Antibody Binding Alters the Characteristics and Contents of Extracellular Vesicles Released by *Histoplasma capsulatum*

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**ABSTRACT** *Histoplasma capsulatum* produces extracellular vesicles containing virulence-associated molecules capable of modulating host machinery, benefiting the pathogen. Treatment of *H. capsulatum* cells with monoclonal antibodies (MAbs) can change the outcome of infection in mice. We evaluated the sizes, enzymatic contents, and proteomic profiles of the vesicles released by fungal cells treated with either protective MAb 6B7 (IgG1) or nonprotective MAb 7B6 (IgG2b), both of which bind *H. capsulatum* heat shock protein 60 (Hsp60). Our results showed that treatment with either MAb was associated with changes in size and vesicle loading. MAb treatments reduced vesicle phosphatase and catalase activities compared to those of vesicles from untreated controls. We identified 1,125 proteins in vesicles, and 250 of these manifested differences in abundance relative to that of proteins in vesicles isolated from yeast cells exposed to Hsp60-binding MAbs, indicating that surface binding of fungal cells by MAbs modified protein loading in the vesicles. The abundance of upregulated proteins in vesicles upon MAb 7B6 treatment was 44.8% of the protein quantities in vesicles from fungal cells treated with MAb 6B7. Analysis of orthologous proteins previously identified in vesicles from other fungi showed that different ascomycete fungi have similar proteins in their extracellular milieu, many of which are associated with virulence. Our results demonstrate that antibody binding can modulate fungal cell responses, resulting in differential loading of vesicles, which could alter fungal cell susceptibility to host defenses. This finding provides additional evidence that antibody binding modulates microbial physiology and suggests a new function for specific immunoglobulins through alterations of fungal secretion.

**IMPORTANCE** Diverse fungal species release extracellular vesicles, indicating that this is a common pathway for the delivery of molecules to the extracellular space. However, there has been no study reporting the impact of antibody binding to the fungal cell on extracellular vesicle release. In the present work, we observed that treatment of *H. capsulatum* cells with Hsp60-binding MAbs significantly changed the size and cargo of extracellular vesicles, as well as the enzymatic activity of certain virulence factors, such as laccase and phosphatase. Furthermore, this finding demonstrates that antibody binding can directly impact protein loading in vesicles and fun-
gal metabolism. Hence, this work presents a new role for antibodies in the modification of fungal physiology.

**KEYWORDS:** *H. capsulatum*, Hsp60, monoclonal antibodies, vesicles

**Histoplasma capsulatum**, a dimorphic fungus, is the etiologic agent of histoplasmosis, a systemic mycosis with a worldwide distribution. *H. capsulatum* infections are common in North America, mainly in the United States (1, 2), and are also highly prevalent in some Latin American countries, such as Brazil, Venezuela, Ecuador, Paraguay, and Argentina (3, 4). Infection occurs after inhalation of microconidia or hyphal fragments from the environment by a susceptible host, and the lung is the primary organ of infection (5, 6). Containment of the infection involves the activation of cell-mediated immunity with uptake of fungi by phagocytic cells such as neutrophils and macrophages (5, 7). Interestingly, *H. capsulatum* yeast cells subvert the intraphagosomal milieu, maintaining an environment that is permissive to fungal multiplication (5, 8). Although the role of humoral immunity in the pathogenesis of histoplasmosis is uncertain, monoclonal antibodies (MAbs) have been shown to significantly improve survival after a lethal challenge in a murine infection model (9, 10). Interestingly, we previously demonstrated that two competing MAbs to heat shock protein 60 (Hsp60) of different subtypes had dramatically different effects on disease pathogenesis, with MAb 6B7 (IgG1) producing a protective response and MAb 7B6 (IgG2b) enhancing the disease (9).

Over the past decade, several studies have shown that fungi produce extracellular vesicles. This remarkable process involves the transport of macromolecule-containing vesicles across the complex fungal cell wall, a secretory machinery that is utilized by diverse ascomycetes and basidiomycetes, including *H. capsulatum*, *Candida albicans*, *Cryptococcus neoformans*, *Malassezia sympodialis*, *Paracoccidioides brasiliensis*, and *Alternaria infectoria* (11–16). Analyses of the contents of vesicles from these different fungi have revealed the presence of lipids, phospholipids, polysaccharides, nucleic acid, proteins, and virulence factors, such as laccase and urease (11, 17, 18). In *H. capsulatum*, the extracellular vesicles contain important proteins involved in fungal pathogenesis and stress responses, including Hsp60, which suggest the participation of fungal extracellular vesicles in the establishment and progression of disease (15).

It is notable that several of the described virulence factors of *H. capsulatum* that have been identified in the secreted vesicles are unconventional cell wall components. For example, the chaperone Hsp60 is a major ligand involved in phagocytosis by mediating the attachment of *H. capsulatum* cells to macrophage/monocyte integrin CR3 (CD11b/CD18), whereas M antigen, another surface antigen, is a catalase involved in the protection of fungal cells from oxidative stress (9, 19). In addition, phosphatase and laccase are enzymes involved in protein dephosphorylation and melanin synthesis, respectively (19, 20). Given the finding that MAbs can modify disease pathogenesis, we determined the effects of a protective MAb and a nonprotective MAb on the production and contents of extracellular vesicles from *H. capsulatum*.

**RESULTS**

**DLS analysis of extracellular vesicles released after treatment of *H. capsulatum* cells with a protective (6B7) or nonprotective (7B6) antibody.** Dynamic light scattering (DLS) was used to evaluate the vesicle sizes in each sample (Fig. 1A and B). The results show that incubation of *H. capsulatum* cells with MAbs 6B7 and 7B6 significantly changed the size of the vesicles released by the fungal cells in comparison with that of vesicles released by untreated yeast cells (Fig. 1A and B). Vesicles collected from untreated control cells were found to occur in two distinct size ranges: a small population varying between 40 and 60 nm and those of a larger size ranging between 170 and 250 nm in diameter. After treatment with MAb 6B7, the sizes of both vesicle populations increased compared with those of the control. The sizes of small and large vesicles ranged between 60 and 80 nm and 240 and 350 nm, respectively. Cells treated
with MAb 7B6 produced small vesicles that varied between 55 and 100 nm and larger vesicles that varied between 200 and 300 nm.

Protein and sterol content quantification after treatment of *H. capsulatum* cells with protective (6B7) and nonprotective (7B6) antibodies. The total protein and sterol concentrations of extracellular vesicles were determined with the Bradford assay and an Amplex Red kit, respectively (Fig. 2A and B; see Table S1 in the supplemental material). The structural differences between fungal and mammalian sterols do not interfere with the kit’s detection activity (14). Analysis of the total protein from extracellular vesicles shows that treatment of *H. capsulatum* with either MAb 6B7 or 7B6 results in a significant increase in protein compared with that in vesicles from untreated control yeast cells (Fig. 2A). The total protein in vesicles collected from *H. capsulatum* incubated with MAbs 6B7 and 7B6 increased 6- and 9.5-fold, respectively,

![FIG 1](image1.png) Distribution of extracellular vesicle dimensions obtained from control *H. capsulatum* yeast cells compared to vesicle size ranges obtained from yeast cells treated with MAb 6B7 (A) or 7B6 (B). Control: *H. capsulatum* cells not treated with MAbs.

