Review

Fungal Extracellular Vesicles: Isolation, Characterization, and the Immune System Response

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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 fungal EVs, their cargo, how they are obtained, and their role during the infectious process, which is extremely important for understanding this neglected mycosis.

Keywords: extracellular vesicles; immune response; mycosis

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1. Introduction

1.1. Extracellular Vesicles (EVs)

In 1967, Peter Wolf wrote what many consider today to be 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". This material is referred to as "platelet-dust" [1]. 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 [2].

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, in 1973, vesicles ranging from 50 to 150 nm and larger multivesicular bodies, suggesting their role in mediating the exportation of intracellular content towards the extracellular space through the membrane [3]. After this discovery, interest in extracellular vesicles (EVs) seemed to decline in the following years, until the 1900s, when EVs were proven to be biologically functional and there was an exponential growth in interest in the field [4].

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 [5]. 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 directly shed from the plasma membrane. Apoptotic bodies are larger still and are released during apoptosis [6].

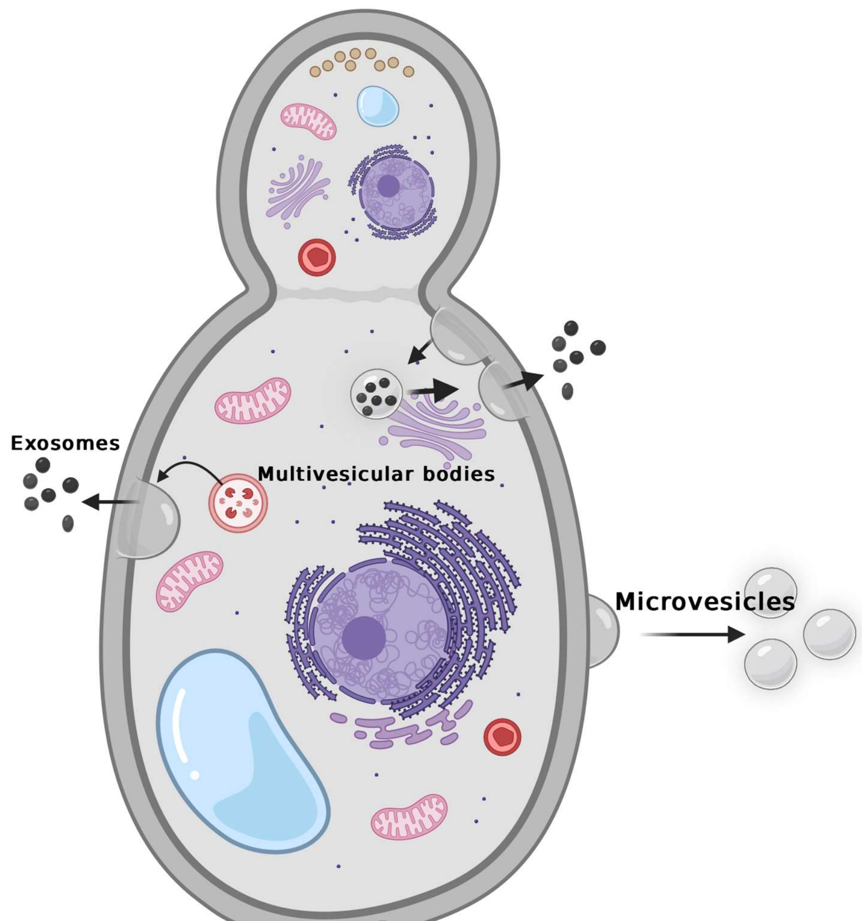

Figure 1. Extracellular vesicles in yeast cells. The figure shows the microvesicles and exosome formation. Created with BioRender.com.

