Review

Fungi extracellular vesicles: extraction, cargo, and the immune system response

Marcelo Augusto Kazuo Ikeda , Renato Massis Souza Campos , Jennifer Lacerda Da Silva , Karen Spadari Ferreira *

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Review

Fungi Extracellular Vesicles: Extraction, Cargo, and the Immune System Response

Ikeda MA [1], Campos RMS [2], da Silva JL [2] and Ferreira KS [1*]

[1] Instituto de Ciências Ambientais, Químicas e Farmacêuticas da Universidade Federal de São Paulo—Brazil—Department of Pharmaceutical Sciences
[2] Universidade de São Paulo—Brazil—Department of Clinical and Toxicological Analyses
* Correspondence: karen.spadari@unifesp.br

Abstract: Like other organisms, fungi produce extracellular vesicles (EVs) that are involved in various biological processes, including intercellular communication and the transport of molecules between cells. These EVs can be applied in fungal pathogenesis, virulence, and interactions with other organisms, including host cells, in the case of fungal infections. While some types of mycoses are relatively common and easily treatable, certain neglected mycoses pose significant public health challenges, such as sporotrichosis, chromoblastomycosis, and paracoccidioidomycosis. These infectious diseases can cause significant morbidity and disability, leading to a reduced quality of life for the patients. So, research about the virulence factor is essential to understand how fungi escape the immune system. In this context, this manuscript reviews the study of fungi EVs, their cargo, their obtaining, and their role during the infectious process, which is extremely important for understanding this neglected mycosis.

Keywords: extracellular vesicles; immune response; mycosis

Introduction

Fungal Extracellular Vesicles

In 1967, Peter Wolf wrote what many consider today one of the first descriptions of round-shaped structures resembling small vesicles in human plasma. He mentions, "The purpose of the present communication is to provide evidence for the occurrence in normal plasma, serum, and fractions derived from coagulant material in minute particulate form, sedimentable by high-speed centrifugation and originating from platelets, but distinguishable by from intact platelets." It is suggested that this material, hereafter referred to as 'platelet-dust'" (Wolf, 1967). This report would guide future research and find similar structures in other organisms. In 1972 Gibson and Peberdy observed a fungus of vesicle-like structures near the *Aspergillus nidulans* protoplasts' wall.

Furthermore, they also observed a structure pushing the membrane outwards, resembling yeast budding. These structures were outpouchings of the plasma membrane that were eventually pinched from the fungal cell, forming 'subprotoplasts'". Takeo and colleagues also found 1973 vesicles ranging from 50-150 nm and larger multivesicular bodies, suggesting their role in mediating the exportation of intracellular content towards the extracellular space through the membrane (Takeo et al., 1973). After this finding, interest in extracellular vesicles (EVs) seemed to decline in the following years.

EVs are small membrane-bound structures released by cells into the extracellular space. They are produced by almost all cell types in the body, including cells of the immune system and fungal cells. EVs are involved in various physiological and pathological processes and are crucial in intercellular communication (Yáñez-mó et al., 2015). There are three main types of EVs: exosomes, microvesicles, and apoptotic bodies. Exosomes are the smallest and most extensively studied vesicles, typically ranging from 30 to 150 nanometers. They are formed within the endosomal system and are released from cells upon fusion of multivesicular bodies with the plasma membrane (Figure 1). Microvesicles, also known as ectosomes or shedding vesicles, are larger than exosomes and are

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directly shed from the plasma membrane. Apoptotic bodies are larger still and are released during apoptosis. (Santavanond et al., 2021).

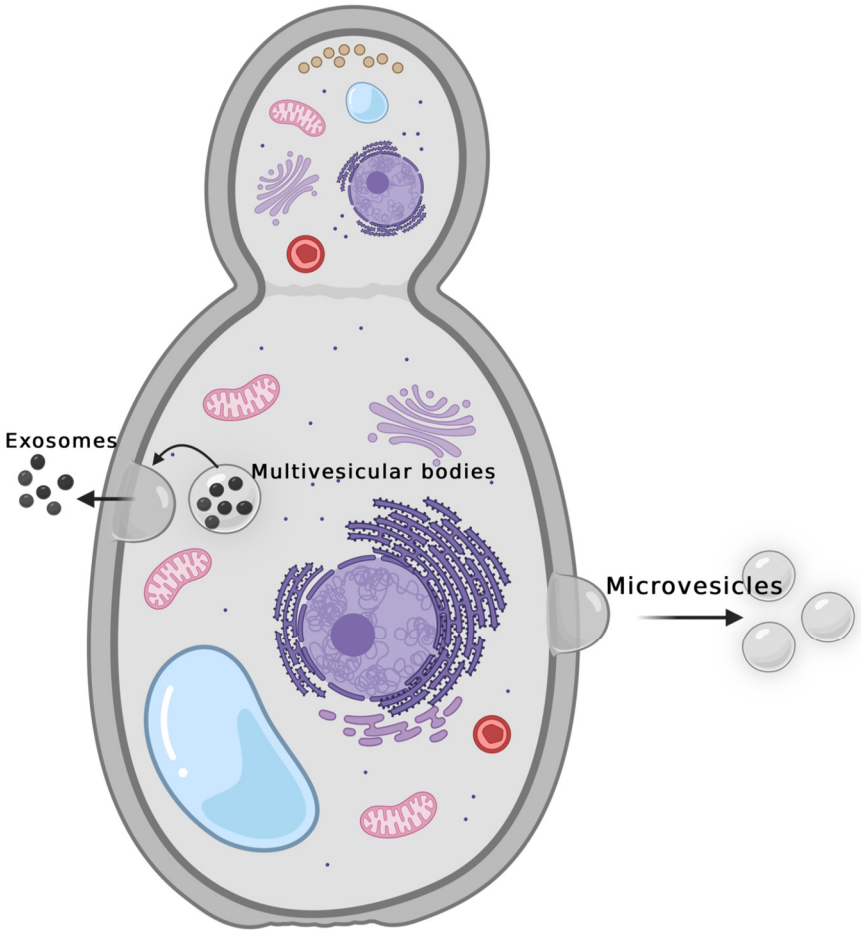

Figure 1. Extracellular vesicles in yeast cells. The figure shows the microvesicles and exosome formation. Created with BioRender.com.