![FIG 2](image2.png) Total protein analysis and sterol content quantification in vesicles from *H. capsulatum* yeast cells with or without treatment with MAb 6B7 or 7B6. (A) Bradford assay for protein quantification. (B) Sterol content quantification. *H. capsulatum* cells were grown in Ham’s F12 medium for 7 days. The vesicles were collected and suspended in 0.5 ml of PBS. All of the analyses were performed in duplicate. **, *P* < 0.05 compared to the untreated control (*H. capsulatum* cells not treated with MAbs); ##, *P* < 0.05 compared to MAb 6B7.
in comparison with that in untreated control vesicles. In addition, the amount of protein in vesicles from *H. capsulatum* treated with MAb 7B6 was greater than that of protein in vesicles from *H. capsulatum* treated with MAb 6B7 (Fig. 2A). Interestingly, the amount of fungal sterol in the vesicles did not change after treatment with MAbs 6B7 and 7B6 compared to that in the untreated control (Fig. 2B).

**Enzymatic assay of vesicles derived from *H. capsulatum* cells with or without MAb treatment.** To detect urease, phosphatase, laccase, and catalase activities, suspensions of vesicles were added to an enzyme reaction solution specific to each enzyme evaluated. The activities of these enzymes were detected in extracellular vesicles isolated from *H. capsulatum* yeasts with or without MAb treatment (Fig. 3A and B). Although the urease activity levels were similar in control and antibody-treated vesicles (Fig. 3A), phosphatase, laccase, and catalase activities were modified by antibody treatment (Fig. 3B to D). Both MAbs 6B7 and 7B6 significantly decreased the phosphatase activity in vesicles compared to that in the untreated control (Fig. 3B). Laccase activity was significantly lower in vesicles from cells incubated with MAb 6B7 than in either untreated control (Fig. 3C). There was also a trend toward lower laccase and catalase activities in vesicles isolated from cells treated with MAb 7B6 than in vesicles isolated from the untreated control. In addition, the catalase activity levels were similar in control and MAB 6B7-treated vesicles.

**Proteomic analysis of extracellular vesicles of *H. capsulatum* cells treated with MAb 6B7 or 7B6.** Protein analysis was performed after vesicle purification and enzymatic digestion. Identification of individual peptides was achieved by searching
tandem mass spectra against a sequence database containing the *H. capsulatum* complete proteome set from the UniProt Knowledge Base and common contaminant sequences with the Paragon tool of the Protein Pilot software (AB Sciex). Complete proteomic analysis of the *H. capsulatum* extracellular vesicles isolated from each of the conditions examined led to the identification of a total of 1,125 proteins that were separated into 1,117 groups (see Tables S2 and S3 in the supplemental material), in which a protein group is defined by isoforms that have the same peptides. Of the 1,117 protein groups, 699 had peptide intensities above the limit of quantification (see Table S4 in the supplemental material) and 250 proteins in this subset were differentially abundant (Table 1; see Table S5 in the supplemental material). Figure 4 depicts the best-represented protein categories organized according to their biological processes. This classification based on biological processes shows that the most plentiful of the proteins are related to amino acid/protein metabolism (20%), followed by proteins associated with sugar metabolism (7.2%), nuclear proteins, and lipid metabolism (both 4%). In addition, 27.6% of the proteins were grouped together as miscellaneous and 12.4% were uncharacterized proteins.