1.2. Fungal EVs

Due to their ability to carry bioactive molecules, EVs have gained significant attention in biomedical research. However, it was in 2007, after more than thirty years of EVs discovery, that the first work where fungal EVs were successfully isolated was published by Rodrigues and colleagues describing the EVs from the fungus *Cryptococcus neoformans* as responsible for transporting glucuronoxylomannan through the cell wall [7]. After Rodrigues' publication, 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 their 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. 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 into growth media. *Saccharomyces cerevisiae*, *Candida albicans*, *Candida parapsilosis*, and *Sporothrix schenckii*

secreted vesicles around 100 nm, similar to those produced by *Histoplasma capsulatum* and *Cryptococcus neoformans* [8].

Other studies investigating EVs from *Cryptococcus neoformans* and *Cryptococcus gatti* found more virulence factors within their vesicles [9,10]. In 2011, two reports confirmed the production of EVs by *Malassezia sympodialis* [11], along with the description of EVs released from the pathogenic fungus *Paracoccidioides brasiliensis* [12]. In 2018, Ikeda et al. isolated EVs from the pathogenic fungus *Sporothrix brasiliensis*, responsible for the epidemy of zoonotic sporotrichosis [13]. In the upcoming years, EVs were isolated from other pathogenic genera such as *Aspergillus* [14,15], *Pichia* [16], *Rhizopus* [17], *Trichophyton* [18], *Exophiala* [19] and *Fonsecaea* [20]. Also, EVs were found in some phytopathogens such as *Alternaria infectoria* [21], *Fusarium oxysporum* sp. *vasinfectum* [22], *Trichoderma reesei* [23], *Penicillium digitatum* [24], and *Colletotrichum higginsianum* [25].

A major question regarding fungal EVs is their ability to cross the complex cell wall present in these microorganisms, composed of cross-linked glycoproteins, glucans and chitins, in addition to other species-specific components. It is hypothesized that EVs carry enzymes capable of temporarily modeling cell walls, which would demonstrate an efficient vesicular transport mechanism [26]. Also, Rodrigues and colleagues proposed a third route of release of fungal EVs by cytoplasmic subtraction originating from membrane reshaping with consequent plasma membrane invaginations and formation of an isolated membranous compartment located in the periplasmic space [27].

2. Fungal EVs' Isolation and Analysis

2.1. EVs' Isolation

The first description of fungal EVs being isolated was made by Rodrigues and colleagues in 2007 [7], 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 $4000\times g$ to remove the heavy portion of cells and at $15{,}000\times g$ to remove most of the apoptotic bodies, debris, and molecules with higher density than the EVs. The pellet was discarded and the supernatant was concentrated with an Amicon® ultrafiltration system of 100 kDa cutoff (Merck KGaA, Darmstadt, Germany). They were finally ultracentrifuged at $100{,}000\times g$ for 1–2 h, repeating this step five times to wash the pellet. Such a 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 1×10^8 to 10^{11} particles/mL could be easily obtained [13,18,23]. 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 [28,29].

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 [30,31]. 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 [32–34]. 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 150 nm, affecting the success of the physical separation by gravitational force and generating a pellet that will generally contain a pool of exosomes, microvesicles and other nonexosomal particles [34,35]. 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 EV 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 [31,36,37]. 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 is obtained [34].

Similarly, in studies regarding fungal EVs, specific ultracentrifugation tubes (Amicon®, Vivapsin®, Merck KGaA, Darmstadt, Germany) are often employed, which consists of a centrifuge tube varying in size (2 or 15 mL) accoupled with filters with different molecular weight cutoffs (MWCO) ranging from 3–100 kDa. After simple centrifugation at 6000× *g*, the sample is concentrated 20-fold and collected at the bottom along with any particle that has a size smaller than the chosen MWCO [12,38]. Since EV isolation from fungal cultures usually requires large volumes of liquid media [10,14,19], this procedure drastically reduces the number of ultracentrifugation steps needed and yields a higher-purity sample.

In 2019, Reis and colleagues [39] 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. Then, 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 carried out 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 [40,41], Magnetic bead separation [28], Immunoaffinity-based capture [42], Size-exclusion chromatography (SEC), and ExoQuick precipitation agent, these approaches are most seen used in the extraction of EV's from human samples [43–45]. Furthermore, these methods are more efficient with the use of EVs' protein markers that are only available for mammalians cells. One study by Dawson and colleagues evaluated two putative targets for *C. albicans* that may be used as a specific marker for this fungi species [46].