Due to their ability to carry bioactive molecules, EVs have gained significant attention in biomedical research. However, it was when in 2007, after more than thirty years of EVs discovery, Rodrigues and colleagues described EVs from the fungus *Cryptococcus neoformans* as responsible for transporting glucuronoxylomannan through the cell wall (Rodrigues et al., 2007). After Rodrigues's publication (2007), we saw an increase in publications describing new species of fungi that could produce EVs.

In 2008, Albuquerque et al. made the first report on EVs in *Histoplasma capsulatum*. Further investigations regarding its content and composition revealed that these vesicles carry dozens of proteins, with functions varying from cell wall assembly and cell signaling to nuclear proteins and cell growth/division (Albuquerque et al., 2008). After confirming that *Histoplasma capsulatum* also released EVs during these experiments, the researchers observed EVs in *Ascomycetes* after their culture supernatant was ultracentrifuged and analyzed by transmission electron microscopy (TEM). Four new species also had EVs released to the growth media. *Saccharomyces cerevisiae*, *Candida albicans*, *Candida parapsilosis*, and *Sporothrix schenckii* secreted vesicles around 100 nm, similar to those produced by *Histoplasma capsulatum* e *Cryptococcus neoformans*.

Other studies investigating EVs from *Cryptococcus neoformans* and *Cryptococcus gatti* found more virulence factors within its vesicles (Rodrigues et al., 2008; Bielska et al., 2018). In 2011 two new reports confirmed the production of EVs by *Malassezia sympodialis* (Gehrmann et al., 2011), along with the description of EVs released from the pathogenic fungus *Paracoccidioides brasiliensis* (Vallejo et al.,

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2011). In 2018 Ikeda et al. isolated EVs from the pathogenic fungus *Sporothrix brasiliensis*, responsible for the epidemy of zoonotic sporotrichosis. (Ikeda et al., 2018). In the upcoming years, EVs were isolated from other pathogenic genera such as *Aspergillus* (Souza et al., 2019; Brauer et al., 2020), *Pichia* (Leone et al., 2017), *Rhizopus* (Liu et al., 2018), *Trichophyton* (Bittencourt et al., 2018), *Exophiala* (Lavrin et al., 2020) and *Fonsecaea* (Las-Casas et al., 2022). Also, EVs were found in some phytopathogens such as *Alternaria infectoria* (Silva et al., 2014), *Fusarium oxysporum* sp. *vasinfectum* (Bleakley et al., 2020), *Trichoderma reesei* (De Paula et al., 2019), *Penicillium digitatum* (Costa et al., 2021), *a*nd *Colletotrichum higginsianum* (Rutter et al., 2022).

Fungal EVs Methods of Extraction

The first description of fungal EVs being isolated was made by Rodrigues and colleagues in 2007, where EVs were separated by ultracentrifugation based on the different buoyant densities of cells and particles in the solution. From liquid media, a culture of *C. neoformans* was submitted to two centrifugations in a cold rotor (4°C) at 4.000g to remove the heavy portion of cells and at 15.000g to remove most of the apoptotic bodies, debris, and molecules with higher density than the EVs. They were finally ultracentrifuged at 100.000g for 1-2 hours, repeating this step 5 times to wash the pellet. Such protocol granted a reliable and cost-effective method to isolate EVs from many other fungi until today. In a few hours, with only one ultracentrifuge and a flask of TBE or PBS, a pellet of $1x10^8$ to 10^{11} particles/mL could be easily obtained (Bitencourt et al., 2018; Ikeda et al., 2018; De Paula et al., 2019). However, the main disadvantages regarding centrifugation are working with large volumes and the fact that other molecules, such as proteins, lipoproteins, and nonexosomal particles, will also be isolated given their similar size and density (Gardiner et al., 2016; Mathieu et al., 2019).

Another strategy, the isolation with a density gradient such as a 30% sucrose gradient, has also been employed in many studies in recent years to improve the basic ultracentrifugation protocol (Abramowicz et al., 2016; Reis et al., 2021). This method further purifies the sample solely based on the buoyant density, focusing on refining the isolation of exosomes from other larger vesicles, given their characteristic density of 1.11-1.19 g/mL (Lamparski et al., 2002; Théry et al., 2006; Taylor; Shah, 2015). This procedure is sufficient to recover a high-quality sample in cases where high levels of separation between EV types are unnecessary. In cases where highly-purified and isolated exosomes are required, other methods must be applied. Is it known that exosomes and microvesicles' densities and sizes overlap around 50 and 150nm, affecting the success of the physical separation by gravitational force and generating a pellet that generally will contain a pool of exosomes, microvesicles and other non exosomal particles (Taylor; Shah, 2015; Brennan et al., 2020). Depending on the application or the experimental approach, these "contaminants" need to be considered, where further purifications may be necessary to remove foreign, non-vesicular material.

Filtration is easily one of the most valuable techniques in a laboratory due to its efficiency, low cost, and how long the procedure lasts. The separation of EVs can be performed with the help of different pore-sized membranes, which will retain a specific particle size while allowing smaller particles to pass through. The dimensions usually used to filter EVs samples are 0.8, 0.45, 0.22, and 0.1 μm, retaining particles greater than 800 nm, 450 nm, 220 nm, and 100 nm, respectively (Merchant et al., 2010; Liebana-Jordan et al., 2021; Reis et al., 2021). Large particles are first filtered through the 0.8 μm and 0.45 μm membranes, where the flow-through can then be screened until the smallest pore size (Taylor; Shah, 2015).