Comparison of the proteomes of vesicles of *H. capsulatum* cells treated with MAbs 6B7 and 7B6. Treatment of *H. capsulatum* cells with MAb 6B7 or 7B6 changed the profile of proteins in the vesicles in relation to that in untreated control vesicles (Fig. 5A; see Table S5 in the supplemental material). After treatment with MAb 6B7, 46.8% of the proteins were reduced in quantity and about half (45.8%) were increased (Fig. 5B). Although MAb 7B6 treatment also changed the expression profile, there were increases in 31.2% of the proteins and 43.6% were reduced (Fig. 5C). Treatment with MAb 6B7 most significantly reduced proteins associated with amino acid/protein metabolism (22.2%), followed by sugar metabolism, nuclear, ribosomal, and lipid metabolism proteins (5.6%) and cell wall architecture proteins (4.2%). Among the proteins with higher abundance after treatment with MAb 6B7, 20.8% were associated with amino acid/protein metabolism, 10.4% were associated with sugar metabolism, and 8% were ribosomal proteins (Fig. 5B). Analyzing the set of proteins altered after treatment with MAb 7B6, we determined that the quantities were reduced 21.1% for proteins involved in amino acid/protein metabolism, 7.3% for sugar metabolism proteins, and 5.5% for nuclear proteins (Fig. 5C), and the quantities were increased 21.8% for proteins involved in amino acid/protein metabolism, 12.8% for sugar metabolism proteins, and 9% for ribosomal proteins (Fig. 5C). We also compared the abundance of proteins in vesicles isolated from yeast cells after treatment with MAb 7B6 in relation to that of proteins in vesicles from cells treated with MAb 6B7. The analysis showed that most proteins were increased (44.8%) in the MAb 7B6 vesicles compared to the MAb 6B7 vesicles, with proteins associated with amino acid/protein metabolism having the most abundance (21.4%), followed by sugar metabolism proteins (10.7%) and cytoskeleton proteins (7.1%) (Fig. 5D). This comparison also showed that treatment with MAb 7B6 reduced more proteins, with 20.6% of these proteins being associated with amino acid/protein metabolism and 14.7% being cell wall architecture-related proteins. Interestingly proteins related to cell wall architecture, such as cell wall remodeling protein, cell wall synthesis protein, and 1,3-β-glucananosyltransferase, were decreased in vesicles isolated from yeasts treated with MAb 6B7 and increased in MAb 7B6-treated vesicles. Furthermore, MAbs 6B7 and 7B6 changed the abundance of polyphenol oxidase and alkaline phosphatase enzymes in relation to that in untreated control vesicles, as both were increased according to proteomic analysis (see Table S5 in the supplemental material). Notably, alteration of the abundance of vesicle proteins also occurred after treatment with the Hsp60-binding control MAb 12D3, where 50% of the proteins were increased and 32.4% were reduced (see Table S5 in the supplemental material). MAb 12D3 produces biological and protective responses similar to those of MAb 6B7 (8) and was thus utilized to support our evidence that protective MAbs produce differential loading of vesicles compared to the one nonprotective MAb to Hsp60 that we currently have.
| Protein type and hit no. | Accession no. | Identification | Function |
|-------------------------|---------------|---------------|----------|
| **Chaperone-like proteins** | | | |
| 1 | C0NEZ9 | Receptor-associated protein | Intracellular protein transport |
| 2 | CONS16 | DnaK-type molecular chaperone BipA | Chaperone |
| 3 | CONBV8 | Heat shock protein | Chaperone |
| 4 | CONYC6 | Hsp70-like protein | Chaperone |
| 5 | COP0B3 | Hsp60-like protein | Chaperone |
| 6 | COP152 | Heat shock protein | Chaperone |
| **Endocytic-route proteins** | | | |
| 7 | C0NI41 | VHS domain-containing protein | Intracellular protein transport |
| 8 | CONKH9 | ADP-ribosylation factor | GTP binding |
| 9 | CONA79 | Prenylated Rab accepter 1 | Involved in transport between ER and Golgi complex |
| 10 | CONL9 | Vacuolar-sorting-associated protein | Vesicular protein sorting |
| 11 | CONXJ2 | Secretory pathway GDP dissociation inhibitor | Rab GDP-dissociation inhibitor activity |
| 12 | CONRE5 | e-COP | Retrograde vesicle-mediated transport, Golgi to ER |
| **Cytoskeleton/motility proteins** | | | |
| 13 | COP0B4 | Cofilin | Actin binding |
| 14 | CONA44 | Coronin | Actin-associated protein |
| 15 | CONBZ7 | F-actin-capping protein subunit β | Actin binding |
| 16 | CONMF2 | Fimbrin | Protein binding |
| 17 | CONTH2 | Septin | Cytokinesis |
| 18 | P53455 | Actin | Cytoskeleton assembly |
| 19 | CONKB3 | Tubulin β chain | Structural constituent of cytoskeleton |
| 20 | COP0S2 | Tubulin α chain | Structural constituent of cytoskeleton |
| 21 | COP074 | Tubulin α-1 subunit | Structural constituent of cytoskeleton |
| **Cell growth/division proteins** | | | |
| 22 | CONBG1 | DNA damage checkpoint protein Rad24 | DNA damage checkpoint |
| 23 | CONC23 | dUTPase | |
| 24 | CONFW3 | RNA polymerase Rpb1 C-terminal repeat domain-containing protein | Transcription of DNA |
| 25 | CONQN9 | Septin | GTP binding |
| 26 | CONF61 | Cell division control protein | ATP binding |
| 27 | CONXU1 | Flap endonuclease 1 | DNA binding |
| 28 | CONML9 | Mitogen-activated protein kinase | MAP kinase activity |
| **Cell signaling proteins** | | | |
| 29 | CONFM9 | Small G-β protein GPB | Protein binding |
| 30 | CONFNS | Ran-specific GTPase-activating protein | Intracellular transport |
| 31 | CONC1 | PH domain-containing protein | Intracellular signaling |
| 32 | COP083 | GTP-binding protein ypt3 | Small GTPase-mediated signal transduction |
| **Nuclear proteins** | | | |
| 33 | CONC10 | Uracil-DNA glycosylase | Uracil DNA N-glycosylase activity |
| 34 | CONRN4 | Histone H2A | |
| 35 | CONZ94 | Histone H2B | DNA binding |
| 36 | CONL60 | Histone H3 | DNA binding |
| 37 | COP057 | RuvB-like helicase | DNA helicase activity |
| 38 | CONJ22 | RuvB-like helicase 1 | DNA helicase activity |
| 39 | COP170 | Cap-binding protein | RNA metabolic process |
| 40 | CONQX1 | DNA ATP-dependent helicase | DNA binding, ATP binding |
| 41 | CONP2 | DNA damage-binding protein 1a | Nucleic acid binding |
| 42 | CONFM3 | XPG I region protein | DNA repair |
| 43 | COP05 | Woronin body major protein | Translation elongation factor activity |
| **Cell wall architecture** | | | |
| 44 | CONDA4 | Cell wall synthesis protein | Cell wall synthesis |
| 45 | CONL12 | Glucanoyltransferase | Cell wall assembly |
| 46 | CONK9 | β-Glucosidase | Carbohydrate metabolic process |
| 47 | CONW75 | Chitinase | Chitinase activity |
| 48 | CONSG6 | Extracellular cell wall glucanase Crf1 | Hydrolase activity, hydrolyzing O-glycosyl compounds |
| 49 | CONH39 | 1,3-β-Glucanotransferase | Carbohydrate metabolic process |
| 50 | CONIP3 | GPI-anchored cell wall organization protein Ecm33 | |
| **Antioxidant proteins** | | | |
| 51 | CONAP3 | Polyphenoloxidase | Oxidoreductase activity |
| 52 | CON23 | Glutathione peroxidase | Glutathione peroxidase activity |
| 53 | CONMI3 | Thiol-specific antioxidant | Antioxidant activity |

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| Protein type and hit no. | Accession no. | Identification | Function |
|-------------------------|---------------|----------------|----------|
| Proteasome proteins     |               |                |          |
| 54                      | C0NV29        | Proteasome subunit α type  | Endopeptidase activity |
| 55                      | C0P150        | Proteasome subunit β type  | Endopeptidase activity |
| 56                      | C0NYE5        | 26S proteasome regulatory subunit | Protein binding |
| Lipid metabolism proteins |              |                |          |
| 57                      | C0NMK6        | Acyl-CoA dehydrogenase | Acyl-CoA dehydrogenase activity |
| 58                      | C0NM0         | 3-Ketoacyl-CoA thiolase | Catalytic activity |
| 59                      | C0NW7         | 3-Ketoacyl-CoA thiolase peroxisomal A | Catalytic activity |
| 60                      | C0NY84        | Glycerocephosphoryl diester phosphodiesterase | Glycolytic activity |
| 61                      | C0NZ5         | Enoyl-CoA hydratase/isomerase | Catalytic activity |
| 62                      | C0NEL5        | δ-9 fatty acid desaturase | Insertion of double bond at δ position of fatty acids |
| 63                      | C0PO87        | Long-chain fatty acid CoA ligase | Catalytic activity |
| 64                      | C0NUX2        | Oxyyster-binding protein | Ergosterol synthesis |
| 65                      | C0NAZ6        | Oxyyster-binding protein | Ergosterol synthesis |
| 66                      | C0NTW1        | Oxidosqualene:lanoster cyclase | Intramolecular transferase activity |
| Sugar metabolism proteins |              |                |          |
| 67                      | C0NP4         | N-Glycosyltransferase | Catalysis of glycosyl group transfer |
| 68                      | C0PO90        | Citrate synthase | Citrate (Si)-synthase activity |
| 69                      | C0NHJ7        | Glucosidase I | Mannosyl-oligosaccharide glucosidase activity, catalytic activity |
| 70                      | C0NGE0        | Acetate hydratase | Tricarboxylic acid cycle |
| 71                      | C0NRA2        | Sugar transporter | Transporter activity |
| 72                      | C0NB1         | Phosphoglycerate kinase | Phosphoglycerate kinase activity |
| 73                      | C0NR6         | Ribose 5-phosphate isomerase A | Ribose-5-phosphate isomerase activity |
| 74                      | C0NQ1         | Fructose 1,6-biphosphatase aldolase | Fructose-bisphosphatase aldolase activity |
| 75                      | C0NQ1         | Fructose 1,6-bisphosphatase | Fructose 1,6-bisphosphatase 1-phosphatase activity |
| 76                      | C0ND4         | β-Glucosidase | Carbohydrate metabolic process |
| 77                      | C0NF3         | Triosephosphate isomerase | Glycolytic process |
| 78                      | C0NR1         | Glyceraldehyde-3-phosphate dehydrogenase | Glucose metabolic process |
| 79                      | C0PO46        | Malate dehydrogenase | Malate metabolic process |
| 80                      | C0NDH1        | Malate dehydrogenase | Malate metabolic process |
| 81                      | C0NH60        | Acetate | Tricarboxylic acid cycle |
| 82                      | C0NAG1        | Pyruvate carboxylase | Pyruvate metabolic process |
| 83                      | C0NV40        | N-Acetylglucosamine-phosphate mutase | Carbohydrate metabolic process |
| 84                      | C0NH7         | Isoctuate lyase | Isocitrate lyase activity |
| Ribosomal proteins      |               |                |          |
| 85                      | C0NC9         | 60S acidic ribosomal protein P0 | Structural constituent of ribosome |
| 86                      | C0NE75        | 60S ribosomal protein L23 | Structural constituent of ribosome |
| 87                      | C0NK2         | 60S ribosomal protein L1 | Structural constituent of ribosome |
| 88                      | C0NL3         | 40S ribosomal protein S4 | Structural constituent of ribosome |
| 89                      | C0MA2         | Ribosomal protein L19 | Structural constituent of ribosome |
| 90                      | C0RD6         | 60S ribosomal protein L5 | Structural constituent of ribosome |
| 91                      | C0ND0         | 40S ribosomal protein S3 | Structural constituent of ribosome |
| 92                      | C0NE9         | 40S ribosomal protein S12 | Structural constituent of ribosome |
| 93                      | C0HH49        | Ribosomal protein L14 | Structural constituent of ribosome |
| 94                      | C0NP9         | 60S ribosomal protein L13 | Structural constituent of ribosome |
| 95                      | C0KWW         | 40S ribosomal protein S0 | Structural constituent of ribosome |
| 96                      | C0DC6         | Large-subunit ribosomal protein L3 | Structural constituent of ribosome |
| 97                      | C0CE3         | 60S ribosomal protein L20 | Structural constituent of ribosome |
| 98                      | C0RHS         | 60S ribosomal protein L24 | Structural constituent of ribosome |
| 99                      | C0NC6         | 40S ribosomal protein S17 | Structural constituent of ribosome |
| Amino acids/proteins involved in metabolism | | | |
| 100                     | C0NQ9         | Probable dipeptidyl-aminopeptidase B | Serine-type peptidase activity |
| 101                     | C0NM4         | Glutamate dehydrogenase | Cellular amino acid metabolic process |
| 102                     | C0NXA3        | Eukaryotic translation initiation factor 3 subunit C | Translation initiation factor activity |
| 103                     | C0NAB3        | Probable carbboxypeptidase HCBG_00059 | Hydrolase activity |
| 104                     | C0NAK7        | Aspartyl aminopeptidase | Aminopeptidase activity |
| 105                     | C0NWA2        | Eukaryotic translation initiation factor 3 subunit I | Protein synthesis |
| 106                     | C0NC9         | Protein disulfide-isomerase | Protein folding |
| 107                     | C0NE1         | Fumarylacetoacetase | Aromatic amino acid family metabolic process |
| 108                     | C0NIW4        | Cobalamin-independent methionine synthase | Methionine biosynthetic process |

