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REVIEW

Various brain-eating amoebae: the protozoa, the pathogenesis, and the disease

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Abstract **Among various genera of free-living amoebae prevalent in nature, some members are identified as causative agents of human encephalitis, in which *Naegleria fowleri* followed by *Acanthamoeba* spp. and *Balamuthia mandrillaris* have been successively discovered. As the three dominant genera responsible for infections, *Acanthamoeba* and *Balamuthia* work as opportunistic pathogens of granulomatous amoebic encephalitis in immunocompetent and immunocompromised individuals, whereas *Naegleria* induces primary amoebic meningoencephalitis mostly in healthy children and young adults as a more violent and deadly disease. Due to the lack of typical symptoms and laboratory findings, all these amoebic encephalitic diseases are difficult to diagnose. Considering that subsequent therapies are also affected, all these brain infections cause significant mortality worldwide, with more than 90% of the cases being fatal. Along with global warming and population explosion, expanding areas of human and amoebae activity in some regions lead to increased contact, resulting in more serious infections and drawing increased public attention. In this review, we summarize the present information of these pathogenic free-living amoebae, including their phylogeny, classification, biology, and ecology. The mechanisms of pathogenesis, immunology, pathophysiology, clinical manifestations, epidemiology, diagnosis, and therapies are also discussed.**

Keywords free-living amoebae; central nervous system infection; primary amoebic meningoencephalitis; granulomatous amoebic encephalitis

Introduction

As an easily neglected source of infection, free-living protists exist worldwide and potentially cause infections in humans and other animals, leading to serious clinical problems [1]. Their infection has low morbidity but is usually characterized by a relatively high mortality rate, thereby becoming a huge challenge for efficient diagnosis and therapy [2]. Among these pathogenic and opportunistic protozoa, free-living amoebae (FLA) are a conspicuous group with a pattern of primary existence as free-living organisms in nature. They occasionally invade a host and live as parasites within the host tissue, which explains why they are also called amphizoic amoebae [3]. FLA can cause localized systemic diseases and disseminated infections. The skin, eyes, lungs, kidneys, and sinuses are all predilection sites. However, the most misleading and almost always fatal infections caused by FLA are those "brain-eating" ones of the central nervous system (CNS) occurring in immunocompetent and immunocompromised individuals, such as patients with AIDS [4].

From the numerous genera of FLA in nature, some are extensively known to cause CNS diseases in humans and animals. Among them are several species of *Acanthamoeba* (such as *Acanthamoeba castellanii* and *Acanthamoeba culberstoni*) and only one species of three genera, namely, *Balamuthia* (*Balamuthia mandrillaris*), *Naegleria* (*Naegleria fowleri*) and *Sappinia* (*Sappinia pedata*). The former three genera are also the dominant FLA pathogens for humans and animals [3,5]. Known as a "brain-eating" amoeba, *N. fowleri* is the causative agent for the invasive and fulminating fatal form of meningoencephalitis called primary amoebic meningoencephalitis (PAM), which primarily occurs in healthy children and young adults [6]. By contrast, *Acanthamoeba* and *B. mandrillaris* are responsible for a chronic infection in immunocompetent and immunocompromised hosts known as granulomatous amoebic encephalitis (GAE). *S. pedata* has been identified

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in only one case of an immunocompetent male in Texas, USA, causing a unique GAE encephalitis with a favorable clinical outcome. The causative agent of this amoeba-associated encephalitis case was originally identified as *Sappinia diploidea*, whereas an affiliation to *Sappinia pedata* has later been proven through molecular biology [7,8]. Some recently described FLA species have also been discovered to invade humans and other animals occasionally. Among them, *Paravahlkampfia francinae* is the only species in its genus known to cause infection in the CNS. The species was isolated from the cerebrospinal fluid (CSF) of a patient with PAM-like symptoms, which is usually caused by *N. fowleri* [9].

Phylogeny and classification

Amoebae were first described shortly after the invention of the microscope. Since then, increasing numbers of amoebae have been named, described, and classified [10]. Nevertheless, before the innovation of molecular phylogeny, a great variety of FLA were generally described and sorted in the Rhizopoda clade of the Sarcodina supergroup in the original classification [11]. Morphological traits such as pseudopodium type, cell shape at different life stages, and amoeboid movements were extensively used for the subdivided FLA classification at that time [12]. In recent years, molecular evidence and an associated re-evaluation of morphology have enabled a more molecular-based and a more validated morphological-based classification on the relationships among higher-level groups of amoebae, leading to the revision and improvement in FLA classification [13,14]. Given the much richer databases of 18S rRNA gene sequences, they have become the most extensively used marker gene for the single-gene phylogeny investigation of FLA. Moreover, the burgeoning technology of mitochondrial genome sequencing has been implemented in a series of studies and shown to serve as a potent and scientific source of information for an evolutionary and phylogenetic classification. However, available data remain insufficient to this day, with only several dozens of mitochondrial genomes for FLA [15,16]. As a result, FLA have been classified into four clades in the eukaryotic tree of life, focusing primarily on Amoebozoa, Excavata, and to a lesser extent, Rhizaria and Opisthokonta [14,17].

Among the four clades of amoeba, Amoebozoa is the most studied and diverse clade with a gross estimate of about 17 000 species [18]. Along with the systematic implementation of high-throughput sequencing technology, a reconstructed phylogenetic classification enables a more detailed overview of Amoebozoa supergroup diversity and taxonomic relationships [19,20]. *Acanthamoeba* spp. and *B. mandrillaris*, as single-celled, flattened naked amoebae causing CNS diseases, belong to Amoebozoa in the line of Order Acanthopodida, Class Centramoebia, Phylum Discosea, Amoebozoa clade in the Amorphea domain of eukaryotic organisms. Among Excavata, Class Heterolobosea encompasses approximately 140 described species [21]. Compared with Amoebozoa, molecular approaches that contribute to confirm a detailed taxonomy within the clade remain lacking for these Heterolobosea groups. Heterolobosea are widely known mostly because of the representative *N. fowleri* with the line of Family Vahlkampfiidae, Order Schizopyrenida, Class Heterolobosea, Phylum Percolozoa, Excavata clade in the Amorphea domain of eukaryotic organisms [22]. Molecular and morphological evidence also systematically and extensively supports the diversity of other FLA's phylogenetic classification. There are two remaining genera that cause CNS infection, *S. pedata* (Order Thecamoebida, Class Flabellini*a*, Phylum Discosea) and *P. francinae* (Family Vahlkampfiidae, similar to the orientation of *N. fowleri*).

Lacking distinctive morphological or behavioral characters, FLA such as filose and reticulose cercozoans are found in the Rhizaria clade with a scattered distribution in different phylogenetic groups. A clear estimate or reliable formulation of FLA diversity affiliated with Rhizaria remains lacking [23,24]. According to the revisions of classification and diversity in 2019, FLA in Opisthokonta are incorporated in two well-supported clades named Holozoa and Nucletmycea [14,25].

Biology and ecology

Most FLA such as the above-mentioned *Acanthamoeba* spp., *B. mandrillaris*, and *S. pedata* have two developmental stages: the trophozoite as a nutrition feeding form and the cyst as a resting form. With a general size of some dozens of microns, the trophozoite is an infective stage engaged in amoeboid locomotion, whereas a cyst is a dormant stage against the harsh environment with a smaller size of about more than 10 μm [26]. Some amoebae like *Naegleria* spp. have an additional flagellate stage; the trophozoite transforms into this temporary motile stage when a nutritional deficiency occurs in the environment, but water is present [27]. With a length ranging within 10–16 μm, *N. fowleri*'s flagellate stage is usually pyriform. They neither divide nor feed, generally reverting to trophozoites within an hour or less. As a metabolically active stage, the trophozoite feeds primarily on Gram-positive and Gram-negative bacteria, as well as on algae, fungi, and other protozoa [28]. Sometimes they even feed on relatively large organisms, such as nematodes and planktonic rotifers in aquatic systems [29,30]. The trophozoite multiplies by binary fission and sometimes actively and constantly changes its size and shape [26]. In most FLA such as *Acanthamoeba* and *Naegleria*, cysts generally have two layers: the ectocyst and the endocyst. The ectocyst forms during the initial stage of encystment

and appears as an amorphous and discontinuous layer. Although the endocyst varies in shape such as polygonal or spherical, it has a tiny granular texture with a more consistent and thicker structure than the former [31,32]. Flush pores have been found in the cyst wall of *N. fowleri*. Some species like *B. mandrillaris* even have a third layer between the outer and inner ones as the mesocyst in an amorphous shape. These structures may contribute to the resistance to biocides contained in contact lenses and bronchoscope disinfectants, as well as chlorination and sterilization agents of domestic water systems and hospital water-treatment networks [33–36]. Encystment occurs under the conditions of imbalanced pH, unsuitable osmotic pressure, or inadaptable temperature; it also occurs under nutritional deficiency and in the presence of antiamoeba agents [37]. By contrast, FLA excyst when environmental conditions become favorable again.