Similarly, in studies regarding fungal EVs, it is often seen ultracentrifugation units (Amicon®, Vivapsin®) being employed, which consists of a centrifuge tube varying in size (2 or 15 mL) carrying filtering units with different molecular weight cutoffs (MWCO) ranging from 3 -100 kDa. After simple centrifugation at 6.000g, the sample is concentrated 20-fold and collected at the bottom along with any particle that has a size smaller than the chosen MWCO (Vallejo et al., 2011; Da Silva et al., 2015). Since EV isolation from fungal cultures usually requires large volumes of liquid media (Bielska et al., 2018; Souza et al., 2019; Lavrin et al., 2020), this procedure drastically reduces the number of ultracentrifugation steps needed and yields a higher purity sample.

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In 2019, Reis and colleagues (Reis et al. 2019) developed an even more efficient strategy, where fungal cultures were grown on solid media after a step of enrichment in extract-peptone-dextrose (YPD) media for two days under shaking. Cells were counted and diluted to a desired concentration, and aliquots of 300 µL were plated onto YPD plates. With these petri dishes, it is just a matter of scraping the cells onto a tube with the desired volume of 0.22 µm-filtered PBS and proceeding for ultracentrifugation. 20 or 30 mL of PBS can be used to resuspend the cells. A procedure that once took hours, usually utilizing all the slots on the centrifuge rotor, reloading the same sample over and over to concentrate the large volume of liquid, can now be done in one single round of ultracentrifugation with only a few tubes.

Although there are many other techniques for EV isolation and purification, such as Polyethylene glycol precipitation (Kim et al., 2015; Deregibus et al., 2016), Magnetic bead separation (Gardiner et al., 2016), Immunoaffinity-based capture (Ingato et al., 2016), Size-exclusion chromatography (SEC), ExoQuick precipitation agent, these approaches are most seen used in the extraction of EV's from human samples (Musante; Tataruch; Holthofer, 2014; Guerreiro et al., 2018; Zhu et al., 2020). In the case of fungal extracellular vesicles, simple ultracentrifugation alone or coupled with either ultrafiltration systems or density gradient/cushion is sufficient to generate high yields of EVs from a single flask of cultured yeast while being significantly more affordable than other techniques. Figure 2, a resume from extraction and analysis of fungal EVs.

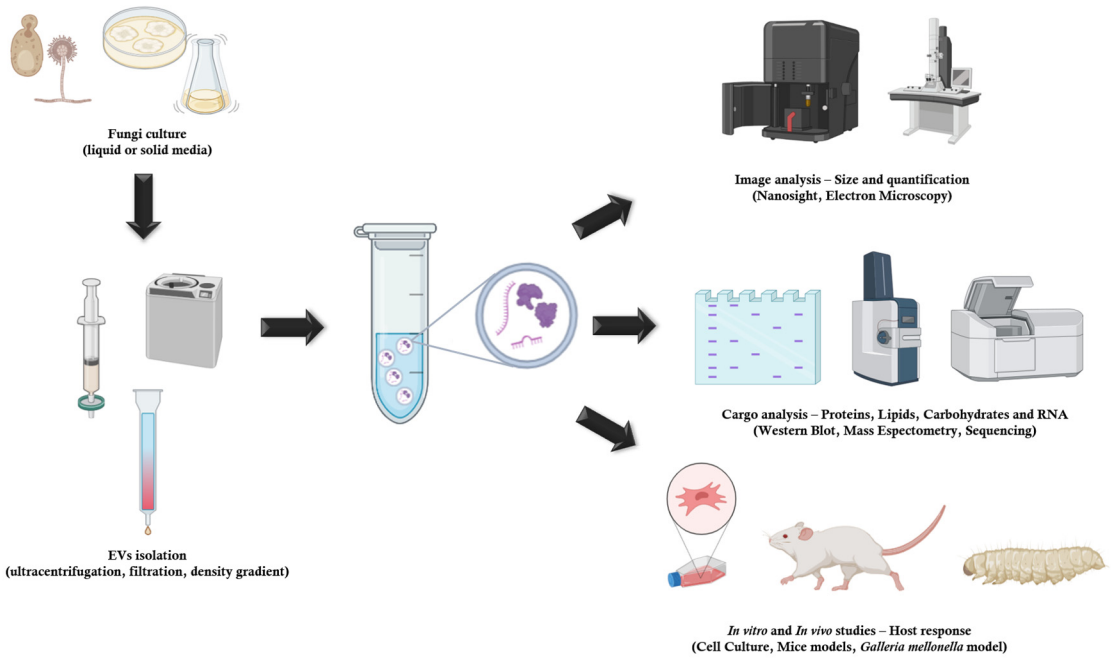

Figure 2. Evs—Fungi culture isolates EVs from several methods, such as ultracentrifugation, filtration, or density gradient. Then, the EV are analyzed and can be studied *in vitro* or *in vivo*.

Cargo of Fungal Extracellular vesicles

Due to their cargo, EVs have been implicated in numerous physiological processes, including the immune system. They can be used as diagnostic biomarkers, therapeutic delivery vehicles, and targets for therapeutic intervention (Yáñez-mó et al., 2015). EVs contain various molecules, including proteins, lipids, nucleic acids, and metabolites. These cargo molecules can reflect the state of the cell of origin and can be selectively packaged and transferred to recipient cells. EVs can act as carriers of biological information and can transmit signals to nearby or distant cells, influencing their behavior and function. Recipient cells can take them up through various mechanisms, allowing the transfer of their cargo and subsequent modulation of cellular processes (Zamith-Miranda et al., 2018).

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Fungal EVs can carry raw material for the growth and cell wall remodeling of some types of fungi, which can interact with the immune host (Nimrichter et al., 2016). The *C. neoformans* EVs can carry various immunomodulating molecules such as glucuronoxylomannan (Rodrigues et al., 2007), a component of the cryptococcal capsule, and melanin (de Sousa et al., 2022). In *P. brasiliensis*, EVs carry highly immunogenic α-Gal epitopes (Vallejo et al., 2011). *E. dermatitis* EVs contain melanin (Lavrin et al., 2020).