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| Accession no. | Identification Function |
|--------------|------------------------|
| 109 | CONKL7 Peptidyl-prolyl cis-trans isomerase Leucine biosynthetic process |
| 110 | CONNC2b Aminopeptidase Metallopeptidase activity |
| 111 | COP0D5 Aminopeptidase Metallopeptidase activity |
| 112 | CONND5 Thioredoxin Protein disulfide oxidoreductase activity |
| 113 | CONJC3 Adenosylhomocysteinate Adenosylhomocysteinate activity |
| 114 | CONQ08 Saccharopine dehydrogenase (NAD\(^+\), \(\alpha\)-lysine forming) Activity |
| 115 | CONRP4 Peptidyl-prolyl cis-trans isomerase Peptidyl-prolyl cis-trans isomerase activity |
| 116 | CONS19 Carboxypeptidase Serine-type carboxypeptidase activity |
| 117 | CONSN4 Elongation factor 2 GTP binding, GTPase activity |
| 118 | CONSU0 Serine/threonine phosphatase Hydrolase activity |
| 119 | CONT48b A-pheromone-processing metalloproteinase Ste23 Catalytic activity, metal ion binding |
| 120 | CONUD2 Phosphoprotein phosphatase A Binding |
| 121 | CONNE2 3-Isopropylmalate dehydratase Leucine biosynthetic process |
| 122 | CONVD3 Kynureninase Hydrolyase activity |
| 123 | CONVD9b Eukaryotic translation initiation factor 3 subunit L Eukaryotic translation initiation factor 3 complex |
| 124 | CONBB3 Peptidyl-prolyl cis-trans isomerase Seryl-tRNA synthetase Aminoacyl-tRNA ligase activity |
| 125 | CONE91 Seryl-tRNA synthetase Aminoacyl-tRNA ligase activity |
| 126 | CONJ54 Elongation factor Tu Translational elongation factor activity during protein biosynthesis |
| 127 | CONPC9 Ubiquitin-activating enzyme Small-protein-activating enzyme activity |
| 128 | CONVW8 Peptidyl-prolyl cis-trans isomerase Protein folding |
| 129 | CONXH6b Ornithine aminotransferase Pyridoxal phosphate binding |
| 130 | CONXL8b Argininosuccinase lase Arginine biosynthetic process via ornithine |
| 131 | CONZA7 Cytosolic nonspecific dipeptidase Hydrolyase activity |
| 132 | CONZE4 d-Tyrosyl-tRNA(Tyr) deacylase Aromatic amino acid family biosynthetic process |
| 133 | CONN56 L-Leucine synthase Glutamate-ammonia ligase activity |
| 134 | P04911 Elongation factor 1-α Translational elongation factor activity during protein biosynthesis |
| 135 | CONAN2 ATP-dependent RNA helicase EIF4A Nucleic acid binding |
| 136 | CONZL2 Ketol-acid reductoisomerase Branched-chain amino acid biosynthetic process |
| 137 | CONEM8a 3-Isopropylmalate dehydrogenase Leucine biosynthetic process |
| 138 | CONX46a Carboxypeptidase Y homolog A Serine-type carboxypeptidase activity |
| 139 | CONL66 Isocitrate dehydrogenase, cytoplasmic Aminoacyl-tRNA ligase activity |
| 140 | CONJ5ua Saccharopine dehydrogenase Oxidoreductase activity |
| 141 | CONBP4a Aromatic amino acid aminotransferase Pyridoxal phosphate binding |
| 142 | CONG7y Aspartyl-tRNA synthetase tRNA aminoacylation for protein translation |
| 143 | CONLE3 Metalloproteinase MepB Metalloendopeptidase activity |
| 144 | CONXZ3 Serine/threonine-protein kinase DCLK1 Protein phosphorylation |
| 145 | CONQD6 Phospho-2-dehydro-3-deoxyheptonate aldolase Aromatic amino acid family biosynthetic process |
| 146 | CONB7a Calcium/calcimodulin-dependent protein kinase Phosphatase PP1 regulatory subunit sds22 Protein binding |
| 147 | CONFN7b α-1,2-Mannosyltransferase Kr6 Transferase activity, transferring phosphorus-containing groups |
| 148 | CONV96b Phospho-2-dehydro-3-deoxyheptonate aldolase Aromatic amino acid family biosynthetic process |
| 149 | COP028 DUF895 domain-containing protein Transmembrane transporter activity |
| 150 | COP096 Plasma membrane ATPase ATP biosynthetic process |
| 151 | CONDZ9 Pyridoxine biosynthesis protein PyrO Pyridoxine biosynthesis activity |
| 152 | CONDZ7 Probable Xaa-Pro aminopeptidase P Hydrolyase activity |
| 153 | CONB22b RNA-binding protein Nucleotide binding |
| 154 | CONB64b Short-chain dehydrogenase/reductase Oxidoreductase activity |
| 155 | CONBU6 Esterase S-Formylglutathione hydrolase activity |
| 156 | CONBV1a Cyclin-dependent protein kinase PhoA Transferase activity, transferring phosphorus-containing groups |
| 157 | CONCA6a Serine/threonine-protein phosphatase Hydrolyase activity |
| 158 | CONCC8a Phosphatase PP1 regulatory subunit sds22 Protein binding |
| 159 | CONQ2 Fumarate reductase flavoprotein subunit Succinate dehydrogenase activity |
| 160 | CONE90b Fibrillarin RNA binding |
| 161 | CONFD1b Armadillo repeat protein Protein binding |
| 162 | CONG75 ATP synthase subunit α ATP binding |
| 163 | CONIZ7b Nicotinate-nucleotide pyrophosphorylase (carboxylating) NAD biosynthetic process |
| 164 | CONHZ2b Prohibitin DNA synthesis inhibition |
| 165 | CONJV2 Aha1 domain family Chaperone binding |
| 166 | CONJV7 V-type proton ATPase subunit A Hydrogen ion transmembrane transporter activity |