FLA are omnipresent in soils, freshwater, marine waters, inside vertebrates, and on the aerial parts of plants and animals [38]. They have also been isolated from human volunteers' nasal mucosa and CSF [39,40]. The abundance and diversity of these protists in the environment are strongly related to temperature, moisture, precipitation, pH, and nutrient availability [41]. As the main predators controlling bacterial populations in soils, FLA are more abundant in the rhizospheric zone and the surrounding bulk soil because plants allow the growth of various plant parasites such as bacteria and fungi on which amoebae feed. However, they may also further penetrate the vadose zone of groundwater systems, especially where bacterial populations have reached a high density. In water, a flagellate stage enables some FLA to swim at liberty, whereas others have to be attached to suspended particulates [38]. Attached FLA are also spread throughout water columns in the first 30 μm of the water surface or on the bodies of submerged animals and plants. Given the need for attachment, FLA often live on biofilms and at water–soil, water–air, water–plant interfaces. This life habit of living at interfaces further results in difficulty distinguishing their attributive environments. Biofilms such as those on contact lenses and dental-unit waterlines are supportive to FLA growth [42,43]. Some genera such as *Acanthamoeba* spp. and *Naegleria* spp. have been detected as well in treated waters like drinking water, tap water, cooling towers, swimming pools, hydrotherapy pools, and domestic water systems [35,44–46]. Unconventional water sources such as sewage and aquaria are also not spared from them [47].

Among all pathogenic FLA leading to human encephalitis, several species such as *Acanthamoeba*, *B. mandrillaris*, and *N. fowleri* are the dominant ones of concern and are thus massively studied (Table 1). In two forms of encephalitis, *N. fowleri* acts as the agent causing PAM, whereas *Acanthamoeba* spp. and *B. mandrillaris* are identified to be the causative organisms of GAE.

Naegleria fowleri in PAM

The genus name *Naegleria* was first coined by Alexeieff in 1912 [48]. Forty-five years later, the earliest case of *N. fowleri* infection was found in Australia [49]. Up to 2019, around 430 cases of PAM have been reported worldwide, with the USA and South Asia having the two most conspicuous infection areas [35,50,51]. Evaluation of the origin and evolution of *Naegleria* has revealed more than 40 species within the genus, whereas *N. fowleri* is the only known species to infect humans and cause disease in the CNS. Numerous studies on the molecular and genetic characteristics of *Naegleria* spp. have been conducted in recent years; some *N. fowleri* genomes such as ATCC 30863 and ATCC 30894 are already publicly available, and others can be obtained by requesting the authors [52,53]. Based on the length of the internal transcribed spacer 1 and a 1 bp transition in the 5.8S rDNA, the most accepted system for the identification of *N. fowleri* species was created in 2011, which revealed the existence of at least eight different genotypes unevenly distributed throughout different continents [54,55]. Without evidence of virulence differences among various *N. fowleri* types, three genotypes (I, II, and III) are found in the USA, seven genotypes (II, III, IV, V, VI, VII, and VIII) are found in Europe, two genotypes (II and III) are found in mainland Asia, and only one genotype (V) is found in the Oceania and Japan. Five of these eight genotypes (I, II, III, IV, and V) have been confirmed to cause PAM in patients [56,57].

Invasion pathways and pathogenesis

PAM targets the human CNS as water is identified to be the most frequent route of infection in most PAM cases [51,58]. During recreational water activities such as swimming, diving, and water skiing, *N. fowleri* has the ability to intrude into the human body by entering the nose due to splashing or forcing of contaminated water within the nasal cavity. In the form of trophozoites, infectivity is initiated with the attachment onto the nasal mucosa, followed by the locomotion along the olfactory nerve and cribriform plate. The chemotactic response to nerve-cell components then leads to the arrival on the olfactory bulbs in the CNS [59–61]. As a result, the tissue necrosis and neuron destruction caused by *N. fowleri* are reflected in the ingestion of brain tissue, the release of cytolytic molecules, and the fierce immune response of the host, giving rise to severe inflammation [62,63]. However, individuals can also be infected through a dry pathway of entering the nasal passages by cyst-laden dust followed by excysting and reaching the CNS similar to water infection [51,64]. When contaminated dust alights on the eye, cysts may also enter the nasal part through the nasolacrimal ducts [51]. As these dry infections tend to occur in regions with higher temperatures like those in South Asia, people living in

Table 1 Comparative features of three free-living amoebae and their pathogenic role in amoebic encephalitis

	Naegleria fowleri	*Acanthamoeba* spp.	*Balamuthia mandrillaris*
Trophozoite stage	Diameter 10–30 μm, speed about 1.0 μm/s	Diameter 15–35 μm, speed 0.3–0.4 μm/s	Diameter 10–60 μm, speed about 0.25 μm/s
Cyst stage	Diameter 7–15 μm, cysts not formed in brain tissue	Diameter 10–15 μm, cysts formed in brain tissue	Diameter 10–30 μm, cysts formed in brain tissue
Flagellate stage	Transformed from trophozoites	Not found	Not found
Environmental habitat	Warm fresh waters, soil, dust	Freshwater, soil, dusty air, hospital and household environments	Soil, freshwater
CNS infection	Primary amoebic meningoencephalitis (PAM)	Granulomatous amoebic encephalitis (GAE)	GAE
Susceptible host	Immunocompetent children and young adults	Mainly immunocompromised individuals	Immunocompetent and immunocompromised individuals
Portal of entry	Olfactory neuroepithelium	Nasopharyngeal or cutaneous epithelium	Mainly in cutaneous epithelium
Incubation period	Days	Weeks to months	Weeks to months, even years
Clinical manifestations	Headache, fever, nausea, nuchal rigidity, personality changes, seizures, coma, behavioral abnormality	Headache, irritability, fever, nausea, seizures, confusion, ataxia, hemiparesis, abnormal behavior	Headache, irritability, fever, nausea, stiff neck, sinus infection, behavioral abnormality
CSF	Elevated WBCs, generally low glucose level and high protein concentration, detected trophozoite, no flagellate or cyst	Elevated WBCs and protein, hydrocephalus, generally low glucose level	Elevated WBCs and protein, generally low glucose level
Neuroimaging	Cerebral edema, multifocal parenchymal and pseudotumor lesions, nonspecific and unhelpful	Single and multiple space-occupying or ring-enhancing lesions, not specific	Single and multiple space-occupying or ring-enhancing lesions, not specific
Diagnosis	CSF examination for trophozoites and polymorphonuclear leukocytes, neuroimaging CT and MRI, polyclonal and monoclonal antibodies, PCR assays	Microscopic staining, immunofluorescent microscopy, neuroimaging CT and MRI, PCR assays, trophozoite and cyst	Microscopic staining, immunofluorescent microscopy, neuroimaging, PCR assays, metagenomic deep sequencing, unsuitable for isolation and culture *in vitro*, trophozoite and cyst
Epidemiology	Worldwide distribution especially warm regions, hot summer months	Worldwide distribution, any time of year	Mainly on American continent
Estimated cases	>300	>200	~200
Case fatality rate	>95%	>90%	>90%
Therapy	Amphotericin B, azithromycin, chlorpromazine, miltefosine, rifampin, miconazole and fluconazole	Voriconazole, sulfadiazine, fluconazole, pentamidine, itraconazole, rifampin, meropenem, flucytosine, liposomal amphotericin B, and miltefosine	Fluconazole, pentamidine, sulfadiazine, itraconazole, rifampin, azithromycin, flucytosine, linezolid, liposomal amphotericin B, and miltefosine
Prognosis	Poor	Poor	Poor

WBC, white blood cell; CSF, cerebrospinal fluid; CT, computerized tomography; MRI, magnetic resonance imaging; PCR, polymerase chain reaction.

these areas are particularly at risk because they can do little to avoid inhaling cysts. Fortunately, due to the considerably higher incidence of nasal contact than the relevant cases of PAM dry infection, a threshold effect seems to exist because numerous activated amoebae are required for valid penetration into the epithelium and invasion into the brain. Thus, a high probability of cyst exposure may not equate with a high risk of PAM disease [51].