Several virulence-related carbohydrates, proteins, and lipids were found in EVs from *A. fumigatus* (Rizzo et al., 2020), *C. albicans* (Gil-Bona et al., 2015; Vargas et al., 2015; Wolf et al., 2015), *C. auris* (Amatuzzi et al., 2022) *C. neoformans* (Rodrigues et al., 2008; Wolf et al., 2014), *H. capsulatum* (Albuquerque et al., 2008; Baltazar et al., 2016; Cleare et al., 2020), *P. brasiliensis* (Vallejo et al., 2012; Vallejo et al., 2013; Peres da Silva et al., 2015), *S. brasiliensis* and *S. schenckii* (Ikeda et al., 2018).

Also, fungal EVs carry functional RNA that can affect the physiology of host cells (Bitencourt et al., 2022) as described for *C.albicans*, *C. neoformans*, and *P. brasiliensis* (Peres da Silva et al., 2015), *C. gattii* (Reis et al., 2019), *C. auris* (Munhoz da Rocha et al., 2021; Amatuzzi et al., 2022), *H. capsulatum* (Alves et al., 2019), *P. lutzii* (Peres da Silva et al., 2019). and *M. sympodialis* (Rayner et al., 2017).

Fungi EVs and host immune system

Since the discovery of EVs, several studies have demonstrated the interaction of EVs produced by microorganisms with the host cells (Rodrigues and Nimrichter, 2022). In mycology, we have works demonstrating the ability of fungal EVs to interact with the host immune system (Table 1), as seen in *A. flavus* (Brauer et al., 2020), *A. fumigatus* (Souza et al., 2019; Freitas et al., 2023), *C. albicans* (Vargas et al., 2015; Wolf et al., 2015; Vargas et al., 2021; Zamith-Miranda et al. 2021; Honorato et al., 2022; Wei et al., 2023), *C. auris* (Zamith-Miranda et al. 2021), *C. haemulonii* var. *vulnera* (Oliveira et al., 2022), *C. glabrata*, *C. parapsilosis*, *C. tropicalis* (Kulig et al., 2022), *C. deuterogatti* (Castelli et al., 2022); *C. gatti* (Bielska et al., 2018), *C. neoformans* (Oliveira et al., 2010, Huang et al., 2012; Colombo et al., 2019; Marina et al., 2020; Rizzo et al., 2021), *F. pedrosoi*, *F. nubica* (Las-Casas et al., 2022), *H. capsulatum* (Baltazar et al., 2018), *M. sympodialis* (Gehrmann et al., 2011), *P. brasiliensis* (Peres da Silva et al., 2019; Baltazar et al., 2021; Octaviano et al., 2022), *S. brasiliensis* (Ikeda et al., 2018; Campos et al., 2021), *T. marneffei* (Yang et al., 2021) and *T. interdigitale* (Bitencourt et al, 2018).

Most mycoses are considered neglected diseases with few therapeutic options available, so immunotherapy is an option to reduce the occurrence of these emerging threats (B R Da Silva et al., 2021). The EVs released by fungi contain a range of immunogenic molecules that can serve as a delivery tool, such as vaccines (Freitas et al., 2019). By western blot, sera of infected animals or patients were able to react with components from EVs of *A. fumigatus* (Souza et al., 2019), *C. albicans* (Gil-Bona et al., 2015), *C. neoformans* (Rodrigues et al., 2008), *H. capsulatum* (Albuquerque et al., 2008) *M. sympodialis* (Gehrmann et al., 2011), *P. brasiliensis* (Vallejo et al., 2011), *S. brasiliensis* (Ikeda et al., 2018) and *S. schenckii* (Ikeda and Ferreira, 2021), demonstrating the capacity of EVs interact with host cells.

A diversity of *in vitro* assays shows the immunomodulatory effects of fungi EVs. Neutrophils are the first line of immune defense recruited to the tissue against some fungal pathogens (Desai and Lionakis, 2018). In *A. fumigatus*, the interaction of mice bone marrow-derived neutrophils with EVs allowed an increase in the phagocytic index and reduction of fungal burden in the fungal challenge, associated with an increase in the production of TNF-α and IL-1β cytokines (Souza et al., 2019). However, *A. fumigatus* EVs could not induce the release of neutrophil extracellular traps by human neutrophils, nor the cytokine production by human peripheral blood mononuclear cells (Freitas et al., 2023).

Macrophages are another cell that plays a vital role in controlling fungi infection (Heung, 2020). Fungi EVs were able to modulate these cells, increasing the fungicidal capacity and/or production of inflammatory mediators as observed in *A. flavus* (Brauer et al., 2020), *A. fumigatus* (Souza et al., 2019; Freitas et al. 2023), *C. albicans* (Zamith-Miranda et al., 2021), *C. neoformans* (Oliveira et al., 2010), *P. brasiliensis* (da Silva et al., 2016), *S. brasiliensis* (Campos et al., 2021) and *Trichophyton interdigitale* (Bitencourt et al., 2018). Otherwise, in *H. capsulatum* (Baltazar et al., 2018) and one strain of *C. auris*

(Zamith-Miranda et al., 2021), EVs reduced the fungicidal rate of macrophages, revealing different effects of EVs on host cells.

Dendritic cells are professional antigen-presenting cells that can induce adaptive immune responses that promote fungal clearance (Heung, 2020). In *C. albicans* (Vargas et al., 2015; Vargas et al., 2020; Zamith-Miranda et al., 2021) and *C. auris* (Zamith-Miranda et al., 2021), EVs were able to activate dendritic cells increasing production of cytokines and expression of surface markers. In *S. brasiliensis* (Ikeda et al., 2018), dendritic cells were stimulated with EVs and challenged with yeasts, resulting in increased phagocytic index but inability to eliminate the fungus. Although the cells did not have an excellent fungicidal capacity, the production of cytokines could activate the immune system. In a trans-well co-culture model of *P. brasiliensis* yeasts with dendritic cells (Peres da Silva et al., 2019), EVs downregulated Pknox1 and Gbpb2 transcription factor, that regulates IL-7 and IL-10 production.