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| Protein type and hit no. | Accession no. | Identification | Function |
|-------------------------|--------------|---------------|----------|
| 167                     | C0NLZ4       | Isochorismatase domain-containing protein | Catalytic activity |
| 168                     | C0P0E1       | NADH-ubiquinone oxidoreductase | ATP synthesis-coupled electron transport |
| 169                     | C0NP32a      | DUF221 domain-containing protein | |
| 170                     | C0NP52       | Cytochrome b-c1 complex subunit Rieske, mitochondrial | Ubiquinol-cytochrome c reductase activity, oxidoreductase activity |
| 171                     | C0NQQ6a      | MYG1 protein | |
| 172                     | C0NQX6       | Alcohol dehydrogenase | Oxidoreductase activity |
| 173                     | C0NR06b      | Adenosine kinase | Adenosine kinase activity |
| 174                     | C0NRW1       | Endonuclease/exonuclease/phosphatase | |
| 175                     | C0NT49       | Cleavage- and polyadenylation-specific factor 5 | mRNA binding |
| 176                     | C0NSJ0       | Vacuolar ATP synthase subunit B | ATP hydrolysis-coupled proton transport |
| 177                     | C0NTN5       | Nucleoside diphosphate kinase | Nucleoside diphosphate kinase activity |
| 178                     | C0NUW2       | Hydroxymethylglutaryl-CoA synthase | Hydroxymethylglutaryl-CoA synthase activity |
| 179                     | C0NVG5       | Sterigmatocystin 8-O-methyltransferase | O-Methyltransferase activity |
| 180                     | C0NB0        | Enolase | Phosphoenolpyruvate hydratase activity |
| 181                     | C0NF22b      | Oxidoreductase | Oxidoreductase activity |
| 182                     | C0NLK3b      | Glutathione-dependent formaldehyde dehydrogenase | |
| 183                     | C0NSW3       | Aldehyde dehydrogenase | |
| 184                     | C0NTQ2       | CRAL/TRIO domain-containing protein | |
| 185                     | C0NH0        | 2-Methylcitrate dehydratase | 2-Methylcitrate dehydratase activity |
| 186                     | C0NX78       | Phosphoribosylformylglycinamidine cyclo-ligase | Purine nucleobase biosynthetic process |
| 187                     | C0NYQ7a      | Xanthine phosphoribosyltransferase | Nucleoside metabolic process |
| 188                     | C0NZ16a      | FAD-dependent oxidoreductase superfamily | Oxidoreductase activity |
| 189                     | C0NZ33       | Choline sulfatase | Sulfuric ester hydrolase activity |
| 190                     | C0P037b      | NAD^{+}-dependent betaine aldehyde dehydrogenase | Oxidoreductase activity |
| 191                     | C0P0C5       | ATP synthase subunit β | ATP binding |
| 192                     | C0P0H6       | 3-Methylcrotonyl-CoA carboxylase | Biotin carboxylase activity |
| 193                     | C0NTZ5       | RNA-binding protein Snd1 | Transcription cofactor activity |
| 194                     | C0N02        | Transketolase | Transketolase activity |
| 195                     | C0P141       | Allergen Asp4 | Allergen |
| 196                     | C0NCZ4      | Carnitine acetyltransferase | Transferase activity, transferring acyl groups |
| 197                     | C0NP90       | Alkaline phosphatase | Phosphatase activity |
| 198                     | C0NB7        | Alkaline phosphatase | Phosphatase activity |
| 199                     | C0P0T3b      | NADH-ubiquinone oxidoreductase | |
| 200                     | C0NTA4       | Farnesyl-pyrophosphate synthetase | Isoprenoid biosynthetic process |
| 201                     | C0NN26       | Calnexin | Calcium ion binding |
| 202                     | C0NUH0       | KH domain RNA-binding protein | Nucleic acid binding |
| 203                     | C0NC54       | ATP synthase subunit gamma | ATP synthesis-coupled proton transport |
| 204                     | C0NH9b       | Amidohydrolase | Nitrogen compound metabolic process |
| 205                     | C0NZ24a      | 2-Nitropropane dioxygenase | Nitrate monoxygenase activity |
| 206                     | C0NC22a      | Pyridoxine kinase | Pyridoxal kinase activity |
| 207                     | C0NKA1       | Vacuolar ATP synthase subunit C | ATP hydrolysis-coupled proton transport |
| 208                     | C0NQK3       | Adenylosuccinate lyase | Purine ribonucleotide biosynthetic process |
| 209                     | C0NTY6b      | Serine/threonine-protein phosphatase | Hydrolase activity |
| 210                     | C0NW72a      | RNase T2-like protein | RNase T2 activity |
| 211                     | C0NCF6       | NADP-dependent mannitol dehydrogenase | Oxidoreductase activity |
| 212                     | C0NA3        | DUF757 domain-containing protein | |
| 213                     | C0NK86b      | DUF255 domain-containing protein | Catalytic activity |
| 214                     | C0NF8b       | Ribonucleotide reductase M2 B | Deoxyribonucleoside diphosphate metabolic process |
| 215                     | C0NP11       | Golgi apyrase | Hydrolase activity |
| 216                     | C0NZS2       | MOAT family protein | |
| 217                     | C0NMY3       | Ubiquitin | Protein binding |
| 218                     | C0NBS0a      | Indoleamine 2,3-dioxigenase | Heme binding |
| 219                     | C0NZM7       | S import receptor | Intracellular protein transport |