In the case of fungal EVs, 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.

2.2. Fungal EVs' Characterization

The characteristics of EVs of a small-sized heterogeneous population, with a low refractive index, and the possibility of aggregation of proteins and glycoproteins of similar size with EVs do not allow for the use of more conventional analysis techniques. In the study of EVs, the most used single-particle analysis techniques are nanoparticle-tracking analysis (NTA), electron microscopy (EM), atomic force microscopy, high-resolution microscopy, resistive pulse sensing, high-resolution flow cytometry, and Raman spectroscopy [47]. In mycology, works mainly use NTA and EM techniques for EVs quantification and size measurement. Most studies show that fungal EVs vary in size between 30 nm and 600 nm [37], but there are different fungal growth conditions, EV isolation technique and EV analysis techniques, not allowing for an accurate comparison between the studies.

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 for the transfer of their cargo and subsequent modulation of cellular processes [26,48].

Fungal EVs can carry raw material for the growth and cell wall remodeling of some types of fungi, some of which act as virulence factors. The *C. neoformans* EVs can carry various immunomodulating molecules such as glucuronoxylomannan (GMX), which is related [7], a component of the cryptococcal capsule, and melanin [49]. In *P. brasiliensis*, EVs carry highly immunogenic α-Gal epitopes [12]. *E. dermatitis* EVs contain melanin [19].

Several virulence-related carbohydrates, proteins, and lipids were found in EVs from *A. fumigatus* [50], *C. albicans* [51–53], *C. auris* [54], *C. neoformans* [9,55], *H. capsulatum* [8,56,57], *P. brasiliensis* [58–60], *S. brasiliensis* and *S. schenckii* [13].

Also, fungal EVs carry functional RNA that can affect the physiology of host cells [61] as described for *C. albicans*, *C. neoformans*, and *P. brasiliensis* [61–63], *C. gattii* [39], *C. auris* [54,64], *H. capsulatum* [65], *P. lutzii* [63] and *M. sympodialis* [66].

By Western blot, the sera of infected animals or patients were able to react with components from EVs of *A. fumigatus* [14], *C. albicans* [51], *C. neoformans* [9], *H. capsulatum* [8], *M. sympodialis* [11], *P. brasiliensis* [12], *S. brasiliensis* [13] and *S. schenckii* [67], demonstrating the capacity of EVs to interact with the immune system.

Figure 2 sums up the isolation and analysis of fungal EVs.