Regarding *in vivo* effects of EVs, some studies have demonstrated the ability to reduce mortality in the insect *Galleria mellonella* infection model with previous stimulation with EVs, as seen in *A. flavus* (Brauer et al., 2020), *A. fumigatus* (Freitas et al., 2023) and *C. albicans* (Vargas et al., 2015; Vargas et al., 2020). Except for *C. neoformans* and *C. deuterogatti*, EVs exacerbated the infection (Colombo et al., 2019; Castelli et al., 2022). Reis and colleagues (2021) isolated a peptide from EVs of *Cryptococcus gattii* and improved the survival of *G. mellonella* lethally infected with *C. gattii* or *C. neoformans*. This model allows a preliminary evaluation of potential candidates for immunotherapy, complementing *in vitro* assays with cells and reducing the use of animals (Curtis et al., 2022).

Other studies looked for EVs effects in animal models. In the commensal fungi *C. albicans*, Vargas and colleagues (2020) performed a mice immunization model with three intraperitoneal applications of EVs. After the third application, immunosuppression with cyclophosphamide was performed, followed by an intraperitoneal infection with a lethal inoculum of *C. albicans* yeasts. Compared with the untreated group, vaccination with EVs reduced the fungal burden in evaluated organs (kidneys, spleen, and liver) and allowed mice to survive against lethal infection. These results were accompanied by an increased antibody production with a predominance of IgG1 and high levels of cytokines involved with inflammation and protective role in candidiasis (IFN-γ, IL-4, IL-6, IL-10, IL-12p70, TGF-β, and TNF-α).

For *Sporothrix brasiliensis*, subcutaneous vaccination with EVs promoted increased fungal load and skin lesion diameter in a subcutaneous infection model in Balb/C mice. The results were accompanied by an increase in the cytokines IL1-β and TNF-α, which could explain an exacerbation of the inflammatory response, favoring the establishment of the fungus in the lesion. (Ikeda et al., 2018).

In the endemic dimorphic fungi *P. brasiliensis*, two studies in mice using EVs as vaccines were conducted. Baltazar and colleagues (2021) performed an immunization scheme with two applications of EVs subcutaneously, followed by an intratracheal infection with the fungus in C57BL/6 mice. In the treated group, the fungal load in the lung tissue was reduced, and a lower score of histopathological alterations was observed. These results were accompanied by increased recruitment of activated T cells (CD4+ and CD8+) and NK cells, production of antibodies IgM and IgG, and high levels of cytokines TNF-α, IFN-γ, and IL-17. Otherwise, in another study, the use of EVs from two strains of *P. brasiliensis*, one attenuated and the other virulent, was evaluated with the subcutaneous application of three doses of EVs followed by intratracheal route infection in Balb/C mice. In both strains, an increase in the fungal load and a worsening in the macroscopic and microscopic lesions of the lungs associated with an increase of the inflammatory mediators TNF-α, IFN-γ, and MCP-1 were observed. These are higher with EVs from attenuated strain (Octaviano et al., 2022). The authors cite that the discrepancies in the results may have occurred due to several factors, such as differences in the culture medium of the fungi, animal model, number of vaccine doses, and observed infection time.

In opportunistic fungi *C. neoformans*, immunization with peritoneal injection of EVs obtained from both wild-type strains and mutants without capsules, with subsequent intranasal infection, allowed a longer survival time of the Balb/C mice accompanied by an increase in the production of

antibodies (Rizzo et al. al., 2021). On the other hand, Huang and colleagues (2012), in a hematogenic disseminated infection model in C57LB/6 mice, showed an increase of fungal burden in the brain of animals that received intravenous EVs, which can be explained by *in vitro* assays where EVs altered the distribution of membrane lipid raft components of brain microvascular endothelial cells, and enhanced *C. neoformans* adherence and traversal across the barrier of cells.

For the ubiquitous fungi *A. fumigatus*, which can cause severe pulmonary infection in immunocompromised individuals, the immunization of C57BL mice with Fungi EVs before infection with *A. fumigatus* conidia resulted in decreased inflammatory cells infiltrate in lungs, mainly of neutrophils, reduced production of pro-inflammatory mediators IL-1β and IL-6, and reduced pulmonary tissue damage. Also, an increased production of specific IgG and increased phagocytic index of immune cells obtained from bronchoalveolar lavage was observed, associated with decreased fungal burden of the lungs. In this work (Souza et al., 2022), immunized animals did not alter survival rates, but the EVs immunization in association with amphotericin treatment showed an increased survival of the animals.

The use of adjuvants in combination with the application of EVs can induce a more robust immune system response. Vargas and colleagues (2020) showed in the *C. albicans* model that the combination of EVs with Freund's adjuvant, when compared to the use of EVs alone, allowed a more significant reduction in fungal load, more outstanding production of IgM and IgG and induction of higher antibodies levels of IFN-γ, IL-4, IL-6, IL-12p70, and TNF-α. As well, an oil-based adjuvant was evaluated in *P. brasiliensis* (Baltazar et al., 2021), where EVs immunization associated with Montanide adjuvant promoted induction of higher levels of IgM and IgG compared to the group without adjuvant. In addition, on *ex vivo* cell proliferation assay, only splenocytes from animals treated with the combination produced detectable levels of IFN-γ, indicating a proliferation response of memory and effector T cells.

An important factor in using EVs as vaccines is the ability to preserve their structural integrity and function, so the storage condition is essential. Vargas and colleagues (2020) showed that EVs stored at different temperatures kept their ability to stimulate IL-6 production in dendritic cells and decrease the mortality of *G. mellonella* larvae; however, EVs held at -80 °C had a lower level of IL-6 compared to fresh EVs and EVs stored at -20 °C, and fresh EVs lead to highest survival rates in *G. mellonella* model.