Putative uncharacterized proteins

| Protein type and hit no. | Accession no. | Identification | Function |
|-------------------------|--------------|---------------|----------|
| 220                     | C0NB0a       | Putative uncharacterized protein | |
| 221                     | C0NA95       | Putative uncharacterized protein | |
| 222                     | C0NBS0a      | Putative uncharacterized protein | |
| 223                     | C0NUB2       | Putative uncharacterized protein | |
| 224                     | C0NVK4       | Putative uncharacterized protein | |
| 225                     | C0NA87b      | Putative uncharacterized protein | |
Fungal extracellular vesicle orthologues. To understand whether or not 6B7 and 7B6 MAb treatments would differentially affect conserved functions, we additionally compared *H. capsulatum* extracellular vesicle proteins with orthologous proteins carried by vesicles from *P. brasiliensis*, *C. neoformans*, *Saccharomyces cerevisiae*, and *C. albicans* (Fig. 6). We assumed that the more conserved the functions is, the more

| Protein type and hit no. | Accession no. | Identification | Function |
|-------------------------|---------------|----------------|----------|
| 226                     | C0NH90        | Putative uncharacterized protein | Catalytic activity |
| 227                     | C0NF7         | Putative uncharacterized protein | Endo-DNase activity, producing 5’-phosphomonoesters |
| 228                     | C0NS9         | Putative uncharacterized protein | Protein binding |
| 229                     | C0NJ6D        | Putative uncharacterized protein | Protein binding |
| 230                     | C0P165        | Putative uncharacterized protein | Protein binding |
| 231                     | C0NK6S        | Putative uncharacterized protein | Endo-DNase activity, producing 5’-phosphomonoesters |
| 232                     | C0NLZ9        | Putative uncharacterized protein | Endocytosis |
| 233                     | C0NG6S        | Putative uncharacterized protein | Protein binding |
| 234                     | C0NNW9S       | Putative uncharacterized protein | Protein binding |
| 235                     | C0NQ22        | Putative uncharacterized protein | Transport |
| 236                     | C0QA6         | Putative uncharacterized protein | Structural constituent of ribosome |
| 237                     | C0QQ6         | Putative uncharacterized protein | Carbohydrate metabolic process |
| 238                     | C0QA6         | Putative uncharacterized protein | Nucleic acid binding |
| 239                     | C0P1A8        | Putative uncharacterized protein | Protein binding |
| 240                     | C0NQ33        | Putative uncharacterized protein | Protein binding |
| 241                     | C0Q51S        | Putative uncharacterized protein | Transport |
| 242                     | C0NF6         | Putative uncharacterized protein | Structural constituent of ribosome |
| 243                     | C0NST1        | Putative uncharacterized protein | Carbohydrate metabolic process |
| 244                     | C0NUK9        | Putative uncharacterized protein | Nucleic acid binding |
| 245                     | C0NTJ9        | Putative uncharacterized protein | Protein binding |
| 246                     | C0P1A8        | Putative uncharacterized protein | Structural constituent of ribosome |
| 247                     | C0P1C6        | Putative uncharacterized protein | Carbohydrate metabolic process |
| 248                     | C0ND33        | Putative uncharacterized protein | Nucleic acid binding |
| 249                     | C0NS2         | Putative uncharacterized protein | Protein binding |
| 250                     | C0NW09        | Putative uncharacterized protein | Protein binding |

*Protein found in the *H. capsulatum* vesicles only after treatment of *H. capsulatum* with MAb 6B7.

*Protein found in the *H. capsulatum* vesicles only after treatment of *H. capsulatum* with MAb 7B6.

CoA, coenzyme A.

ER, endoplasmic reticulum.

FAD, flavin adenine dinucleotide.

**FIG 4** Differentially abundant proteins in vesicles isolated from *H. capsulatum* yeast cells incubated with MAb 687 or 786 compared to those in vesicles isolated from untreated yeast cells.
FIG 5 Ranking of differentially abundant proteins in extracellular vesicles after treatment of *H. capsulatum* yeast cells with MAb 6B7 or 7B6.
commonly would protein orthologues related to these functions be found in more different species. The network layout was according to proteins commonly found in different species, with each subnetwork containing only proteins shared by common species (represented by yellow diamonds). Each rectangle represents a protein orthologue and is colored according to its differential abundance in vesicles released by cells treated with different MAbs. A total of 11 common protein orthologues were found in all of the species analyzed, and most of them were upregulated after treatment with MAb 7B6 (Fig. 6). Comparison of the etiologic agents of pulmonary fungal infections (H. capsulatum, P. brasiliensis, and C. neoformans) showed that 48 proteins were common to all three of these fungal species (see subnetworks of all species; H. capsulatum, P. brasiliensis, S. cerevisiae, and C. neoformans; and H. capsulatum, P. brasiliensis, and C. neoformans) (Fig. 6). In this case again, the protein orthologues common to the pulmonary pathogen species were differentially abundant in extracellular vesicles derived from cells treated with MAbs 6B7 and 7B6, reinforcing the idea that these antibodies differentially regulate conserved fungal pathways although they bear the same epitope.

**DISCUSSION**

We previously demonstrated that opsonization of H. capsulatum cells with Hsp60-binding MAbs 6B7 (IgG1, protective antibody) and 7B6 (IgG2b, nonprotective antibody) significantly altered their phagocytosis rate and survival within macrophages, as well as modified the course of infection in a murine disease model (9). In the present work, we observed that treatment of H. capsulatum cells with these MAbs also changed the size of the extracellular vesicles produced by the fungus. This study builds upon our prior description of extracellular vesicle production by H. capsulatum (15) and the information regarding vesicle production by other fungi by demonstrating that treatment with different MAbs significantly alters the size and content of these biologically important vesicles. Hence, this is a new role for antibodies in fungal pathobiology. Furthermore, the finding adds another dimension to the observation that some antibodies can have direct effects on the physiology of microbes (21, 22).

Fungal cells produce a heterogeneous population of extracellular vesicles that vary notably in size and content (23). Our analysis of the protein contents of vesicles isolated from H. capsulatum cells treated with or without MAb 6B7 or 7B6 reveals that MAb exposure significantly increases the protein loading of vesicles. Treated vesicles are larger and contain more proteins than untreated control vesicles. Thus, opsonization with these MAbs appears to change the quality and quantity of vesicle cargo loading.

The binding of MAbs to the surface of a microorganism can modify the complex relationship between a host and a pathogen (9). Interestingly, our results reveal that treatment of H. capsulatum with MAbs 6B7 or 7B6 results in differences in the activities of phosphatase, laccase, and catalase in vesicles, suggesting that these antibodies modulate the production, trafficking, and release into the extracellular space of important fungal virulence factors. The alteration of several proteins concomitantly is important, as modification of single proteins may not significantly impact pathogenicity. For example, the loss of the catalase CatB has no deleterious effect on Histoplasma virulence in vivo or in vitro (23), indicating that several enzymes are involved in the protection of the fungus from reactive oxygen species. Laccase catalyzes melanin synthesis, and the resultant pigment protects the fungal cells from oxidative stress, as well as from phagocytosis by macrophages. The reduction of laccase activity in vesicles from yeast cells treated with MAb 6B7 is an example of a specific factor whose presence is required for virulence, as best demonstrated in C. neoformans (24, 25), where smaller alterations in activity may translate to significant biological differences.