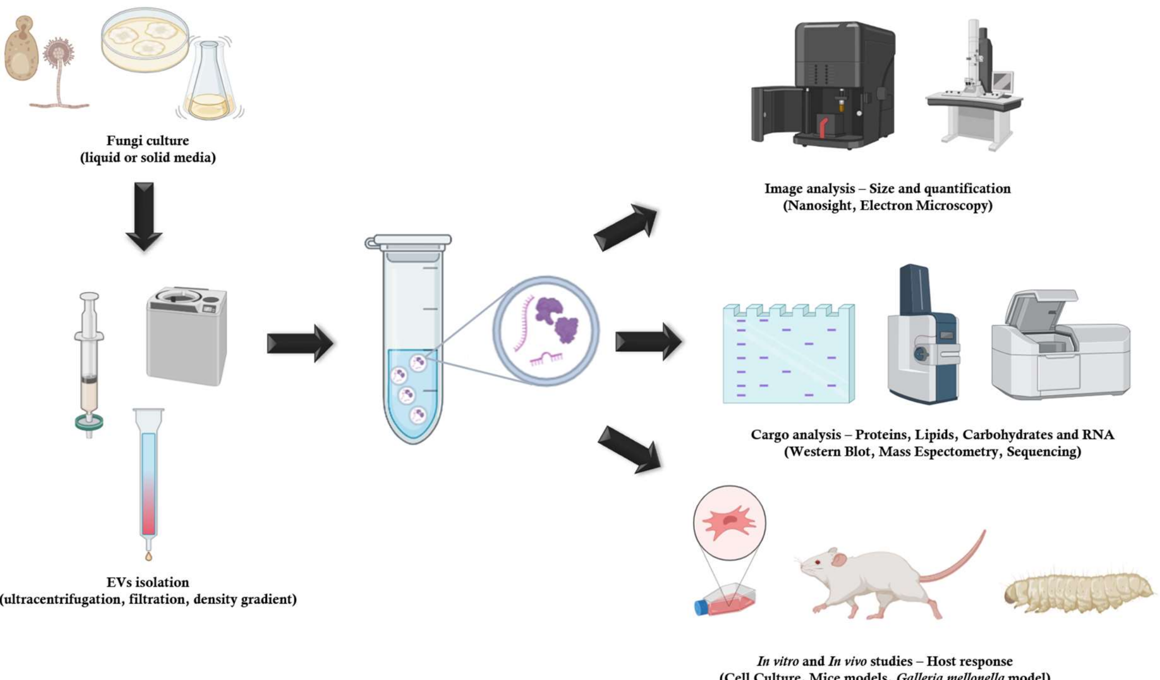

Figure 2. EVs—Fungi culture isolates EVs using several methods, such as ultracentrifugation, filtration, or density gradient. Then, the EVs are analyzed and can be studied in vitro or in vivo. Created with BioRender.com.

3. Impacts of Fungal EVs on Host Immunity

Since the discovery of EVs, several studies have demonstrated the interaction of EVs produced by microorganisms with the host cells [68]. In mycology, we have works (Table 1) demonstrating the ability of fungal EVs to interact with the host immune system as seen in *A. flavus*, *A. fumigatus*, *C. albicans*, *C. auris*, *C. haemulonii* var. *vulnera*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. deuterogatti*, *C. gatti*, *C. neoformans*, *F. pedrosoi*, *F. nubica*, *H. capsulatum*, *M. sympodialis*, *P. brasiliensis*, *S. brasiliensis*, *T. marneffei* and *T. interdigitale*.

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

Fungi	Model		EVs Effects
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 [15].
	In vivo	*Galleria mellonella* larvae infection after EV treatment	Decrease in larvae fungal burden and enhanced survival of the larvae [15].
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) [14].
		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) [69].
		Human neutrophils of healthy volunteers	Increased NETosis or the release of NETs was not evident when neutrophils were stimulated with EVs [69].
		Human peripheral blood mononuclear cells of healthy volunteers	EVs induced the release of IL-6 when stimulated with 1 × 1010EVs/mL for 24 h, but EVs did not induce TNF-α or IL-10 production [69].
	In vivo	C57BL/6 male mice infection by the intranasal route after EVs intranasally treatment	Decrease in 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 and increased survival rate when immunization was associated with amphotericin treatment [70].
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 [52].
		Bone-marrow-derived dendritic cells of Balb/C female mice	Production of IL-6 [71].
		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 [53].
		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 damage in Lactate dehydrogenase cytotoxicity assay [72].
		Bone-marrow-derived dendritic cells of C57BL/6 mice and RAW 264.7 murine macrophage cell line	Reduced killing rate of macrophages. Higher expression of MHCII, CD80, and CD86. Production of IL-6 and TGF-β on dendritic cells [73].
	In vivo	*Galleria mellonella* larvae	EV treatment before infection reduced the mortality of the infected larvae [52,71] and reduced the fungal burden [52]. Infection with EVs-treated yeasts resulted in reduced mortality of larvae in comparison with non-treated yeasts [74].
		Balb/C female immunosuppressed mice infection by intraperitoneal 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 [71].
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. Higher expression of MHCII, CD80, and CD86. Production of IL-6 and TGF-β on dendritic cells [73].
Candida glabrata *Candida parapsilosis* *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 [75].
Candida haemulonii var. *vulnera*	In vitro	RAW 264.7 murine macrophage cell line	RAW 264.7 showed increased ROS production, NOX-2 Expression, and H_2O_2 Levels [76].
Cryptococcus deuterogatti	In vivo	*Galleria mellonella* larvae	Infection with attenuated 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 EVs [77].
Cryptococcus gatti	In vitro	Murine macrophage cell line J774A.1	Pre-treating macrophages with EVs of a virulent strain of *C. gatti* before infection with yeasts increased the fungi intracellular replication in a dose-dependent manner [10].