The factors associated with the pathogenesis of *N. fowleri* infection can be direct and indirect [65]. Direct factors include contact-dependent mechanisms involving adherence and phagocytosis and contact-independent mechanisms involving various cytopathic enzymes. Conversely, indirect factors include phenotypic switching, morphology, ubiquity, physiologic tolerance, chemotaxis, and drug resistance. As the primary step for parasite

cytopathogenicity, adherence onto target cells is one of the first events during the invasion of *N. fowleri* [66,67]. A comparison between pathogenic *N. fowleri* and another nonpathogenic *Naegleria* reveals a differential ability of adhesion and invasion [68]. Mediated by adhesins expressed on the surface of *N. fowleri*, fibronectin (FN) is considered an important extracellular matrix (ECM) protein involved in the adherence onto epithelial cells [69]. Furthermore, FN receptors are known to be integrins, so two integrin-like amoebic proteins are described as co-localized to the focal adhesion-like structures assisting in the adherence of *N. fowleri* [68]. In the study, a 60 kDa FN binding protein is observed to play a significant role in amoeba-mediated host cell cytotoxicity, and protein kinase C in *N. fowleri* is identified to improve the ability of adherence and cytotoxicity on host cells as a downstream component of integrin-like protein. With the impact of anti-integrin antibody, the reduced ability of *N. fowleri* to bind to ECM further supports this point [68]. A 23 kDa plasma membrane protein is probably involved in the cytotoxicity of *N. fowleri* [70]. In binding assays of different multivalent lectins, carbohydrates are also identified to participate in adherence and cytotoxicity [71,72]. The carbohydrate expression of *N. fowleri* and *N. gruberi* are confirmed to be differential, among which the expression of mannose residues is essential for *N. fowleri* adherence onto the nasal mucosa.

Cytopathic enzymes play a crucial role in PAM progression. Early in the initial stage of penetrating the mucous layer, enzymes with mucinolytic activity like a 37 kDa cysteine protease may contribute to the avoidance of the host response of *N. fowleri* [73,74]. Mucin secretion is suggested to be an important protective barrier against infection, whereas one study has shown that the mucinolytic activity of *N. fowleri* is prominently higher than *N. gruberi* that leads to evasion. Naegleriapores A and B, two pore-forming polypeptides of *N. fowleri* processed from separate multipeptide precursor structures, have been found to share similar structural properties with antimicrobial and cytolytic polypeptides [75]. The glycosylation degree of these naegleriapores also reflects their stability against degradation by proteases [76]. Phospholipases are found to be related to the extensive demyelinization in the white matter of PAM patients, and lysophospholipase and sphingomyelinase are identified as factors inflicting damage to the lipid-rich cytoplasmic membrane of cells and the demyelinization of nerve tissue after several years [77,78]. Neuraminidase activity, detected in pathogenic *N. fowleri*, is related to reported glycolipid alterations in demyelinating diseases, and the activity is maximal at pH 4.5–5.0 and is ion independent [79]. Although the role of cysteine proteases in *N. fowleri* has not been clearly defined, they are also suggested to participate in PAM progression [80]. The capability of *N. fowleri* to induce lactate dehydrogenase release and intracellular reactive oxygen species (ROS) accumulation reportedly gives rise to the death of host target cells [81]. Another study has revealed that electrodense granules are secreted by *N. fowleri* trophozoites in the course of brain-tissue invasion, and then they make contact with epithelial cells or collagen substrates to cause damage [82]. Trophozoites of *N. fowleri* are found to produce nitric oxide (NO) *in vitro* and react to the NOS_2 antibody, suggesting that NO may participate in PAM pathogenesis [83].

N. fowleri's capacity of active locomotion and phagocytosis of various host cells also involve host-cell damage [66,84]. As a sucker apparatus protruding from the surface of *N. fowleri*, food cups play an important role in the piecemeal consumption of target cells [85,86]. Along with polymerization from monomeric G-actin into filamentous F-actin, the consumption process is actin dependent, and a 360 bp *nfa1* gene has been identified to be expressed on pseudopodia encoding Nfa1 protein with a size of 13.1 kDa [87]. Several years later, one study has further supported the idea that anti-Nfa1 antibody and gene silencing of *nfa1* could reduce host-cell damage induced by *N. fowleri* [88–90]. As phagocytosis depends on the dynamics of cytoskeleton rearrangements, myosin and tubulin have been identified in trophozoites, whereas actin exists in the cytoplasm, pseudopodia, and food-cup structures [91]. Consisting of a 1.2 kbp coding sequence, the nf-actin gene produces a 50 kDa recombinant fusion protein (Nf-actin), which has been found to cluster in the food-cup structures of *N. fowleri* under fluorescence microscopy [92]. The phagocytic activity of nf-actin-overexpressing *N. fowleri* is sharply increased compared with that of control groups comprising wild-type ones.

Host immune response

N. fowleri is an FLA existing worldwide, so numerous individuals are exposed to *N. fowleri* in the course of their lives, either through direct contact such as soil or water or through wind-blown cysts landing on the nasal mucosa. In a study implemented among Czechoslovakian students and psychiatric patients, the positive antibody to *Naegleria* is detected to range from 1% to 4%. Conversely, another study implemented in the USA exhibits a much higher percentage of positive responses to the pathogenic *N. fowleri* and the nonpathogenic *Naegleria lovaniensis*, i.e., more than 80% of serum samples from hospitalized patients [93,94]. In general, because of the swiftness of PAM and the rarity of surviving patients, the comprehension of the mechanisms underlying early immune failure and the factors leading to subsequent fulminant inflammation is challenging. Fortunately, a remarkable similarity exists between PAM animal models and human infections [63].

Although the mechanism of PAM pathogenesis is not well understood, innate and adaptive immune responses

have been identified to participate in the defense against *N. fowleri* invasion [95]. However, *N. fowleri* belongs to eukaryotes instead of bacteria or viruses, so most mammalian pattern-recognition receptors do not recognize it as foreign. During the initial stages of infection, the host response is activated by the secretion of mucus trapping the trophozoites, and mucus contains mucin as the major component [61,73]. Mucus is considered as an effective protective barrier resisting most PAM erosion, and invasion occurs only when the number of amoebae is overwhelmingly sufficient to the innate immune response (Fig. 1A). Early in respiratory epithelial cells, the activation of innate defense has been induced by *N. fowleri*, leading to ROS production and then expressing the MUC5AC gene and protein, as well as the pro-inflammatory mediators interleukin-8 (IL-8) and interleukin-1β (IL-1β) [96]. The canonical Toll-like receptor (TLR) 4 pathway has also been demonstrated to express and produce the proinflammatory cytokines and β defensin-2 in a time-dependent manner [97]. Eosinophils and neutrophils participate in the inflammatory process induced by trophozoite invasion, and activated neutrophils play notable roles in early-stage infections [61]. Neutrophils surround *N. fowleri* through contact-dependent and contact-independent mechanisms and then engulf them [62,65,98]. Moreover, although a single neutrophil is insufficient for phagocytosing an entire *N. fowleri*, the cluster of several neutrophils is adequate to rupture *N. fowleri* by pinching off and engulfing them part by part. Complement activation especially that mediated by antibodies has been identified to enhance neutrophil activity against amoebae, and the cleavage products of complement also play a role as a chemotactic impetus for immune-cell recruitment [99,100]. Tumor necrosis factor-α (TNF-α) augments neutrophil activity by enhancing the production of oxygen radicals that destroy *N. fowleri* [101]. DNA, myeloperoxidase, histones, and elastase enzymes are all found to play roles in the definitive mechanism of neutrophils [102]. The feature of macrophages in the host defense against *N. fowleri* has also been demonstrated, among which microglial cells are the ones located in the brain [103]. During exposure to *N. fowleri* lysates, microglial cells release TNF-α, IL-1β, and IL-6, whereas astrocytes lead to AP-1 activation and the subsequent expression of IL-1β and IL-6 in an extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase (MAPK) dependent pathway [104].

Due to the swift and lethal disease course of PAM, research on the adaptive immune response to *N. fowleri* infection is difficult (Fig. 1B). Circulating antibodies have been identified as the dominant protective adaptive immune mechanism by immune serum-transfer experiments [105,106]. Among them, immunoglobulin M (IgM) is the primary antibody isotype generated by *N. fowleri* infection [2]. Although IgM contributes to the agglutination of *N. fowleri* and complement activation, its function is severely hindered by its high molecular weight of about 900 kDa, which confers difficulty in crossing the blood–brain barrier (BBB) [107]. Together with polymorphonuclear cells (PMNs), IgA and IgG antibodies could reportedly avoid the attachment of *N. fowleri* to the nasal epithelium [108–110]. Secretory immunoglobulin A (SIgA) can inhibit the binding of *N. fowleri* to collagen type I and block the proliferation of *N. fowleri* [111,112]. By forming neutrophil extracellular traps, IgG mediates PMN activation, which may be a crucial antimicrobial response against *N. fowleri* [113]. The ability to stimulate the migration and maturation of antigen-presenting cells (APCs) such as macrophages and dendritic cells is related to the enhanced adaptive immune response against *N. fowleri* [114]. Amoebic surface antigens seem to favor T-independent responses, and cell-mediated immunity against *N. fowleri* is observed in the form of delayed-type hypersensitivity [115]. Unfortunately, further studies on the functionality of amoeba-specific $CD4^+$ T cells are still lacking.