Some strategies can be carried out to modulate the biogenesis and cargo of EVs, directly affecting their biological role. In *H. capsulatum*, it was demonstrated that EVs released by yeasts treated with monoclonal antibodies have different protein compositions (Baltazar et al., 2016) and were able to have a more significant inhibition activity on the phagocytosis and killing rates in bone marrow-derived macrophages (Baltazar et al., 2018).

The nutrition conditions of fungi can alter the cargo and effects of isolated EVs. Cleare and colleagues (2020) showed that EVs from *H. capsulatum* cultivated in the rich medium had more protein content and altered protein expression than cultures in a less nutritional medium. In *C. neoformans* (Marina et al., 2020), EVs from fungi cultivated in a rich medium induced less response of cytokines in bone marrow-derived dendritic cells and macrophages compared with EVs from fungi grown in a less rich medium. Also, *in vivo*, intranasal treatment with EVs from a rich medium in C57Bl/6 mice after intratracheal infection resulted in a reduction of the fungal burden of lungs after five days of illness but an increase of fungal burden with 15 days post-infection. Reduced cytokine levels and downregulation of inflammasome genes accompanied these results.

Genetic modification can result in mutant fungi that release EVs with different composition that impacts host cell interaction. In *C. albicans* (Wolf et al., 2015), EVs from mutants for phospholipid biosynthesis showed decreased NF-κB activation of macrophages.

Another possibility is intraspecies modulation based on EVs, where the alteration of fungal cell mechanisms with EVs of the species itself can occur. Bitencourt and colleagues (2022) demonstrated in different fungal species the ability of fungal EVs to perform gene regulation in fungi of the same species. In *A. fumigatus* and *P. brasiliensis*, EVs were isolated from cultures submitted to treatments that increased the expression of genes related to the stress response. Fungi of the same species not

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introduced to the therapy could assimilate these EVs and then showed a higher expression of stress-related genes. In *C. albicans*, yeasts were exposed to EVs from cultures with yeast-hypha transition; after that, they began upregulating gene expression related to the hypha transition. Another work on *P. brasiliensis* (Octaviano et al., 2022) showed that yeasts from attenuated strains after incubation with EVs isolated from the virulent strain changed to an antioxidant gene expression, which could convert the virulence profile of the fungus. Another work (Honorato et al., 2022) showed that EVs from *C. albicans* in interaction with yeast cells inhibited biofilm development and affected yeast-to-hypha differentiation, impacting in reduced death of *G. mellonella* larvae infected with EVs-treated yeasts.

It is also possible to use vaccines from EVs obtained from immune system cells activated by the microorganism or its products. In *C. neoformans*, bone marrow-derived macrophages from C57BL/6 mice were activated with yeast, and after that, EVs were obtained from these cells. These EVs allowed *in vitro* an increase in the phagocytosis percentage and killing capacity and a shift to the pro-inflammatory M1 phenotype of naïve macrophages. Also, there was an upregulation of Immune-related pathway genes. *In vivo*, the intraperitoneal injection of these EVs before an intranasal infection in C57BL/6 mice promoted a reduction of fungal burden in the brain and lungs but with a squeeze of survival rate of animals (Zhang et al., 2021). In other work (Reales-Calderón et al., 2017), EVs from THP-1 monocytes cultured with *C. albicans* yeasts were able to stimulate THP-1 macrophages to produce pro-inflammatory cytokines TNF-α, IL-12p40, and IL-8 and increased the fungicidal activity.

The exact mechanisms by which EVs promote changes in the immune system's response are still uncertain; more studies are necessary to elucidate the composition and immunomodulatory and improve the development of new immunotherapies for fungi infection. In Figure 3, we left a summary of EVs and immune responses studied until now.

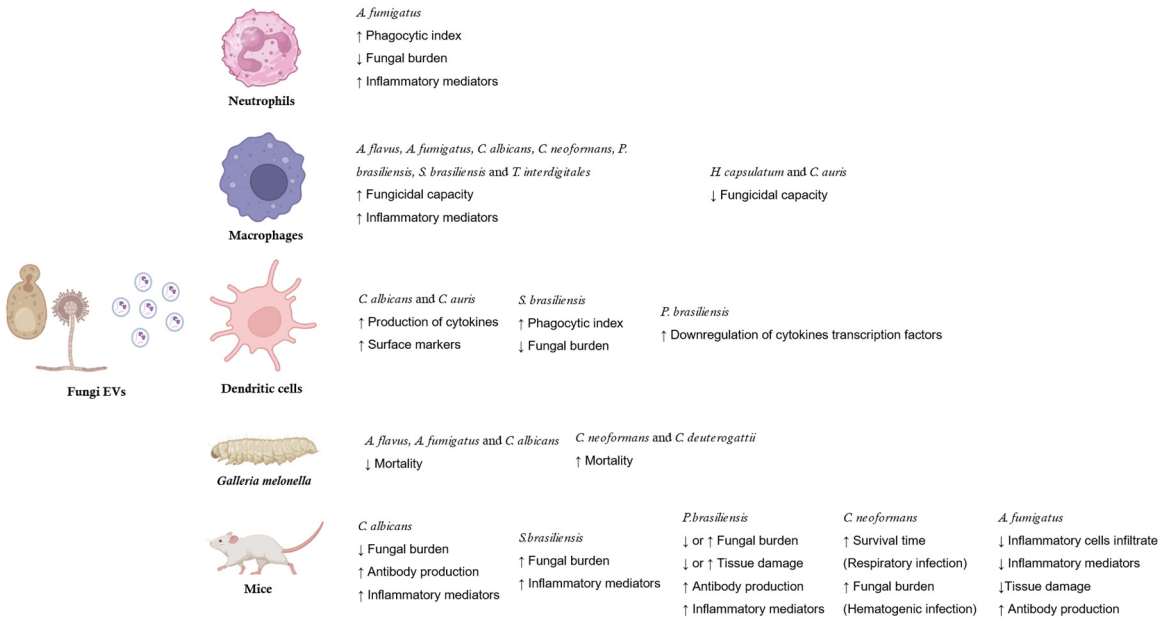

Figure 3. EVs Plasticity in cells, mice, and *Galleria*. Fungal EVs modulate the immune response in different models of infection.

