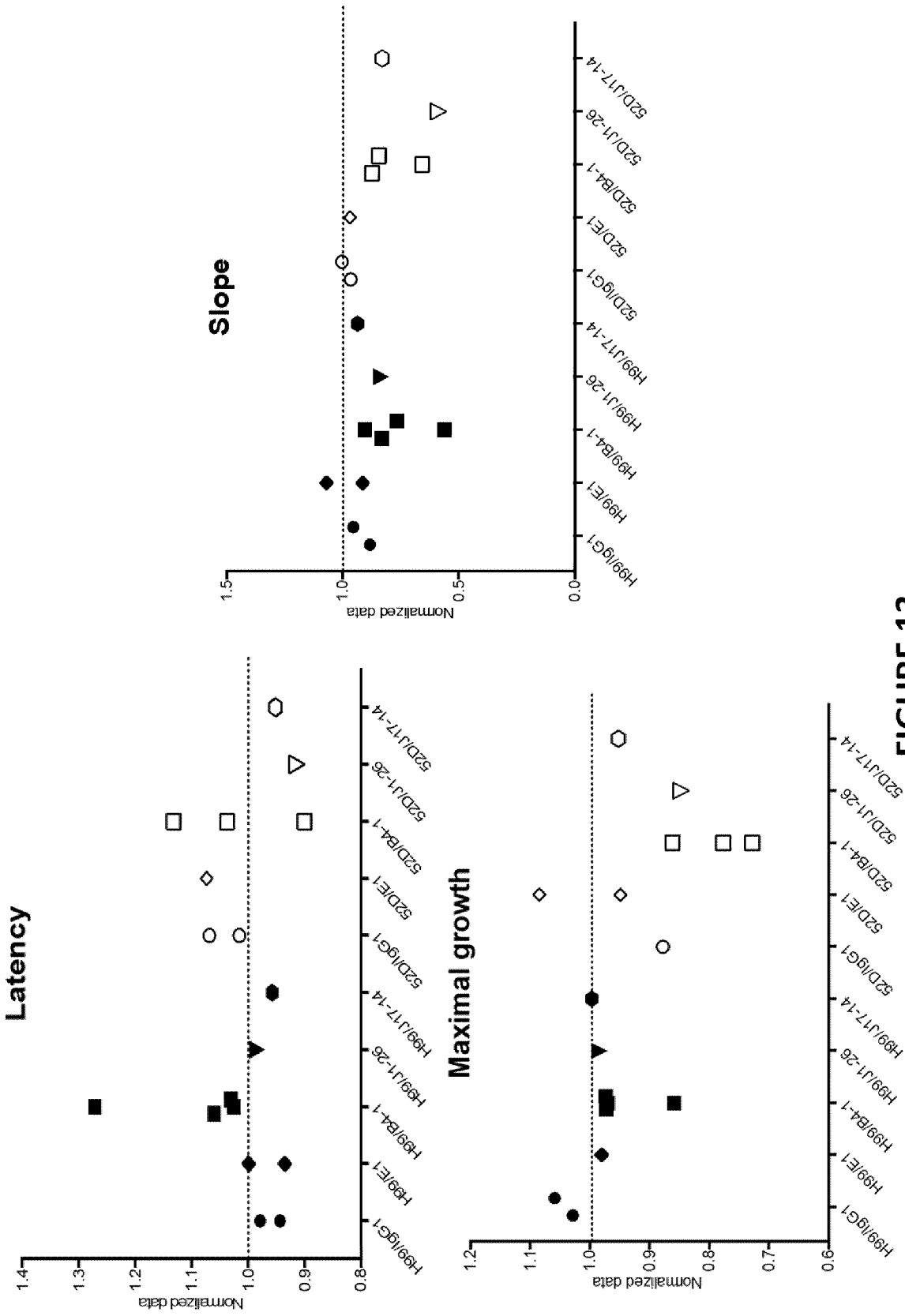


FIGURE 13

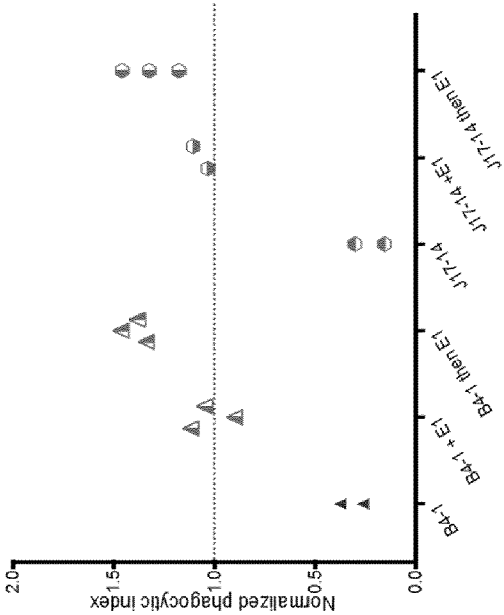


FIGURE 14

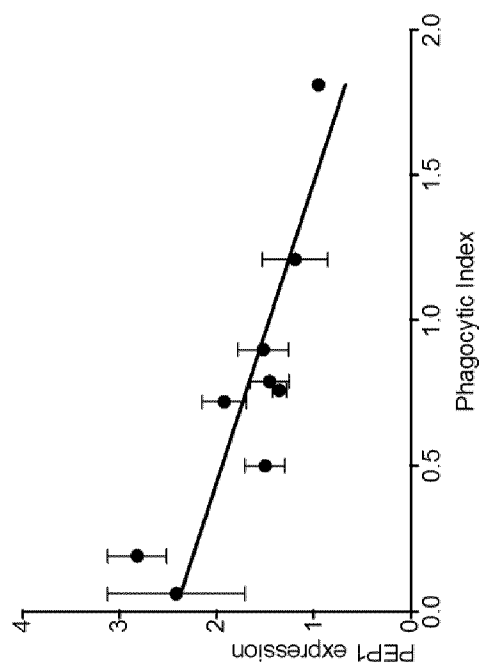


FIGURE 15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2016/082297

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☒ forming part of the international application as filed:
- ☒ in the form of an Annex C/ST.25 text file.
- ☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
- ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
- ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/082297

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/00 C07K16/40
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CA 2 354 100 A1 (PASTEUR INSTITUT [FR]) 2 February 2003 (2003-02-02) cited in the application page 7, line 20 - page 8, line 6 page 10, line 10 - line 18 page 10, line 28 - page 11, line 26 page 27, line 1 - page 28, line 3 page 30, line 1 - line 20 claims 17-46; figures 8,10 ----- -/--	1-10



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

22 March 2017

Date of mailing of the international search report

12/05/2017

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Bayer, Annette

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/082297

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BOZZA SILVIA ET AL: "Immune sensing of Aspergillus fumigatus proteins, glycolipids, and polysaccharides and the impact on Th immunity and vaccination.", JOURNAL OF IMMUNOLOGY (BALTIMORE, MD. : 1950) 15 AUG 2009, vol. 183, no. 4, 15 August 2009 (2009-08-15), pages 2407-2414, XP002757108, ISSN: 1550-6606 page 408, left-hand column, paragraph 2 - right-hand column, paragraph 1; tables 1,2 results; figures 1-4 -----	1-10
A	F Dromer ET AL: "Protection of mice against experimental cryptococcosis by anti-Cryptococcus neoformans monoclonal antibody", Infection and Immunity, 1 March 1987 (1987-03-01), pages 749-752, XP055357661, UNITED STATES Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC260405/pdf/iai00087-0261.pdf the whole document, in particular page 752, left-hand column, last paragraph -----	1-10