N. fowleri has evolved a series of effective mechanisms to cause the evasion of the host immune system, so early failure occurs in the detection of parasites and the use of effective antiamoeba mechanisms [62]. Except for the mucolytic activity toward nasal mucosa, virulent *N. fowleri* are resistant to subsequent complement-mediated lysis by expressing complement-regulatory proteins and shedding a membrane-attack complex from C5b to C9 on vesicles [106,116,117]. *N. fowleri* can also evade host immune defenses by internalizing surface-bound antibodies. For example, the antigen–antibody complex of SIgA can be eradicated from the surface of *N. fowleri* by capping and then internalizing surface-bound antibodies [112,118,119]. As opposed to the early stage, the host immune system activates an acute inflammatory reaction against *N. fowleri* in the later course of PAM, which plays a crucial role in inflicting damage to CNS tissue [2,65]. At this stage, macrophages of the olfactory region are inclined to recruit an intense neutrophil influx [120]. With all the acute inflammatory cytokines produced by local and recruited leukocytes, the release of various tissue-destructive lysosomal products induces extensive cerebral edema and neuronal tissue damage. Acute inflammatory cells that are recruited as part of chemotaxis exhibit a release reaction of lysosomes and cytokines, causing the activation of the complement cascade. This process contributes to the BBB breach and further results in extensive cerebral damage in PAM patients (Fig. 1C). After BBB breach, neutrophils and macrophages become the predominant leukocytes infiltrating in the neural tissue. Stimulated by fowlerstefin, microglial cells also produce proinflammatory cytokines through the nuclear factor-κB- and AP-1-dependent MAPK signaling pathways, and then they can

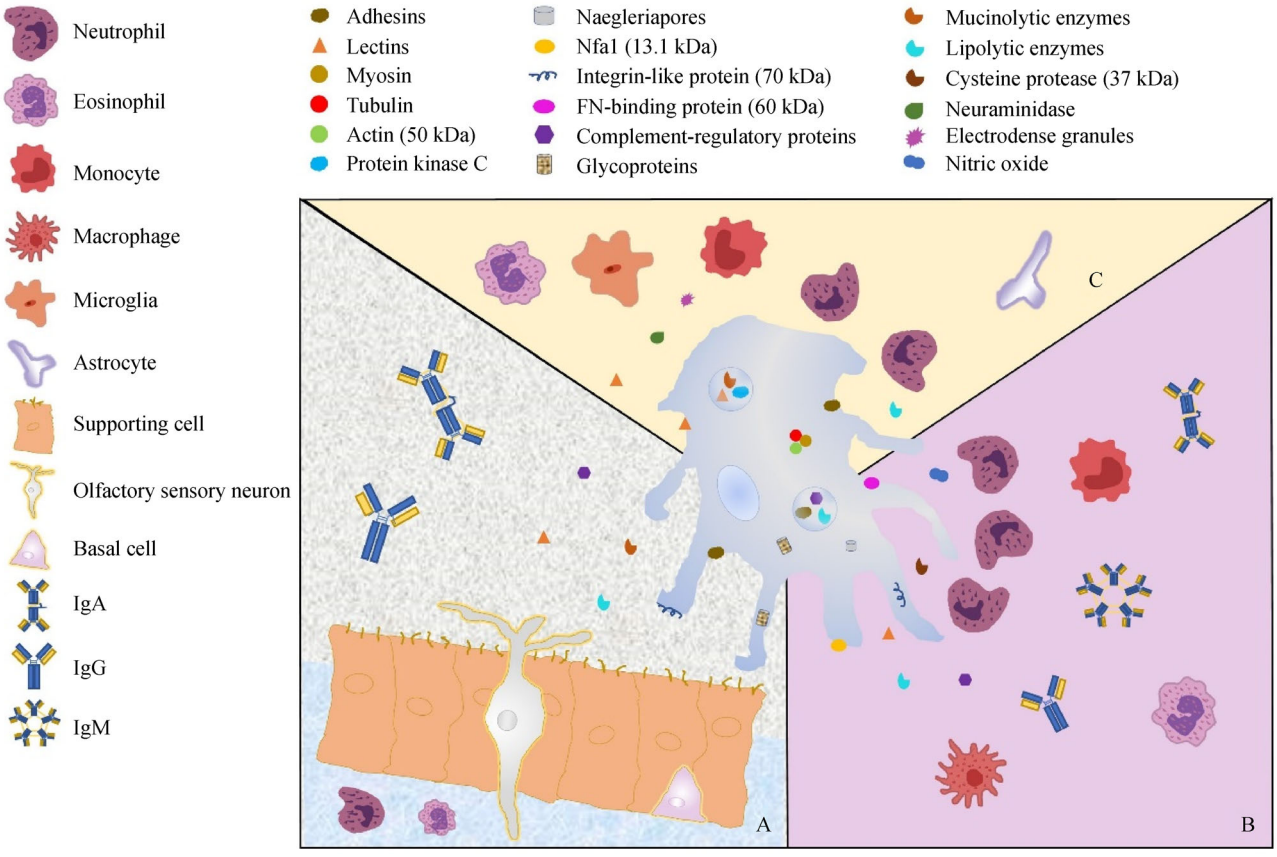

Fig. 1 Model of parasite molecules and host components in invasion by *N. fowleri*. (A) Representative scheme of olfactory epithelium invasion by trophozoites. As the primary step of contact, the penetration into the mucous layer occurs when trophozoite number is overwhelmingly sufficient in the presence of parasite molecules, including adhesins, lectins, and mucinolytic enzymes. Once the barrier is disrupted, trophozoites attach, separate, and lyse epithelial cells moving toward the basal lamina with the participation of lectins, lipolytic enzymes, complement-regulatory proteins, integrin-like proteins, protein kinase C, naegleriapores, *N. fowleri* antigen-related protein 1 (Nfa1), and actin. IgA, IgG, and IgM with the ability of potentially obstructing the attachment to the olfactory epithelial surface are secreted in the airway. (B) Representative scheme of trophozoites invasion in blood. Trophozoites follow the blood stream and carry out adherence and cytotoxicity toward blood–brain barrier. Similar to those participating in epithelial invasion, the parasite molecules involved in the endothelial invasion process include cysteine protease, fibronectin-binding protein, naegleriapores, carbohydrates, complement-regulatory proteins, lipolytic enzymes, Nfa1, and nitric oxide. Trophozoites induces lactate dehydrogenase release and intracellular reactive oxygen species (ROS), whereas ROS is related to the expression of MUC5AC gene and protein and the proinflammatory mediators interleukin-8 and interleukin-1β. Except for various circulating antibodies, neutrophils, eosinophils, monocytes, and macrophages are activated in response. The canonical Toll-like receptor 4 pathway expresses and produces proinflammatory cytokines and β defensin-2 in a time-dependent manner. (C) Representative scheme of trophozoite invasion in cerebrospinal fluid. After gaining access to neuron and gliacyte, trophozoites proliferate and finally provoke an acute inflammatory reaction consisting of monocytes, neutrophils and eosinophils, resulting in damage to CNS tissue. Parasite molecules such as actin, neuraminidase, and lipolytic enzymes are involved in the process. Considering that a single neutrophil is insufficient for phagocytosing an entire parasite, the cluster of several neutrophils is needful as a reinforcement and activity can be enhanced by tumor necrosis factor-α and complement activation.

exacerbate the inflammatory response in tissues infected with *N. fowleri* [121].

Pathophysiology and clinical manifestations

Neurological symptoms of PAM present as acute and fulminating hemorrhagic meningoencephalitis, which occurs primarily in healthy children and young adults having a contact history with contaminated water and also in other individuals like infants [122,123]. The disease generally starts within 5–7 days from the initial contact and sometimes as short as 24 h [2]. Initially, a sudden onset of PAM symptoms occurs; these symptoms include severe bifrontal or bitemporal headaches, nuchal rigidity, chills, and high fever, followed by nausea, vomiting, weakness, fatigue, or behavioral abnormalities including restlessness and irritability [57,124–126]. Except for nuchal rigidity, other positive meningeal irritation signs including the Kernig sign and Brudzinski sign can also be detected sometimes along with the Babinski sign [127]. As the

disease progresses, it leads to photophobia and later neurological abnormalities, such as lethargy, confusion, coma, diplopia, seizures, and bizarre behavior [2,126,128]. Cranial nerve palsies may be the sign of brain edema and herniation with intracranial pressure for 600 mm H_2O or even higher. Finally, patients die within a week.