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Table 1. Fungal EVs that interact with the host immune system.

Year, Authors	Fungi	Model	EVs effects
2020, Brauer et al.	*Aspergillus flavus*	*In vitro* , Bone marrow-derived macrophages of C57BL/6 mice	Production of inflammatory mediators (TNF-α, Nitric Oxid, IL-6 and IL-1β), increased expression of M1 polarization marker (Inducible nitric oxide synthase mRNA), and enhanced phagocytosis and killing rates.
2020, Brauer et al.	*Aspergillus flavus*	*In vivo* , *Galleria mellonella* larvae infection after EV treatment	Decrease of larvae fungal burden and enhanced survival of the larvae.
2019, Souza et al.	*Aspergillus fumigatus*	In vitro, Bone marrow-derived neutrophils of C57BL/6 mice and RAW 264.7 macrophage cell line	Increased fungicide capacity and production of inflammatory mediators in macrophages (TNF-α and CCL2) and neutrophils (TNF-α and IL-1β)
2015, Vargas et al.	*Candida albicans*	*In vivo* , *Galleria mellonella* larvae infection after EV treatment	Reduced the mortality of the infected larvae and reduced the fungal burden.
2015, Vargas et al.	*Candida albicans*	*In vitro* , Bone marrow-derived macrophage and dendritic cells of Balb/c mice, and RAW 264.7 murine macrophage cell line	Production of inflammatory mediators in macrophages (NO, IL-12, IL-10, TGF-β and TNF-α) and dendritic cells (IL-12p40, TNF-α, TGF-β and IL-10). Increased expression of CD86 and MHC II in dendritic cells.
2020, Vargas et al.	*Candida albicans*	*In vivo,* Balb/C female imunnosupressed mice infection by introperitoneal route after EV intraperitoneally treatment	Production of IgM, IgG, and cytokines (IL-12p70, TNF-α, IL-10, TGF-β and IL-4), decreased fungal burden in tissues, and increased mice survival.
2020, Vargas et al.	*Candida albicans*	*In vitro,* Bone marrow-derived dendritic cells of Balb/C female mice	Production of IL-6.
2020, Vargas et al.	*Candida albicans*	*In vivo* , *Galleria mellonella* larvae infection after EV treatment	Reduced the mortality of the infected larvae.
2021, Zamith-Miranda et al.	*Candida albicans* and *Candida auris*	*In vitro* , Bone marrow-derived dendritic cells of C57BL/6 mice and RAW 264.7 murine macrophage cell line	Increased phagocytosis and killing rates by macrophages in *C. albicans* . Reduced killing rate of macrophages in *C. auris* . Higher expression of MHCII, CD80, and CD86, and production of IL-6 and TGF-β on dendritic cells.
2010, Oliveira et al.	*Cryptococcus neoformans*	*In vitro* , RAW 264.7 murine macrophage cell line	Increased production of TNF-α, IL-10, and TGF-β, and enhanced phagocytosis and killing rates.
2012, Huang et al.	*Cryptococcus neoformans*	*In vivo* , C57BL/6 male mice infection by hematogenous route associated with EV reatment	Increased fungal burden in the brain.
2020, Marina et al.	*Cryptococcus neoformans*	*In vitro* , Bone Marrow-Derived Macrophages and Bone Marrow-Derived Dendritic Cells of C57BL/6 mice	Modulation of the production of inflammatory cytokines TNF-α and IL-1β
2020, Marina et al.	*Cryptococcus neoformans*	*In vivo* , C57BL/6 mice infection by the intratracheal route followed by intranasal EVs treatment	Lower fungal load 5 days post infection and higher fungal load 15 days post infection. Reductions of IL-1β and TNF-α. Downregulations of inflammasome genes.
2020, Rizzo et al.	*Cryptococcus neoformans*	*In vivo* , Balb/C female mice infection by the intranasal route after EV intraperitoneal injection	Production of antibodies and increased survival time.
2019, Colombo et al.	*Cyptococcus neoformas*	*In vivo* , *Galleria mellonella* larvae infection after EV treatment	Increased the mortality of the infected larvae.
2018, Baltazar et al.	*Histoplasma capsulatum*	*In vitro* , Bone marrow-derived macrophage of Balb/c mice	Inhibition of phagocytosis rate and decreased killing rate.
2011, Gehrmann et al.	*Malassezia sympodialis*	*In vitro* , Human peripheral blood mononuclear cells depleted of CD34+ cells and CD14+ monocytes	Induced production of IL-4 and TNF-α
2016, da Silva et al.	*Paracoccidioides brasiliensis*	*In vitro* , murine peritoneal macrophages of C57BL/6 mice and murine macrophage cell line J774A.1	Induce proinflammatory mediators production in murine peritoneal macrophages (NO, IL-12p40, IL-12p70, IL-6, TNF-α, IL-1α, and IL-1β) and J774A.1 cells (TNF-α, IL-6, and IL-12). Promote the polarization of macrophages towards the M1 phenotype (higher expression of iNOs mRNA) and induce switching from M2 to M1 macrophages. High fungicidal activity.
2019, Peres da Silva et al.	*Paracoccidioides brasiliensis*	*In vitro* , Monocyte-Derived CD11c+ Cells of C57BL/6 mice	Downregulation of transcription factors (Gabpb2, Pknox1, and Zfp575) that regulates IL-10 and IL-7 expression.
2021, Baltazar et al.	*Paracoccidioides brasiliensis*	*In vivo,* C57BL/6 male mice infection by the intratracheal route after EV subcutaneous treatment	Reduced fungal burden and histopathological alterations, mobilization of activated T lymphocytes and natural killer cells, production of antibodies and cytokines (TNF-α, IFN-γ and IL-17), IFN-γ production upon *ex vivo* restimulation.