Table 1. *Cont.*

Fungi		Model	EVs Effects
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 [78].
		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 [79].
	In vivo	*Galleria mellonella* larvae infection after EV treatment	Increased mortality of the infected larvae [80].
		C57BL/6 male mice infection by hematogenous route associated with EV treatment	Increased fungal burden in the brain [81].
		C57BL/6 mice infection by the intratracheal route followed by intranasal EVs treatment	Lower fungal load is 5 days post-infection and higher is 15 days post infection. Reductions of IL-1β and TNF-α. Downregulations of inflammasome genes [79].
		Balb/C female mice infection by the intranasal route after EV intraperitoneal injection	Production of antibodies and increased survival time [50].
Fonsecaea pedrosoi *Fonsecaea nubica*	In vitro	Bone-marrow-derived macrophages of C57BL/6 mice	Increased TNF-α, IL-1β and IL-10 production [20].
Histoplasma capsulatum	In vitro	Bone-marrow-derived macrophage of Balb/c mice	Inhibition of phagocytosis rate and decreased killing rate [82].
Malassezia sympodialis	In vitro	Human peripheral blood mononuclear cells depleted of CD34+ cells and CD14+ monocytes	Induced production of IL-4 and TNF-α [11].
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 [38].
		Monocyte-derived CD11c+ Cells of C57BL/6 mice	Downregulation of transcription factors (Gabpb2, Pknox1, and Zfp575) that regulates IL-10 and IL-7 expression [62].
		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) [83].
	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 [84].
		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) [83].
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 [85].
		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 [13].
	In vivo	Balb/c mice infection by the subcutaneous route after EV subcutaneous treatment	Increased fungal burden and diameter skin lesion. Higher production of IL1-β and TNF-α in the skin lesion [13].
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 [86].
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 [18].

3.1. In Vitro Assays

A variety of in vitro assays show the immunomodulatory effects of fungal EVs. Neutrophils are the first line of immune defense recruited to the tissue against some fungal pathogens [87]. In *A. fumigatus*, the interaction of mouse bone-marrow-derived neutrophils with EVs allowed for an increase in the phagocytic index and reduction in fungal burden in the fungal challenge, associated with an increase in the production of TNF-α and IL-1β cytokines [14]. 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 [69].

Macrophages are another cell that plays a vital role in controlling fungal infection [88]. Fungi EVs were able to modulate these cells, increasing the fungicidal capacity and/or production of inflammatory mediators as observed in *A. flavus* [15], *A. fumigatus* [14,19], *C. albicans* [73] *C. neoformans* [78], *P. brasiliensis* [38], *S. brasiliensis* [85] and *Trichophyton interdigitale* [18]. In contrast, in *H. capsulatum* [82] and one strain of *C. auris* [73], 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 [88]. In *C. albicans* [52,71,73] and *C. auris* [73], EVs were able to activate dendritic cells, increasing the production of cytokines and expression of surface markers. In *S. brasiliensis* [13], dendritic cells were stimulated with EVs and challenged with yeasts, resulting in an increased phagocytic index but an 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 [62], EVs downregulated Pknox1 and Gbpb2 transcription factor, which regulates IL-7 and IL-10 production.

In vitro assays are important for a better understanding of the impact of EVs on immunological pathways. However, specific cells obtained from different sources are used and are therefore only a small part of the immune system. Therefore, in vivo assays are important to generally evaluate the host's response.