Analysis of predilection sites in brains of *N. fowleri* infection has revealed that the frontal lobe is the most favorable destination in the majority of cases, followed by the parietal lobe and corticomedullary junction [129]. Hydrocephalus is also observed in 27% of selected cases. The observed lesion sites include the base of the orbitofrontal and temporal lobes, base of the brain, posterior fossa, hypothalamus, midbrain, pons, medulla oblongata, and upper portion of the spinal cord [130,131]. Autopsies of PAM cases demonstrate that the cerebral hemispheres are usually soft, swollen, edematous, and severely congested with severely congested leptomeninges [2,132,133]. Limited purulent exudates can be found microscopically within the cerebral hemispheres, base of the brain, brainstem, cerebellum, and upper portion of the spinal cord, which contain primarily neutrophils followed by eosinophils, macrophages, and lymphocytes [134]. Olfactory bulbs and orbitofrontal cortices reveal changes such as hemorrhagic necrosis surrounded by purulent exudates [2]. Numerous *N. fowleri* trophozoites without the presence of polymorphonuclear leukocytes can be found in edematous and necrotic neural tissues; sometimes they also appear in Virchow–Robin spaces with the ability of surrounding blood vessels but not causing an inflammatory response. Brain tissue, meninges, and CSF are all suitable for *N. fowleri* proliferation, and the amoebae can be detected and cultured from brain tissue and CSF samples obtained postmortem [26,135]. By contrast, flagellates or cysts in brain tissue and CSF have not yet been detected [136].

Diagnosis

Due to the lack of distinctive clinical features, PAM is easily confused with other bacterial or viral meningoencephalitis in most cases; thus, a complete and precise clinical history is essential for diagnosis. Relevant information should contain any recent patient contact with freshwater and history of upper respiratory tract diseases such as rhinitis and allergies, especially in children and young adults [67]. In early-stage *N. fowleri* infection, a computed tomography (CT) scan is usually performed, and then it leads to cerebral edema with obliteration of cisterns as the disease progresses. The sulci and adjacent gray matter are also intensely enhanced with a normal-sized ventricle [137]. Combined with the images of magnetic resonance imaging (MRI), multifocal parenchymal lesions, pseudotumor lesions, hemorrhagic infarcts, meningeal exudates, and necrosis are visible in the brains of PAM patients with edema and hydrocephalus [67,138]. Unfortunately, these neuroimaging methods cannot distinguish meningitis cases with different etiologies from one another [139].

Among PAM cases infected with *N. fowleri*, 63.7% are diagnosed postmortem and 36.3% are diagnosed premortem, whereas microscopy is implemented successfully in 36.4% of postmortem cases [129]. Thus, observing motile amoeba trophozoites in CSF samples by microscopy is the most extensively and successfully used premortem diagnostic method. Generally, CSF may have a relatively high pressure with low glucose and high protein concentration, whereas lumbar puncture can be performed under the condition of low CSF pressure [3]. The CSF of PAM patients exhibits various abnormal colors ranging from gray and yellowish-white in early-stage infection to red in the later stage of infection due to the significant increase of erythrocytes [57,140,141]. During PAM, the count of erythrocytes can increase from 250 cells per mm^3 to 24 600 cells per mm^3 [2]. By staining the fixed samples with Giemsa, Wright, trichrome, periodic acid–Schiff, or hematoxylin and eosin (H&E), microscopic examination can reveal the presence of trophozoites in CSF [26,142,143]. However, Gram staining is not applied in the diagnosis due to its uncharacteristic amoebic nuclear morphology [3,144]. Sometimes *N. fowleri* can be confused with macrophages, so their nucleus that contains a large, central, and round nucleolus starts to become important for distinguishing from host cells [133,142]. Moreover, in the early stage of PAM, *N. fowleri* are probably invisible and polymorphonuclear leukocytes can be observed in the CSF primarily comprising neutrophils. Phase-contrast microscopy is beneficial for optimizing amoeba visualization [139,145]. Monoclonal antibodies with the capability of recognizing a glycosylated epitope on *N. fowleri* have been used to diagnose PAM infections [146]. The technique can specifically identify *N. fowleri* in CSF and serum and distinguish *N. fowleri* from other *Naegleria* species, even from other FLA in various samples [147–149]. Molecular techniques such as polymerase chain reaction (PCR), nested PCR, quantitative PCR, and multiplex PCR assays are more sensitive, rapid, and specific for *N. fowleri* detection in clinical and environmental samples [57,125,150–152]. A PCR assay can also reportedly detect *N. fowleri* successfully in formalin-fixed paraffin-embedded brain sections [153]. Compared with quantitative PCR, next-generation droplet digital PCR already exhibits better specificity [154].

Epidemic situation, therapy, and prognosis

As a globally distributed pathogen, *N. fowleri* is detected throughout all continents except Antarctica, especially in warmer equatorial countries [54]. Generally, most PAM cases are acquired through recreational activities such as swimming, diving, and water sports in freshwaters

containing amoebae [50]. Nasal irrigation and ritual nasal ablution by using tap water are also highly correlated in some countries, such as India or Pakistan [35,155]. In temperate countries such as those in northern and eastern Europe, the growing environments of *N. fowleri* are limited in waters warmed either artificially or naturally. Although *N. fowleri* are inclined to be more active in warmer regions, fewer cases are reported in extremely warm areas [50]. Meanwhile, except for the most frequent water-infection route, dry infection can also occur through cyst-laden dust entering the nasal passages. Dry infections tend to occur in hot regions with a percentage of 8% in the Indian subcontinent, whereas it is almost 0% in the USA [51]. With a highly seasonal life cycle, *N. fowleri* has been proven to grow at 30 °C to 46 °C. Although trophozoites degenerate within several hours below 10 °C, cysts can survive at 4 °C for nearly 6 months [156–158]. Due to global warming, average temperatures have increased and large areas have hugely changed as either increased drought conditions or increased precipitation leads to the erosion and eutrophication of freshwater courses. Considering that habitats favored by *N. fowleri* are increasing, climate change may inevitably result in the geographic spread of the amoebae and increased PAM incidence [159]. Moreover, because of the misdiagnosis and lack of autopsies, PAM is suspected to be much more common than currently indicated, especially in developing countries [58,160]. Through the isolation of *N. fowleri* from the resident's nares, it has been suggested that PAM epidemiology is much more serious in warm and dry regions, such as India and Nigeria [161–163].

Given that PAM is rarely confirmed during early-stage infection, the untimely treatment of this acute and fulminating infection contributes to a high mortality rate of more than 90% [65]. Additionally, because of the low incidence of PAM and economic constraints especially in developing countries, interest in the development of such anti-PAM drugs is lacking despite the utmost need. A deficiency exists in clinical trials assessing the efficacy of one therapeutic method over another [164]. Along with the improvement of research on drug use, amphotericin B has been identified as the primary choice used alone or in combination with other drugs for treating PAM. In an *in vitro* study, an amphotericin B concentration of at least 0.1 μg/mL can suppress more than 90% of *N. fowleri* growth, whereas 0.39 μg/mL can completely suppress its proliferation [165]. As the cornerstone of therapy, the intravenous dose of amphotericin B is 1.5 mg/kg/day in two divided doses for 3 days followed by 1 mg/kg/day once daily for an additional 11 days, whereas the intrathecal dose of amphotericin B is 1.5 mg/day for 2 days followed by 1 mg/day for an additional 8 days as recommended by the Centers for Disease Control and Prevention [164]. Due to the various side effects of amphotericin B such as anemia, fever, nausea, and dose-related nephrotoxicity, new formulations with improved toxicity profiles, such as deoxycholate amphotericin B with a very low minimum inhibitory concentration of 10%, have been developed [65]. To reduce side effects, amphotericin B is also used in combination with other drugs, such as azithromycin, chlorpromazine, miltefosine, rifampin, and fluconazole [80,165–168]. Animal trials on experimental mice exhibit survival rates of 40%, 75%, and 55% with amphotericin, chlorpromazine, and miltefosine, respectively [166]. A recent study has indicated that auranofin may also be effective for PAM treatment either as a monotherapy or in combination with the standard amphotericin B [169]. Furthermore, though there are a series of clinical guidelines for amoebic meningoencephalitis, physicians usually adjusts the combinations of various classes of drugs with different action mechanisms for liberal and individualized therapies [129].

Acanthamoeba spp. and *Balamuthia mandrillaris* in GAE

Genus *Acanthamoeba* was created by Volkonsky in 1931. *Acanthamoeba* species were initially classified into three distinct groups (I–III) according to their morphology and cyst size, but this classification was considered unreliable due to variations in culture environments [170]. Nine years later, a modified classification of three groups (I–III) was proposed by analyzing the following three isoenzymes: hexokinase, esterase, and acid phosphatase [171]. Since then, pathogenic and nonpathogenic *Acanthamoeba* have been differentiated based on the differentiation of protein and antigen profile [172]. To date, 22 genotypes (T1–T22) have been identified and designated according to the comparison of sequences of the nuclear 18S rRNA gene [173]. Genotypes T1, T2, T4, T5, T10, and T12 are responsible for causing meningitis in humans, whereas genotype T4 appears to be the most prevalent and predominant one characterized by increased virulence and decreased sensitivity to chemotherapeutic agents [174–176]. Additionally, about 31 species have been described and placed within genus *Acanthamoeba* up to 2020, among which 18 species are reportedly pathogenic or implicated in the medical field. These species are *A. polyphaga*, *A. palestinensis*, *A. castellanii*, *A. rhysodes*, *A. astronyxis*, *A. culbertsoni*, *A. griffini*, *A. lenticulata*, *A. royreba*, *A. divionensis*, *A. lugdunensis*, *A. quina*, *A. triangularis*, *A. healyi*, *A. stevensoni*, *A. jacobsi*, *A. hatchetti*, and *A. byersi* [173,177–192].