2022, Octaviano et al.	*Paracoccidioides brasiliensis*	*In vitro* , Balb/C macrophage cell line RAW 264.7 and bone marrow derived macrophages of Balb/C	Production of pro-inflammatory response cytokines (TNF-α and IL-6), chemokines (MCP-1) and nitric oxide (dose-dependent manner).
2022, Octaviano et al.	*Paracoccidioides brasiliensis*	*In vivo* , Balb/C male mice infection by the intratracheal route after EV subcutaneous treatment	Exacerbated mice infection (increased lung fungal burden, lung macroscopically and microscopically alterations) and production of pro-inflammatory response cytokines (TNF-α, IFN-γ) and chemokines (MCP-1).
2018, Ikeda et al.	*Sporothrix brasiliensis*	*In vivo* , Balb/c mice infection by the subctuneous route after EV subcutaneous treatment	Increased fungal burden and diameter skin lesion. Higher production of IL1-β and TNF-α in the skin lesion.
2018, Ikeda et al.	*Sporothrix brasiliensis*	*In vitro* , Bone marrow-derived macrophage and dendritic cells of Balb/c mice	Increased phagocytic index and fungal burden. Production of IL-12p40, TNF-α and IFN-γ.
2021, Campos et al.	*Sporothrix brasiliensis*	*In vitro* , J774A.1 murine macrophage lineage	Enhanced phagocytosis and killing rates. Production of IL-12 and IL-6 and higher expression of MHC II and CD86.
2021, Yang et al.	*Talaromyces marneffei*	*In vitro* , RAW 264.7 murine macrophage cell line	Production of inflammatory mediators in macrophages (NO, ROS, IL-6, IL-10, IL1-β and TNF-α) and increased expression of CD80, CD86 and MHC II.
2018, Bitencourt et al.	*Trichophyton interdigitale*	*In vitro,* Bone marrow-derived macrophages of C57BL/6 wild-type, TLR2 Knockout, and TLR4 Knockout mice	Production of proinflammatory mediators (nitric oxide, TNF-α, IL-6, and IL-1β) , increased expression of M1 polarization marker (Inducible nitric oxide synthase mRNA), enhanced phagocytosis and killing rates, suggested dependency of TLR2.
2023, Freitas et al.	*Aspergillus fumigatus*	*In vitro* , Human neutrophils of healthy volunteers.	Increased NETosis or the release of NETs was not evident when neutrophils were stimulated with Evs.
2023, Freitas et al.	*Aspergillus fumigatus*	*In vitro* , Human peripheral blood mononuclear cells of healthy volunteers.	EVs induced the release of IL-6 when stimulated with 10^10 EVs/mL for 24 h, but EVs did not induced TNF-α or IL-10 production.
2023, Freitas et al.	*Aspergillus fumigatus*	*In vitro* , AMJ2-C11 and RAW 264.7 murine macrophage cell lines	RAW 264.7 showed increased TNF-α production, reduced superoxide production, Lower Arginase-1 and Higher iNOS Transcription, and higher Adhesion Molecule Gene Expression (CD11b and CD18).
2018, Bielska et al.	*Cryptococcus gatti*	*In vitro,* murine macrophage cell line J774A.1	Pre-treating macrophages with Evs of virulent strain of *C. gatti* before infection with yeasts increasead the fungi intracellular replication in a dose-dependent manner.
2022, Honorato et al.	*Candida albicans*	*In vivo* , *Galleria mellonella* larvae	Infection with EVs-treated yeasts resulted in reduced mortality of larvae in comparison with non-treated yeasts.
2015, Wolf et al.	*Candida albicans*	*In vitro,* murine macrophage cell line J774A.1 and bone marrow-derived macrophage of Balb/c mice	Increased NF-κB activation of cells treated with EVs from wild-type yeasts. Mutant for phospholipid biosynthesis showed decreased NF-κB activation.
2023, Wei et al.	*Candida albicans*	*In vitro,* RAW 264.7 murine macrophage, human oral keratinocytes (HOK), human squamous carcinoma cells (TR146), and human gingival epithelial cells (HGEC)	Cells cultured with yeasts and EVs had increased cell damages in Lactate dehydrogenase cytotoxicity assay.
2022, Castelli et al.	*Cryptococcus deuterogatti*	*In vivo* , *Galleria mellonella* larvae	Infection with atenuated mutant yeasts and EVs from wild-type yeasts resulted in higher mortality rates of larvae in comparison with mutant yeasts alone or mutant yeasts with their own EVs.
2023, Oliveira et al.	*Candida haemulonii* var. *vulnera*	*In vitro* , RAW 264.7 murine macrophage cell line	RAW 264.7 showed increased ROS production, NOX-2 Expression and H2O2 Levels.
2022, Souza et al.	*Aspergillus fumigatus*	*In vivo,* C57BL/6 male mice infection by the intranasally route after EVs intranasally treatment	Decrease of inflammatory cells in bronchoalveolar lavage. Decreased production of IL-1β and IL-6. Increased production of specific igG against EVs. Increased phagocytic index of cells from bronchoalveolar lavage. Reduced fungal burden and tissue damage of lungs. Increased survival rate when immunization were associated with amphotericin treatment.
2022, Kulig et al.	*Candida glabrata, Candida parapsilosis,* and *Candida tropicalis*	*In vitro* , human monocytic cell line THP-1 differentiated into macrophage-like cells	Increased TNF-α and IL-8 production and reduced IL-10 production.
2022, Las-Casas et al.	*Fonsecaea pedrosoi and Fonsecaea nubica*	*In vitro,* Bone marrow-derived macrophages of C57BL/6 mice	Increased TNF-α, IL-1β and IL-10 production.

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