3.2. In Vivo Assays: Galleria mellonella Model

Galleria mellonella larvae are used as in vivo model that allows for a preliminary evaluation of potential candidates for immunotherapy. The insect has immunological mechanisms similar to the innate immune response of mammals, complementing in vitro assays with cells and reducing the use of animals [89]. Some studies have demonstrated the ability to reduce mortality in the insect *G. mellonella* infection model with previous stimulation with EVs, as seen in *A. flavus* [15], *A. fumigatus* [69] and *C. albicans* [52,71]. Except for *C. neoformans* and *C. deuterogatti*, EVs exacerbated the infection [77,80].

3.3. In Vivo Assays: Animal Model

Some studies looked for EVs' effects in animal models. In the opportunistic fungus *C. albicans*, Vargas and colleagues [71] performed a mouse 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 in inflammation and with a protective role in candidiasis (IFN-γ, IL-4, IL-6, IL-10, IL-12p70, TGF-β, and TNF-α).

For the ubiquitous fungus *A. fumigatus*, which can cause severe pulmonary infection in immunocompromised individuals, the immunization of C57BL mice with fungal EVs before infection with *A. fumigatus* conidia resulted in decreased inflammatory cells' infiltrate in lungs, mainly of neutrophils, reduced the 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 [70], immunized animals did not alter survival rates, but the EVs' immunization in association with amphotericin treatment showed an increased survival of the animals.

In opposition, for *Sporothrix brasiliensis*, subcutaneous immunization 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 [13]. *S. brasiliensis* is notably pathogenic, with strains causing invasive mycosis even in immunocompetent hosts. The immune response to EVs may be different from opportunistic fungi that have a different pathogenesis. Furthermore, some proteins present in the EVs, as well as virulence factors, must be different, depending on the fungus.

In the endemic dimorphic fungi *P. brasiliensis*, two studies in mice using EVs were conducted. Baltazar and colleagues performed an immunization scheme with two applications of EVs subcutaneously, followed by an intratracheal infection with the fungus in C57BL/6 mice [84]. 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 in the inflammatory mediators TNF-α, IFN-γ, and MCP-1 were observed. These are higher with EVs from attenuated strain [83]. The discrepancies in the results obtained in the two studies show how the biogenesis and effects of fungal EVs are impacted by the experimental conditions, as there was a difference in the fungi culture, EVs' isolation protocol, animal strain, and number of immunizations.

In opportunistic fungus *C. neoformans*, immunization with peritoneal injection of EVs obtained from both wild-type strains and mutants without capsules, with subsequent intranasal infection, allowed for a longer survival time of the Balb/C mice accompanied by an increase in the production of antibodies [90]. On the other hand, Huang and colleagues, in a hematogenic disseminated infection model in C57LB/6 mice, showed an increase in 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 [81]. The opposite results may have occurred due to the different route of inoculation of the fungus. *C. neoformans* is known to cause infection in the central nervous system, and therefore the hematogenous route may have facilitated the pathogenesis of the fungus.

The immune response is dynamic and complex; we can observe that each fungal species has its particularities of virulence factors and interaction with immune system cells. Therefore, it is important that studies demonstrate detailed methodologies, as slight differences in the EV isolation method, cell or animal lineage and infection/immunization protocol can lead to discrepant results. Figure 3 shows a summary of the EVs and immune responses studied until now.