B. mandrillaris was initially considered by some people as an innocuous soil organism, and all GAE cases were identified to be caused by *Acanthamoeba*, yet brain tissue from some cases could not react with *Acanthamoeba*-specific immunohistochemical tests [193–195]. As an organism associated with soils, soil exposure has been identified as an important risk factor for *Balamuthia*

infection, but its isolation from soil is especially difficult because only several prior occasions have been reported in Iran, Peru, and USA [196–198]. The first report of *B. mandrillaris* causing disease is described in a pregnant mandrill monkey with meningoencephalitis at the San Diego Zoo in 1986, and then the amoeba was identified as a human pathogen four years later [199]. Except for primates, *Balamuthia* has also been reported to cause infection in various other animals such as horses and dogs [200–202]. As the only known pathogenic species infecting humans within the genus, all *B. mandrillaris* are demonstrated to belong to one single genotype through nuclear and mitochondrial rDNA analysis [203]. With no diversification among different isolates, *B. mandrillaris* exhibits a low genetic-variation level [149,204,205].

Invasion pathways and pathogenesis

Through the inhalation of air or aspiration of water contaminated with invasive forms of these amoebae, *Acanthamoeba* infection generally begins with the penetration of the upper respiratory tract and damaged or ulcerative skin, whereas lesions on cutaneous epithelium act as the most common sites for the initial *B. mandrillaris* infection [206–209]. Ocular cornea, oral mucosa, and intestinal mucosa are also possible sites for *Acanthamoeba* infection, whereas the gastrointestinal tract has been demonstrated as a possible route for the entry of *B. mandrillaris* [2,210,211]. Moreover, *B. mandrillaris* can spread through organ transplantation [212,213]. An important migration route of *Acanthamoeba* and *B. mandrillaris* to the CNS is through the nasal mucous membrane, the endothelium of capillaries in the brain, and the ethmoid bone along olfactory nerves [214–217]. These two amoebae also cause cutaneous and respiratory infections, and *Acanthamoeba* is responsible for an additional ocular infection named amoebic keratitis [218,219]. Unfortunately, the hematogenous spread is a precondition for BBB invasion, so it makes GAE pathogenesis especially complicated and remains unclear to date [220,221].

Similar to *N. fowleri* in PAM, factors determining the CNS pathogenicity of *Acanthamoeba* spp. and *B. mandrillaris* can also be divided into direct and indirect ones [222]. Direct agents are related to adherence, interaction, and the secretion of enzymes that are cytotoxic to human neurons. For *Acanthamoeba*, attachment onto the surface of host tissue is identified as the crucial step to establish infection, later culminating in the host cell's death [223]. With the use of cytochalasin B (CB) and latrunculin B (LB), actin cytoskeleton has been revealed to participate in the adherence onto neuronal cells [224]. The main mechanism of action of cytochalasin and latrunculin is to decrease the polymerization rate of actin. With the CB and LB treatment of trophozoites, the normal distribution of actin filaments changes and the acanthopodia of *Acanthamoeba* disintegrate, thereby presenting remarkable inhibition of trophozoite adhesion. After *Acanthamoeba* trophozoites cross the olfactory epithelium, Schwann cells (SCs) are probably some of the first target cells protecting the olfactory nerve bundles [225]. The study reveals that the interaction between *Acanthamoeba* and SC could result in SC autophagy or necrosis. This interaction is characterized primarily by contact-dependent mechanisms including intimate contact and phagocytosis through the emission of cytoplasmic projections, such as amoebostomes. Appearing as sucker-like structures on various amoebic surfaces, amoebostomes play an active role in lysing and engulfing different targets [226,227]. As the entry sites of *Acanthamoeba* into the brain, the BBB is momentous for studies on invasion [228,229]. Different pathogens own various modes of BBB crossing from intracellular to paracellular. As an efficient method of distinguishing transcellular routes, measuring the integrity of the BBB exhibits high transendothelial electrical resistance (TEER) [230]. Through interaction with human brain microvascular endothelial cells, *Acanthamoeba* decrease TEER values to almost zero, causing a Rho-dependent reduction of ZO-1 and occlusion similar to that in *N. fowleri* [231]. Mannose-binding protein and extracellular serine proteases also play a role in the traversal process of *Acanthamoeba* [232]. Furthermore, evidence including host cell DNA laddering, chromatin condensation, membrane blebbing, and formation of apoptotic bodies suggests that *Acanthamoeba* induce apoptosis in neuroblastoma cells, thereby presenting a mechanism for host cell death other than phagocytosis [233]. One study has shown that *Acanthamoeba* induces apoptosis through caspase-dependent and caspase-independent pathways with the overexpression of the proapoptotic protein Bax. As a highly diverse set of proteolytic enzymes, M28 aminopeptidase secreted by *Acanthamoeba* spp. has sufficiently high virulence to inflict host damage and induce the apoptosis of cell lines [234]. Besides, two 130 and 150 kDa proteases from an *Acanthamoeba* isolate reportedly induce GAE, and both of them exhibit maximal activity at neutral pH and over a range of temperatures [235]. These proteases degrade ECM components in the CNS such as collagen I, collagen III, elastin, and plasminogen, as well as casein and hemoglobin.

Like *Acanthamoeba*, multiple elements may be involved in *B. mandrillaris* pathogenesis, including adhesion to cells, secretion of enzymes, penetration of the BBB, and host inflammatory responses [236,237]. However, information on the pathogenic mechanisms of *B. mandrillaris* remains limited [216]. The amoebic invasion of the brain probably occurs through BBB breakage or migration along nerve fibers, and BBB may act as the most possible site for the invasion of *B. mandrillaris* into the CNS [217]. Adhesion, proteolytic attack, and host inflammatory

responses may all contribute to BBB disruption, whereas normal human serum (NHS) is protective against amoebae [238,239]. In turn, by specifically activating phosphatidylinositol 3-kinase, *B. mandrillaris* can induce human brain microvascular endothelial cells to release IL-6, which later participates in initiating the early inflammatory response as a pleiotropic cytokine [240]. *B. mandrillaris* has been demonstrated to have the capability to recognize and interact with specific ECM glycoproteins, such as collagen-1, laminin-1, and FN [241]. As metalloprotease activity is found in two isolates of *Balamuthia*, the proteolytic activities of amoebae to degrade ECM have also been ascertained [242]. *B. mandrillaris* may possess the ability to hydrolyze extracellular ATP through its surface enzymes like ecto-ATPase [243].

As a multifactorial process, the ability of *Acanthamoeba* and *B. mandrillaris* to induce human encephalitis also depends on their capacity to survive outside the mammalian host for various times and under diverse environmental conditions [222,239]. These indirect factors include encystment ability, morphology, ubiquity, tolerance to unsuitable environmental conditions, chemotaxis, and drug resistance. Except for the biological and ecological ones mentioned above, the pathogenicity of amoebic trophozoites may also be related to the number of pseudopodia that allow connection with host cells [244]. The ability of these amoebae to grow at diverse temperatures, osmolalities, and pH is positively correlated with their prevalence in various environments and pathogenicity. For example, although some nonpathogenic strains with high thermal tolerance have been described, pathogenic species among *Acanthamoeba* spp. exhibit generally better thermal tolerance than nonpathogenic ones [2,245].

Host immune response

On account of the ubiquitous distribution of *Acanthamoeba* and *B. mandrillaris* in nature, contact with humans and other animals with trophozoites, cysts, or their antigens can widely elicit antibodies to these amoebae in serum. For example, contact with *Acanthamoeba* appears to be common because the presence of their antibodies has been proven in serum samples from relevant patients and many asymptomatic healthy individuals [94,124]. As an opportunistic pathogen, the ability of *Acanthamoeba* and *B. mandrillaris* to produce diseases in the CNS depends on their own virulence and on host susceptibility and environmental conditions. A competent immune system is generally sufficient to defeat pathogens under normal circumstances. However, due to the complexity of the host immune system and low numbers of GAE infections, the precise factors that contribute to host resistance and associated mechanisms remain unclear.