_Figure Legend Continued_

in relation to vesicles isolated from untreated control yeast cells. (A) Global profile of proteins present in vesicles after treatment with MAb 6B7 or 7B6 in relation to untreated control vesicles. Red, upregulated proteins; green, downregulated proteins. (B, C) Proteins down- or upregulated after treatment with MAb 6B7 (B) or 7B6 (C). (D) Comparison of regulated proteins in vesicles isolated from yeast cells treated with MAb 7B6 or 6B7. U.A., UniProt accession number; U.F. (unknown function), nonabundant proteins.
Proteomic analysis of the extracellular vesicles revealed a large and complex composition of proteins with diverse biological functions such as cell growth and signaling; protein, lipid, and sugar metabolism; cell wall architecture; the endocytic route; and antioxidant proteins. Interestingly, in contrast to the enzymatic assays, the proteomic analysis did not show differences in phosphatase, laccase, or catalase between the groups examined. This discrepancy could be impacted by several factors, such as (i) low sensitivity in the measurement of all cargo proteins, (ii) the possibility that the proteins identified are only a small fraction of the total proteins found in the vesicles, and (iii) an effect of antibody treatment (11, 17). As described by Albuquerque et al. (15) and Holbrook et al. (26), our data also show the presence of extracellular proteins involved in cell wall assembly (e.g., 1,3-β-glucanosyltransferase), and changes in the membrane environment could be involved in the reduction of enzymatic activity. In addition, there were also changes in the antioxidant proteins (e.g., a thiol-specific antioxidant protein) and chaperone and nucleus-associated proteins such as Hsp70 (15, 27).

The abundance of the same set of proteins in the vesicles was differentially modified, depending upon the MAb used to treat the cells, indicating that these MAbs specifically change the total proteins and their profile of abundance in the extracellular vesicles. Under all of the conditions evaluated, amino acids/proteins involved in metabolism were the most abundant proteins in the vesicles, suggesting that the MAbs profoundly impact the metabolism and transport of proteins (28). However, some protein changes were detected only upon treatment with one isotype of MAB, suggesting specific alterations in fungal physiology. For instance, levels of saccharopine dehydrogenase and oxysterol-binding protein were modified only upon MAb 6B7 treatment. The former protein is involved in lysine metabolism and was found to be sensitive to decreasing iron levels (29). This modulation of metabolism might be

FIG 6 Analysis of orthologous proteins found in different fungal extracellular vesicles. Each subnetwork is rooted by the common species upon which the orthologous proteins were identified, represented by the yellow diamonds. Each subnetwork contains only proteins exclusively common to those species. The colors of the orthologous proteins (rectangles) represent differential abundances in extracellular vesicles treated with MAB 7B6 or 6B7. The orthologous proteins in gray were below the limit of quantification (LOQ).
advantageous for the survival of *H. capsulatum* cells in a nutritionally restricted environment such as the immune cell milieu (29). The latter, oxysterol-binding, protein plays a role in ergosterol synthesis, potentially impacting antifungal targets and membrane stability (30). In addition, downregulation of β-glucan proteins (such as 1,3-β-glucanosyltransferase) after MAB 6B7 treatment indicates modification of the synthesis of β-glucan, a structural constituent of the fungal cell wall and a target for host immune system cells (19). Indeed, changes in the profile of abundance of proteins related to protein metabolism and sterol and β-glucan synthesis suggests important modifications of the *H. capsulatum* cell wall upon MAB 6B7 treatment. Thus, these alterations may impact fungal virulence, the immune response, and treatment with antifungal agents that target sterol (1, 19).

Treatment with disease-enhancing MAb 7B6 induced more alterations in the magnitude of protein abundance than did treatment with protective MAb 6B7. Increases in the abundance of sugar metabolism proteins that were upregulated (such as malate dehydrogenase and aconitase) suggests that opsonization with MAb 7B6 enhances energy acquisition. This change was accompanied by an increase in the abundance of amino acid/proteins involved in metabolism that were upregulated, which is consistent with augmentation of protein metabolism. In addition, the increased abundance of cytoskeleton protein/motility-associated proteins indicates that opsonization with MAb 7B6 also enhances intracellular motility. Thus, the interaction of MAB 7B6 with cell surface Hsp60 may lead to a protective adaptation of fungal cells to stress responses and consequently change the loading of proteins in the secreted vesicles, enhancing cellular resistance to host defenses (9, 19).

The fungal species analyzed are from the phylum *Ascomycota*, and orthologue analyses demonstrate that they release common extracellular components to deliver diverse macromolecules to the extracellular space (13, 22). Heat shock proteins, the most highly evolutionarily conserved proteins, were found to be upregulated in all of the fungal species tested and in *H. capsulatum* vesicles after treatment with MAB 7B6. Heat shock proteins are generally produced in response to challenging conditions (e.g., high temperature, oxidative stress, radiation, and inflammation). The binding of MAB 7B6 to *H. capsulatum* appears to induce a stress situation in the yeast that may prime it for more effective survival of host-pathogen interactions. As previously shown, the proteins in *H. capsulatum* vesicles had many similarities to proteins identified in vesicles of *Saccharomyces cerevisiae* (15, 31) and treatment with MAbs 6B7 and 7B6 did not change the profile of the similar proteins, suggesting that regulation of the concentrations of many of these orthologous proteins may not be essential for survival in vivo. Interestingly among the etiologic agents of pulmonary infections, *H. capsulatum* had more proteins in common with *P. brasiliensis* than *C. neoformans*, suggesting that these endemic fungi share more characteristics than dimorphism, an infection route, and the capacity to cause disease in immunocompetent individuals.

In conclusion, our results reveal that treatment with MAbs 6B7 and 7B6 changes vesicle size and increases the protein loading of the vesicles. We found that urease, phosphatase, laccase, and catalase were present in vesicles isolated from yeast cells grown with or without these MAbs, confirming that *H. capsulatum* vesicles are involved in the delivery of virulence factors to the extracellular space and demonstrating that binding by MAbs can modify the quantity of biologically relevant proteins in vesicles. This finding is clearly in line with prior studies of *C. neoformans* showing that antibody binding can directly impact gene regulation and fungal metabolism (21, 32). Finally, analysis of orthologous proteins showed that different ascomycetes produce similar structures in extracellular supernatants with similar proteins in their milieu, corroborating the idea that vesicles are important effectors involved in the communication between intra- and extracellular spaces. Hence, further studies of modified vesicle production and function in the setting of antibodies may provide insights into novel approaches to modifying the pathobiology of these potentially lethal pathogens.
MAb Treatment Alters *H. capsulatum*-Released Vesicles

**MATERIALS AND METHODS**

**Strain and media.** *H. capsulatum* strain ATCC G2178 was cultivated in Ham’s F12 medium (supplemented with glucose [18.2 g/liter], glutamic acid [1 g/liter], HEPES [6 g/liter], cysteine [8.4 mg/liter], and a penicillin-streptomycin solution [1%] at 37°C in a rotary shaker (150 rpm) for 7 days (33)).

**MAb production.** The generation of MAbs in ascites fluid was approved by the Albert Einstein College of Medicine Institutional Animal Care and Use Committee. Briefly, IgG1 (6B7) and IgG2b (7B6) MAbs were produced by injecting 10⁷ hybridoma cells into the peritoneal cavities of ex-breeder BALB/c female mice (National Cancer Institute) that had previously been primed with Pristane (Sigma-Aldrich). The concentration of MAbs in the ascites fluid was determined by enzyme-linked immunosorbent assay with IgG1 and IgG2b standards at known concentrations (34). The same procedures were performed to generate MAB 12D3 (IgG2a), which binds a different region of *H. capsulatum* Hsp60 (8) and was used as a positive control in the proteomic analyses.