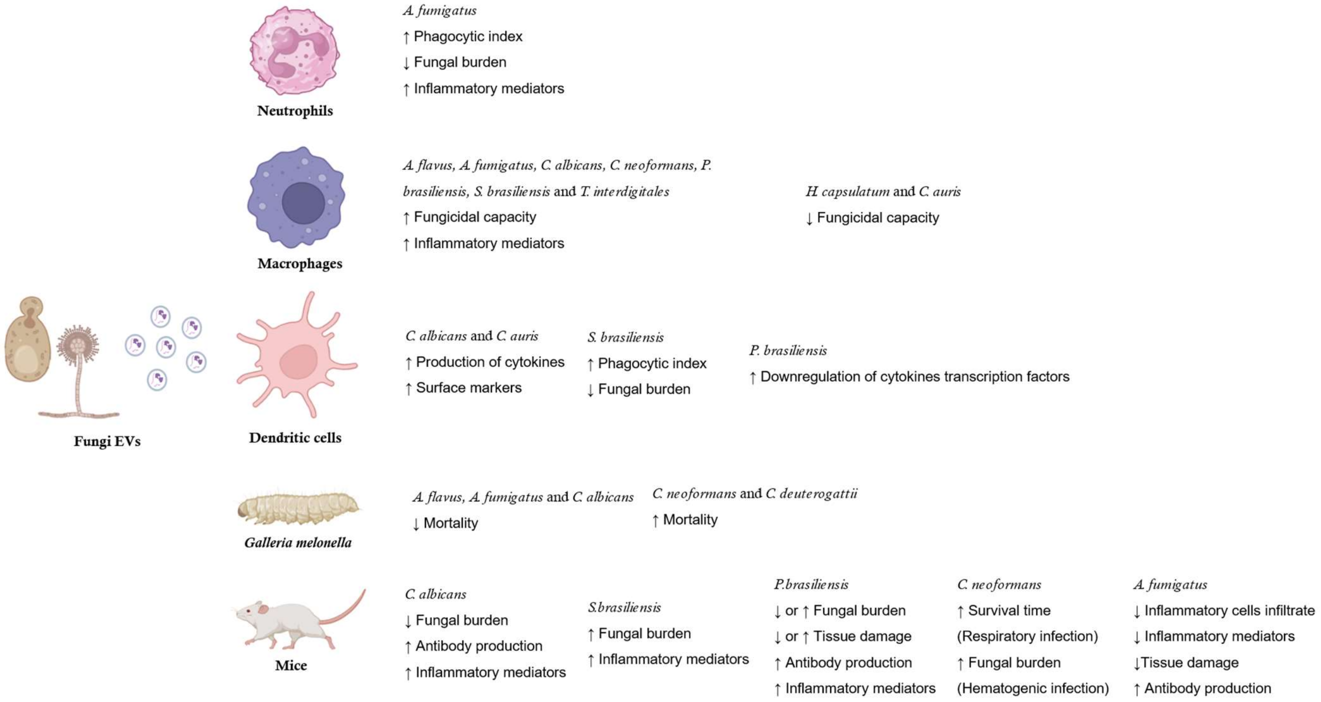

Figure 3. Fungal EVs' plasticity in cells, mice, and *Galleria*. Fungal EVs modulate the immune response in different models of infection. Created with BioRender.com.

3.4. Fungal EVs as Vaccines?

Most mycoses are considered neglected diseases with few therapeutic options available; so immunotherapy, is an option to reduce the occurrence of these emerging threats [91]. The EVs released by fungi contain a range of immunogenic molecules that can serve as a delivery tool, such as vaccines [92]. Reis and colleagues isolated a peptide from EVs of *Cryptococcus gattii* and improved the survival of *G. mellonella* lethally infected with *C. gattii* or *C. neoformans* [31].

The use of adjuvants in combination with the application of EVs can induce a more robust immune system response. Vargas and colleagues showed in the *C. albicans* model that the combination of EVs with Freund's adjuvant, when compared to the use of EVs alone, allowed for 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 [71]. In addition, an oil-based adjuvant was evaluated in *P. brasiliensis* [84], where EVs' immunization associated with Montanide adjuvant promoted the induction of higher levels of IgM and IgG compared to the group without adjuvant. In addition, on an 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 showed that EVs stored at different temperatures kept their ability to stimulate IL-6 production in dendritic cells and decreased 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 led to the highest survival rates in the *G. mellonella* model [71].

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 for an in vitro 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 in fungal burden in the brain and lungs but with a decrease in the survival rate of animals [93]. In other work [94], 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.