Innate and adaptive immune responses have been demonstrated to participate in the *Acanthamoeba* infection [115,246] (Fig. 2A). TLRs play an important role in recognizing amoebae and inducing cytokine production. As an example, increased levels of TLR2 and TLR4 mRNA expression have been found in the brains and lungs of mice infected with *Acanthamoeba*, suggesting the effect of these receptors on immune-response initiation [247,248]. The complement system works as a barrier to infections and activates a cascade system that destroys invading *Acanthamoeba* in GAE. As the first line of defense against protozoa, the complement system is activated through the classical pathway, alternative pathway, or mannose-binding lectin pathway [222]. A recent experiment on mice has suggested that the alternative pathway plays a major role in *Acanthamoeba* lysis, whereas the trigger molecules of the lectin pathway and classical pathway are not essential in complement activation [249]. *In vitro* studies with NHS exhibit the lytic activity of complement and antibodies against *Acanthamoeba*, and the lytic pathway is highly effective in the presence of phagocytes, such as macrophages and neutrophils [250,251]. These interactions further stimulate the secretion of proinflammatory cytokines such as IL-1β and TNF-α released from monocytes and macrophages, leading to the activation of neutrophils and vascular endothelial cells [252,253]. However, the effect exerted by *Acanthamoeba* trophozoites appears to be complex because they can stimulate and inhibit cytokine production [254]. They induce IL-10, IL-8, IL-6, and TNF-α release in monocytes while inhibiting TNF-α and IL-8 production by macrophages. TNF-α has been shown to induce the encystation of *Acanthamoeba*, which in turn makes them resistant to phagocytosis. Macrophages have been found to be involved in initiating and maintaining an effective immune response and play a role in tissue repair [255]. A significant increase in natural killer cells is observed in mice infected with *Acanthamoeba*, suggesting that these cells also participate in the body's protection against the amoebae [256,257]. As a result, complement proteins are induced to be deposited, the opsonization of amoebae is induced and then taken up by phagocytes, and the membrane attack complex ultimately forms, leading to the death of target cells [222].

Antibodies to *B. mandrillaris* have been detected in serum samples of GAE patients and healthy individuals, and cord blood also contains antibodies at a lower titer [258,259] (Fig. 2B). Although serum exerts a protective effect against the combination and destructive activities of *B. mandrillaris*, the amoebicidal effect is relatively limited because only about 40% of trophozoites are killed [239,260]. The protective role of antibodies is still underresearched because several GAE patients with a high titer of antibodies to *B. mandrillaris* have no positive protection response [261]. Regarding the inflammatory response, leukocytes are recruited to the sites of infection by

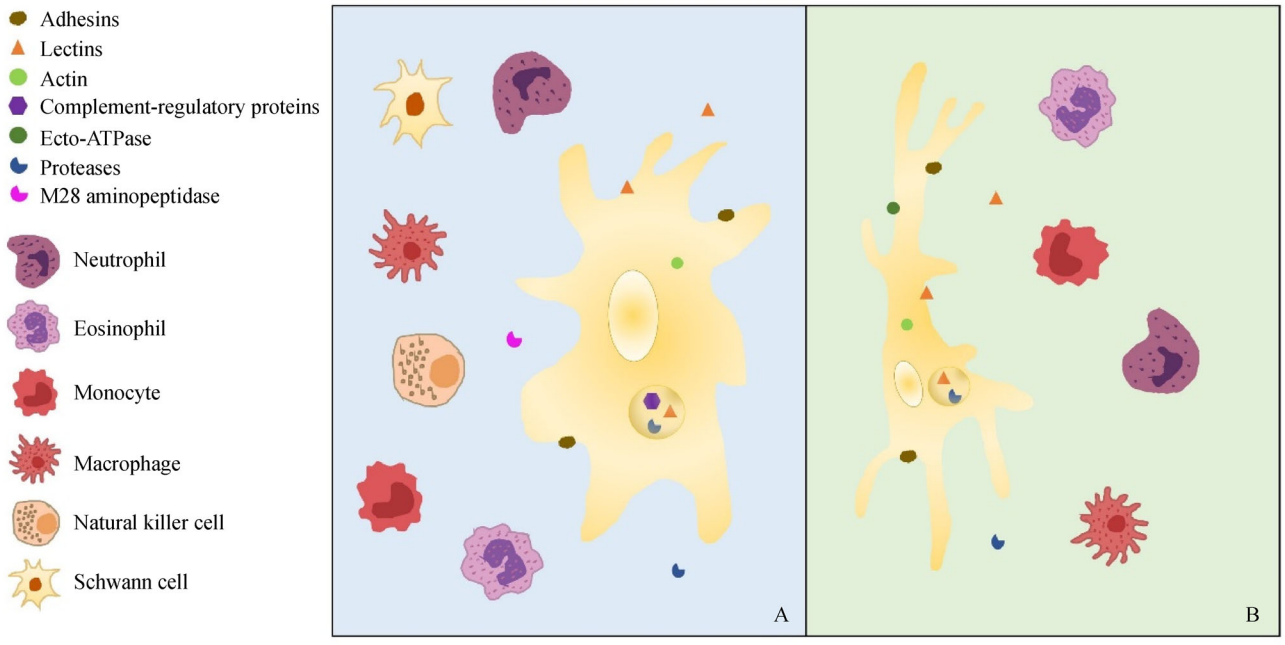

Fig. 2 Model of parasite molecules and host components in invasion by *Acanthamoeba* spp. and *B. mandrillaris*. (A) Representative scheme of invasion by *Acanthamoeba* spp. trophozoites. Infection generally begins with penetration into nasopharyngeal or cutaneous epithelium. Trophozoites attach onto the host-tissue surface and induce apoptosis and phagocytosis in the presence of parasite molecules, such as adhesins, lectins, proteases, complement-regulatory proteins, and actin cytoskeleton. Schwann cells are some of the first target cells with the outcome of autophagy or necrosis. Mannose-binding protein and extracellular serine proteases are related to the traversal process of the blood–brain barrier. Toll-like receptors recognize the trophozoites and induce cytokine production, whereas the alternative pathway plays the major role in *Acanthamoeba* lysis by activating the complement system. Neutrophils, eosinophils, monocytes, macrophages, and natural killer cells are activated in response, among which monocytes are induced to release IL-10, IL-8, IL-6, and TNF-α, whereas macrophages are inhibited to release IL-8 and TNF-α. (B) Representative scheme of invasion by *B. mandrillaris* trophozoites. Parasite molecules involved in the invasion process include adhesins, lectins, proteases, ecto-ATPase, and actin. In the CNS, trophozoites induce the release of IL-6 by specifically activating phosphatidylinositol 3-kinase, and IL-6 later participates in initiating an early inflammatory response as a pleiotropic cytokine. Neutrophils, eosinophils, monocytes, and macrophages are recruited to the sites of infection. Type IV hypersensitivity reaction, including the exocytosis of tissues and the release of matrix-damaging enzymes, occurs in *Acanthamoeba* spp. and *B. mandrillaris* invasion.

modulating the expression of various adhesion molecules, such as intercellular adhesion molecule-1. Among leukocytes, macrophages have recently been identified to have no inhibitory effect on the biological properties of *B. mandrillaris* through *in vitro* experiments, and they even increase the amoebic binding and cell cytotoxicity mediated by protozoa [262]. Furthermore, lectins such as mannose- and galactose-binding proteins are involved in host damage mediated by *Acanthamoeba* and *B. mandrillaris*, respectively [263].

Given that *Acanthamoeba* and *B. mandrillaris* do not have adequate time to be stationed in the cerebral circulation and invade the brain, they can provoke BBB breach despite not actually invading the brain tissue. Brain damage is actually evoked by the amplified host immune responses [120]. The presence of a hardly phagocytable or unphagocytable microorganism and an integrated cellular immunity is critically needed to induce granuloma formation and associated tissue destruction [264,265]. With a general size of about 25 μm, *Acanthamoeba* spp. and *B. mandrillaris* are incapable of phagocytosis, and the amoebic antigens being exposed to the immune system tend to set up an ideal scenario for type IV hypersensitivity reaction, including the exocytosis of tissues and the release of matrix-damaging enzymes [120]. The human immune response plays an important part in breaching the BBB and damaging the brain in GAE.

Pathophysiology and clinical manifestations

Although *Acanthamoeba* spp. rarely leads to CNS infections, their prognosis is very poor, with less than 10 documented survivors out of more than 150 reported cases [266]. *Balamuthia* GAE is also a highly fatal disease with fewer than 10% of patients surviving [208]. GAE caused by *Acanthamoeba* generally occurs among immunocompromised individuals with AIDS and those who are chronically ill, diabetic, having undergone organ transplantation, or debilitated for other reasons (e.g., taking steroids, antibiotics, and chemotherapeutic medications)

[4,135,267–269]. Immunocompetent individuals are much less likely to be infected because only 11 patients are immunocompetent in the approximately 150 documented cases of *Acanthamoeba* GAE [266,270,271]. By contrast, GAE caused by *B. mandrillaris* occurs in healthy and immunocompromised individuals, among which children and young adults are the most vulnerable populations [208,266,272–276]. Similar to viral or bacterial meningitis, the clinical symptoms of GAE usually start with headache, irritability, nausea, dizziness, and low-grade fever. It progresses to other neurological symptoms, including altered mental state, seizures, confusion, aphasia, lethargy, hallucination, focal neurologic signs, diplopia, cranial nerve palsies, ataxia, hemiparesis, stiff neck, and personality changes [2,4,124,208,269,271,275,277–282]. Facial palsy and numbness are also common in *Acanthamoeba* GAE as important causes of facial asymmetry. Moreover, some patients affected with *B. mandrillaris* in Peru exhibit skin lesions as common symptoms, whereas the general symptoms in other regions' cases are relatively absent during the cutaneous stage of GAE [275,283]. As a progressive disease, death due to GAE generally occurs within 1–2 months because of the onset of symptoms owing to the increased intracranial pressure. The disease can also sometimes develop over a period lasting for several years [135,284].