A	SHARADA RAVIKUMAR ET AL: "Optimizing Outcomes in Immunocompromised Hosts: Understanding the Role of Immunotherapy in Invasive Fungal Diseases", FRONTIERS IN MICROBIOLOGY, vol. 6, 26 November 2015 (2015-11-26), XP055357679, DOI: 10.3389/fmicb.2015.01322 the whole document, in particular page 5, right-hand column - page 6, left-hand column, bridging paragraph; page 6, right-hand column, first paragraph -----	1-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/082297

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-10

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-10

An antibody binding to a recombinant antigen for use in the treatment of a fungal disease, wherein said antigen is chosen from:

- a) a recombinant aspartyl protease protein comprising an amino acid sequence having at least 48% identity with SEQ ID NO: 1 and comprising two catalytic motifs, SEQ ID NO: 2 and SEQ ID NO: 3, in positions corresponding to positions 145 to 149 and 327 to 331 of SEQ ID NO: 1 and further comprising at least one protective epitope, said epitope(s) corresponding to one or more of the peptides in positions 1 to 15, 271 to 285, 296 to 310 and 346 to 360 of SEQ ID NO: 1, or a functional variant thereof; and
- b) a polypeptide fragment of the protein in a) comprising at least one of said protective epitope(s), and wherein the antibody is an antibody recognizing specifically at least one of said protective epitope(s) or a functional fragment thereof comprising at least the antibody binding region.

2. claims: 11-20

A recombinant antigen for use in the treatment of a fungal disease, wherein said antigen is chosen from:

- a) a recombinant aspartyl protease protein comprising an amino acid sequence having at least 48% identity with SEQ ID NO: 1 and comprising two catalytic motifs, SEQ ID NO: 2 and SEQ ID NO: 3, in positions corresponding to positions 145 to 149 and 327 to 331 of SEQ ID NO: 1 and further comprising at least one protective epitope, said epitope(s) corresponding to one or more of the peptides in positions 1 to 15, 271 to 285, 296 to 310 and 346 to 360 of SEQ ID NO: 1, or a functional variant thereof; and
- b) a polypeptide fragment of the protein in a) comprising at least one of said protective epitope(s).

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/082297

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CA 2354100	A1	02-02-2003	NONE