EVs can be used as diagnostic biomarkers, therapeutic delivery vehicles, and targets for therapeutic intervention [5]. The exact mechanisms through which fungal EVs promote changes in the immunity-related pathway are still uncertain; more studies are necessary to elucidate the composition and immunomodulatory effects and improve the development of new immunotherapies for fungal infection.

3.5. Modulation of Fungal EVs

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 protective and nonprotective monoclonal antibodies have a larger size and an increased protein cargo, with important changes in metabolic pathways [56]. Also, the treated yeasts' EVs were able to have a more significant inhibition activity on the phagocytosis and killing rates in bone-marrow-derived macrophages [82]. Although it has already been shown in vivo that the treatment with monoclonal antibodies had a protective effect, these antibodies modified the pathobiology of the fungi, and therefore, had an impact on the response of the host's immune system.

The nutrition conditions of fungi can alter the cargo and effects of isolated EVs. Cleare and colleagues showed that EVs from *H. capsulatum* cultivated in a rich medium had more protein content and altered protein expression than cultures in a less nutritional medium [57]. In *C. neoformans* [79], EVs from fungi cultivated in a rich medium induced a lower 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 in the fungal burden of lungs after five days of illness but an increase in fungal burden 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 compositions that impact host cells' interaction. Oliveira and colleagues compared wild-type strains of *C. neoformans* with an acapsular mutant, and it was observed that macrophages stimulated with EVs obtained from the mutant strain had a greater activation of the macrophage response with an increased production of nitric oxide, phagocytosis capacity and fungicidal activity [78]. The same results were obtained in vivo [90] where immunization with EVs from acapsular mutant prolonged survival in a mice infection model.

Also, Colombo et al., demonstrated that a mutant strain of *C. neoformans* that releases EVs enrichened in GXM and sterylglucosides had a protective effect in the *G. mellonella* infection model [80]. In *C. albicans* [53]), EVs obtained from five mutant strains for phospholipid biosynthesis demonstrated different sizes and protein cargo compared to the wild-type strain, and one mutant had a lower activation of NF-κB signaling pathway in bone-marrow-derived macrophage cells. These results show the potential role of using EVs obtained from mutants of important pathways in fungal pathogenesis.

The interactions between EVs from fungal strains with different degrees of virulence can lead to changes in the expression profile of the fungus, impacting the host's response, as shown by Honorato et al., who demonstrated that the EVs from *C. albicans* in interaction with yeast cells inhibited biofilm development and affected yeast-to-hypha differentiation, leading to reduced death of *G. mellonella* larvae infected with EV-treated yeasts [74]. Bitencourt and colleagues demonstrated in different fungal species the ability of fungal EVs to perform gene regulation in fungi of the same species [64]. In *A. fumigatus* and *P. brasiliensis*, EVs were isolated from cultures subjected to treatments that increased the expression of genes related to the stress response. Fungi of the same species not 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* [83] showed that yeasts from attenuated strains after incubation with EVs isolated from the virulent strain changed to an antioxidant gene expression.

With these works, we can observe that changing the fungal strain or EV isolation conditions can be an interesting alternative for modulating the content of fungal EVs, which can lead to promising beneficial responses for the host's immune system.

4. Challenges and Perspectives of Fungal EVs

The study of fungal EVs has advanced a lot in recent years, but several challenges are faced in this process. Regarding the isolation of EVs, despite studies using similar isolation techniques, there is a large variation in the fungal growth conditions, which can cause variations in the size and molecular/biochemical composition of the EVs and consequently have a different activation of the host's immune response.

Concerning the functional capacity of EVs, there are a multitude of options for in vitro and in vivo models, each of which presents particular characteristics. Several studies are necessary to determine the real effect of EVs from each fungus on the immune response. With the advancement of laboratory techniques, more and more information can be obtained about the composition and function of fungal EVs. Furthermore, new bioengineering technologies will make it possible to explore the modulation of EVs and obtain new therapeutic targets and diagnostic markers.

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