As to the predilection sites of *Acanthamoeba* and *B. mandrillaris* infection in the brain, the majority of *Acanthamoeba* cases involve the cerebral cortices, with the frontal and the temporal lobes being the most affected, followed by the parietal and occipital lobes [129]. The temporal lobe is the most vulnerable as observed in *Balamuthia* cases, followed by other sites such as the frontal, parietal, and occipital lobes. The cerebellum and the corticomedullary junction are the most favored targets among extracortical sites in *Acanthamoeba* GAE, whereas the thalamus is the most affected target followed by the corticomedullary junction, cerebellum, and basal ganglia among extracortical sites in *Balamuthia* GAE. Additionally, hydrocephalus is observed in a few *Acanthamoeba* GAE resulting from the blockage of CSF drainage. Unfortunately, premortem diagnosis cannot guarantee survival because the patients have often already suffered from intense damage in the brain before diagnosis. Microscopic examinations of CNS sections obtained from autopsies of *Acanthamoeba* and *B. mandrillaris* GAE cases reveal changes such as edema, encephalomalacia, tonsillar herniation, and multiple necrotic and hemorrhagic areas [135,285]. Hemorrhagic infarcts can be seen in areas such as the brainstem, cerebral hemispheres, and cerebellum. Multinucleated giant cells are also commonly seen in the brainstem, cerebral hemispheres, cerebellum, midbrain, and basal ganglion. Blood vessels may be occasionally seen as being cuffed by amoebic trophozoites and cysts, and angiitis can exist with surrounding perivascular inflammatory cells, among which reactive macrophages are usually mistaken for amoebae [3,26]. Given the deficiency of cellular immune response, a granulomatous response may be absent or minimal in immunocompromised patients [286]. Many of these patients develop a series of skin lesions, abscesses, or erythematous nodules on their body and limbs, especially patients with AIDS. In the skin lesions, amoebic trophozoites and cysts with a single nucleus can be seen. The nodules are usually firm and nontender but are also sometimes ulcerated and purulent [2,124,135,222,287]. In a few cases of immunocompetent patients invaded by *Acanthamoeba*, the infection has not even spread to the CNS [288,289].

Diagnosis

Due to the nondirectional symptoms and rarity of the disease, GAE may usually be confused with other bacterial leptomeningitis or viral meningitis. Brain lesions can be detected by neuroimaging methods such as CT and MRI, but their results are not specific, resulting in limited diagnostic value for GAE [3,290]. Single or multiple enhanced lesions are often seen in the cerebral cortex, basal ganglia, cerebellum, and subcortical white matter through CT, whereas multifocal lesions and ring-like patterns of enhancement are seen in the diencephalon, thalamus, brain stem, and posterior fossa structures through MRI in patients with GAE; edema and hydrocephalus are also visible [4,217,266,269,291–294]. Intralesional hemorrhage has been observed in some GAE neuroimaging findings. Amoebic encephalitis has been suggested to be listed in the differential diagnosis for immunocompromised patients with new brain lesions found on radiographic imaging [295].

Microscopy with some staining such as calcofluor white, acridine orange, or H&E of host brain tissue can be used to detect trophozoites and cysts of *Acanthamoeba* and *B. mandrillaris*. However, morphological features are insufficient for differentiating the exact amoeba genus, which is problematic as it requires expertise. Samples generally originate from CSF, brain-tissue biopsy, sinus or lung biopsy, and skin lesions on the face or extremities obtained either after surgery or postmortem [222,296]. Notably, a negative CSF sample cannot exclude the possibility of GAE infection in suspected patients [297]. As antiamoeba antibodies are found in the serum of healthy and GAE-infected individuals, several immunodiagnostic tests have been well developed and put into application. Immunofluorescent microscopy and indirect immunofluorescent and immunoenzymatic assays (e.g., flow cytometry and enzyme-linked immunosorbent assay) have been successfully used to examine serum and tissue samples [3,298–301]. However, given that *Acanthamoeba* can be isolated from patients' tissues and then cultured *in vitro*, this

method is unsuitable for *B. mandrillaris* because they grow slowly and require the culture of tissue cells as a food source [139,302]. Developing molecular techniques such as PCR, multiplex PCR, and real-time PCR are extensively used to identify *Acanthamoeba* and *B. mandrillaris* in the CSF and brain-tissue samples for many years [4,151,174, 175,269,295,303–305]. These molecular assays are effective diagnostic techniques that can be implemented for rapid and sensitive identification even in formalin-fixed paraffin-embedded brain biopsy specimens, so it is beneficial for the appropriate and timely treatment of patients infected with GAE [306]. Metagenomic deep sequencing has also been applied for the diagnosis of amoebic encephalitis in recent years [307].

Epidemic situation, therapy, and prognosis

The number of either *Acanthamoeba* or *B. mandrillaris* GAE cases worldwide exceeds 200 [124,136]. Among them, *Acanthamoeba* cases reportedly have the highest number of infected patients in North America and India, whereas *B. mandrillaris* cases are found throughout the entire American continent [208,276,284,308–312]. However, compared with the opportunities for humans and other animals to make contact with these amoebae, the number of GAE cases is relatively much smaller. Through breaks in the skin contaminated by soil or through the upper respiratory tract blown by the wind or air currents, infection generally starts with the entry of the protozoa [2,135,311]. Water may also serve as a vehicle for transmission because *Acanthamoeba* and *B. mandrillaris* have been detected in a series of water samples and reported to cause disease in humans and other animals [201,313–315]. *B. mandrillaris* can also be transmitted through organ transplantation [316,317]. *Balamuthia* GAE appears to be more frequent in Hispanics, but further research is still needed to determine whether the unequal incidence is a simple coincidence [318].

Additionally, because of nonspecific symptoms similar to other bacterial or viral diseases and the difficulty in diagnosis at an early stage resulting from the deficiency of reliable diagnostic tests and clinicians' nonfamiliarity with FLA, more than 90% of GAE cases are fatal [284]. The majority of GAE cases are detected postmortem, further resulting in the lack of recommended treatment and management patterns. Except for surgery, the treatment of GAE is still based on limited *in vitro* experimental data and clinical experiences reported in literature [319–321]. According to reported cases with therapeutic success, various drugs are usually given alone or in combination, including voriconazole, fluconazole, itraconazole, rifampin, meropenem, linezolid, liposomal amphotericin B, trimethoprim-sulfamethoxazole, moxifloxacin, caspofungin, and miltefosine, yet treatment may be successful only at early stages [2,26,124,208,222,290,322–325]. Some agents such as quinoline nitroxoline are also considered potent against pathogenic amoebae [326]. Most agents exhibit low sensitivity to the amoebae or are unable to sufficiently cross the BBB into the CNS. Some available agents also exhibit severe side effects, which may result in the disability of GAE survivors [327,328].

Conclusions

The most successful strategy for the survival and multiplication of parasites is sustainably exploiting their hosts, yet many of these free-living organisms generally cause violent infections and kill the patients. Although rare, encephalitis caused by FLA especially *Acanthamoeba*, *Balamuthia*, and *Naegleria* is eliciting attention as an increasing cause of parasitic death worldwide. The low number of infections caused by FLA is probably due to diagnosis difficulty and the lack of experienced caregivers, so many cases may not have been recognized and diagnosed, especially in regions such as Africa and South Asia where facilities are either minimal or lacking. Along with global warming, ample evidence indicates that the available environmental niches for numerous FLA particularly *N. fowleri* can naturally increase. Meanwhile, the population is continuously growing and areas of human activity are also expanding in some regions. Multiple factors conspire to increased contact between human and FLA, leading to further increased number of infections. Patients with amoebic encephalitis in various regions usually have different clinical characteristics and prognoses. Considering that PAM and GAE are almost always fatal as less than 10% of patients are reported to survive, the cure rate urgently needs improvement through immediate diagnosis and early treatment. Studies focused on interactions between pathogenic amoebae and their host contribute to the development of various novel therapeutic drugs against the parasites. However, although numerous new findings have been reported in recent years, the whole picture is far from being complete and a series of topics on the pathogenic role of FLA in encephalitis require further research. Indeed, authoritative standards for the clinical diagnosis and early treatment of FLA encephalitis remain lacking. Future work should aim to address these points with investigations focusing on pathogenesis, clinical manifestations, diagnosis, and therapies.

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Compliance with ethics guidelines

Hongze Zhang and Xunjia Cheng declare no conflict of interest. This

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