**Vesicle purification.** Vesicles were purified according to the protocol described by Rodrigues et al. (35), with minor modifications. *H. capsulatum* yeast cells (2.5 × 10⁶/ml in a volume of 30 ml) were incubated with MAbs 6B7, 7B6, or 12D3 at 6 μg/ml. To maintain the log phase, 10 ml of fresh medium was added to the cells every 48 h (final volume of 50 ml). After 7 days of growth, the yeast cells were removed by centrifugation at 3,000 rpm for 10 min at 4°C and then filtered with a 0.45-μm-pore-size filter (17). Cell-free supernatant was concentrated in an Amicon ultrafiltration system with a membrane with a 100-kDa cutoff. After filtration, the membrane was washed with filtered phosphate-buffered saline (PBS) to collect any remaining vesicles on the membrane surface. The collected vesicles were further centrifuged at 152,813 × g (60,000 rpm, with a TLA 100.3 rotor in a Beckman Coulter ultracentrifuge) for 1 h at 4°C. The supernatant was removed, and the pellets were suspended in 0.1 ml of filtered PBS, combined, and submitted to repeat ultracentrifugation. For proteomic analysis, the pellets were used, whereas vesicles were suspended in 0.5 ml of filtered PBS containing protease inhibitor cocktail (Roche) for DLS analysis, quantification, and enzymatic activity determination. All experiments were performed in duplicate.

**Analysis of extracellular vesicle size by DLS.** The size distributions of extracellular vesicles suspended in PBS supplemented with a protein inhibitor cocktail (Roche) were measured by quasielastic light scattering in a 90Plus/Bi-MAS multianalyte particle sizing analyzer (Brookhaven Instruments). In solution, vesicles undergo Brownian motion that, after illumination by monochromatic laser, produces light scattering fluctuations (i.e., DLS) that provide information about size distribution (17). All experiments were performed in duplicate.

**Protein and sterol quantification.** Protein quantification was performed with Bradford reagent (Bio-Rad, Richmond, CA) by NanoDrop technology (ND-1000 spectrophotometer; Thermo Scientific). Sterol quantification was performed with an Amplex Red kit (Life Technologies).

**Enzyme activity.** To detect urease, phosphatase, and laccase activities in the vesicles, Vesicle suspension volumes of 30 μl with a protein concentration of 10 μg/ml were aliquoted to a 96-well plate (17). One hundred microliters of each enzyme reaction solution was added, and plates were stored at 37°C while protected from the light for 16 h and then read with a spectrophotometer (BioTek). Urease activity was evaluated with an enzyme reaction mixture containing 1% peptone, 0.1% dextrose, 0.5% NaCl, 0.2% KH₂PO₄, 2% urea, and 0.0012% phenol red. The plate was read at 540 nm. To evaluate phosphatase activity, the reaction buffer was prepared with p-nitrophenylphosphate at 1 mg/ml of 100 mM sodium acetate solution. The reaction was read at 405 nm. For laccase evaluation, the solution was prepared with 12.5 mM of L-3,4-dihydroxyphenylalanine in PBS and the plates were read at 450 nm. Finally, catalase activity (with a protein concentration of 10 μg/ml) was evaluated with a catalase assay kit (Cayman Chemical).

**Sample preparation for proteomic analysis.** *H. capsulatum* extracellular vesicle pellets, prepared in biological replicates, were suspended in 100 μl of 50 mM NH₄HCO₃ containing 5 mM dithiothreitol (DTT) and 8 M urea and incubated for 15 min at 37°C in order to reduce disulfide bonds. Free thiol groups were alkylated by adding iodoacetamide (IAA) to a final concentration of 10 mM and incubating the mixture for 30 min at room temperature. DTT was added to a final concentration of 20 mM to terminate the reaction. Samples were then diluted 8-fold with 50 mM NH₄HCO₃, and CaCl₂ was added to a final concentration of 1 mM. Proteins were digested overnight at 37°C with 2 μg of trypsin. Reagents and salts were removed from the samples with solid-phase extraction C₁₈ spin columns (Ultracisor spin columns, C₁₈ 3- to 30-μg capacity; Nest Group). Briefly, 100 μl of each solution was loaded and the column was washed twice with 100% methanol and then washed twice with 0.1% trifluoroacetic acid (TFA). Samples were then loaded and washed four times with 5% acetonitrile (ACN) containing 0.1% TFA before elution with 80% ACN–0.1% TFA. The resulting peptides were dried in a vacuum centrifuge and suspended in a 0.1% formic acid (FA) solution for liquid chromatography-tandem mass spectrometry (MS) analysis.

**Global quantitative proteomic analysis.** Peptides were loaded into a C₁₈ trap column (200 μm by 0.5 mm, ChromXP C₁₈-CL, 3 μm, 120 Å; Eksigent), and separation was carried out in a capillary C₁₈ column (75 μm by 15 cm, ChromXP C₁₈-CL, 3 μm, 120 Å) connected to a nanoHPLC system (Ekspert nanoLC 400; Eksigent). Elution was performed with the following gradient: 1 min in 5% solvent B (solvent A, 0.1% FA; solvent B, 80% ACN–0.1% FA), 5 to 35% solvent B in 60 min, 35 to 80% solvent B in 1 min, 6 min in 80% solvent B, 80 to 5% solvent B in 1 min, and holding at 5% solvent B for 11 min. The flow rate was constant at 200 nl/min over the whole gradient. Eluting peptides were directly analyzed in an electrospray ionization mass spectrometer (5600 TripleTOF; AB Sciex). Full MS spectra were collected in a range of 400 to 2,000 m/z, and the 50 most intense parent ions were submitted for fragmentation for 50 ms with rolling-collision energy.
Peptides were identified by searching tandem mass spectra against a sequence database containing the H. capsulatum complete proteome set from the UniProt Knowledge Base and common contaminant sequences (9,465 total sequences) with the Paragon tool of the Protein Pilot software (AB Sciex). For database searches, trypsin digestion, cysteine residue alkylation with IAA, and biological modifications were considered as factors. Peptides were filtered with a confidence score of >95, which resulted in a false-discovery rate (FDR) of <2% at the protein level on the basis of the reserve sequence database approach.

Peptide and protein quantification was done by extracting peak areas of identified peptides with Skyline (Maclean). For differential-expression (DE) analysis, we used the hierarchical Bayesian model proposed by Wei and Li (36) with the mapDIA software (http://mapdia.sourceforge.net). More importantly, mapDIA allows the analysis of repeated measurements in quantitative proteomic data analysis, such as intensity data from multiple peptides within a protein or transition intensity data acquired from data-independent acquisition MS. We used the peptide isotopic intensity data (M, M + 1, M + 2) as repeated measures of peptide abundance in mapDIA. In the model, two possible probability models of intensity data are proposed for each compound, namely, a DE model and a non-DE model, and the posterior probability of DE is calculated and these scores are used to derive the FDRs for the selection of DE proteins (37).

Statistical analyses. Statistical analyses were performed by one-way analysis of variance or the Newman-Keuls multiple-comparison test with GraphPad Prism software, depending on the data.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://dx.doi.org/10.1128/mSphere.00085-15. Table S1, DOCX file, 0.01 MB. Table S2, XLSX file, 1.2 MB. Table S3, XLSX file, 0.2 MB. Table S4, XLSX file, 0.1 MB. Table S5, XLSX file, 0.1 MB.

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