### Three encephalitis-causing amoebae and their distinct interactions with the host

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#### Naegleria fowleri, Balamuthia mandrillaris, and Acanthamoeba spp.

These amoebae can cause devastating brain infections in humans which almost always result in death. The symptoms of the three infections overlap, but brain inflammation and the course of the disease differ, depending on the amoeba that is responsible. Understanding the differences between these amoebae can result in the development of strategies to prevent and treat these infections. Recently, numerous scientific advancements have been made in the understanding of pathogenicity mechanisms in general, and the basic biology, epidemiology, and the human immune response towards these amoebae in particular. In this review, we combine mechanisms in general, and the basic biology, epidemiology, and the human immune response with limited inflammation, whereas Naegleria fowleri evokes a proinflammatory immune response with excessive brain-tissue damage.

#### Free-living amoebae that go for the brain

**Free-living amoebae (FLA)** (see Glossary) are unicellular eukaryotes ubiquitously present in nature. Human brain infections by these amoebae have devastating effects and almost always result in death. Three different FLA are responsible for human brain infections: *N. fowleri,* *Acanthamoeba* spp., and *B. mandrillaris* [1]. *N. fowleri* causes primary amoebic meningoencephalitis (PAM), a rapid and acute infection characterized by necrotic and hemorrhagic patches in the brain [2–4]. In contrast, *B. mandrillaris* and *Acanthamoeba* spp. cause chronic but fatal granulomatous amoebic encephalitis (GAE), which has a slower onset and disease progression [3–6]. Although the brain infections by these three free-living amoebae cause similar clinical symptoms, the brain inflammation and disease course are quite distinct (summarized in Table 1). It is currently incompletely understood why these different free-living amoebae cause a different type of encephalitis. The host–parasite interaction is probably an important factor as recent advancements have shown that the immune response to the amoeba genera is different. *N. fowleri* induces an acute inflammatory response, mainly involving neutrophils and macrophages, the production of proinflammatory cytokines, and substantial tissue damage [7]. In contrast, the immune response to *Acanthamoeba* spp. and *B. mandrillaris* involves mainly macrophages and T cells and induces the formation of granulomas [3,5]. So far, little is known about the factors that determine the pathogenicity of the amoebae and the host factors that influence the pathogenicity [5–7]. Comparison of the epidemiology, pathogenicity, and clinical features of the three FLA can help to dissect the differences in disease between the three amoebic infections. This review aims to discuss which possible host and parasite factors can contribute to the differences between amoebic encephalitis caused by *N. fowleri,* *Acanthamoeba* spp., and *B. mandrillaris.*

#### The biology of amoebae

The three species of encephalitis-causing free-living amoebae are found in different branches of the eukaryotic evolutionary tree. *Acanthamoeba* spp. and *B. mandrillaris* are evolutionarily closely
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Table 1. Characteristics of N. fowleri, Acanthamoeba spp., and B. mandrillaris

<table>
<thead>
<tr>
<th>Presence in nature</th>
<th>Trophozite appearance</th>
<th>Food</th>
<th>Initial entry point</th>
<th>Route(s) of infection</th>
<th>Tropism</th>
<th>Infection of the brain</th>
<th>Risk factor(s)</th>
<th>Symptom(s)</th>
<th>Histopathology</th>
<th>Type of inflammation</th>
<th>Mortality of brain infection</th>
<th>Cases described in the literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshwater &gt;25°C</td>
<td>Irregular curved shape. Moves with broad pseudopods</td>
<td>Bacteria, algae, and yeast</td>
<td>Nasal mucosa</td>
<td>Through olfactory neuroepithelium</td>
<td>Brain</td>
<td>Primary amoebic meningoencephalitis (PAM)</td>
<td>Recreational activities in warm freshwater and nasal-cleaning rituals</td>
<td>Fever, headache, nausea, seizures, lethargy, coma</td>
<td>Necrosis, hemorrhage, angitis, inflammation</td>
<td>Neutrophilic</td>
<td>&gt;95%</td>
<td>431 [2]</td>
</tr>
<tr>
<td>Freshwater, brackish water, soil, dust, air</td>
<td>Angular shape. Moves with spiky pseudopods</td>
<td>Mainly soil, also freshwater and dust</td>
<td>Ulcerated or broken skin, and nasal passage to lungs</td>
<td>Hematogenous spread from skin or lungs</td>
<td>Skin, eye, brain</td>
<td>Granulomatous amoebic encephalitis (GAE)</td>
<td>Compromised immune system (~40% of patients)</td>
<td>Compromised immune system (~40% of patients)</td>
<td>Granulomatous</td>
<td>90-94%</td>
<td>83 [6]</td>
<td></td>
</tr>
<tr>
<td>Mainly soil, also freshwater and dust</td>
<td>Slender shape. Moves with finger-like pseudopods</td>
<td>Smaller amoebae, fungi</td>
<td>Through olfactory neuroepithelium</td>
<td>Hematogenous spread from skin or lungs</td>
<td>Skin, brain</td>
<td>Granulomatous amoebic encephalitis (GAE)</td>
<td>Soil exposure and (recreational) water activities</td>
<td>Severe encephalitic symptoms to death</td>
<td>Granulomatous</td>
<td>&gt;200 [6,104]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Glossary

Adaptive immune system: part of the immune system that targets a specific pathogen as it mounts an immune response against unique antigens. It is slow to develop, but protection can be very long-lasting.

Central nervous system (CNS): the combination of the brain and the spinal cord, which is described as an area with immune privilege, as it shows an attenuated response to antigens. Access to the CNS is restricted and possible only through certain barriers.

Cytokines: a group of small proteins used in cell signaling, most important in the communication between immune cells. Examples of proinflammatory cytokines are tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin 1 beta (IL-1β), IL-6, IL-8, and IL-12. An example of an anti-inflammatory cytokine is IL-10.

Extracellular matrix (ECM): a three-dimensional network made of fibrous proteins and proteoglycans that provides structural and biochemical support to surrounding cells.

Free-living amoebae (FLA): eukaryotic unicellular organisms that can live freely in the environment but can also enter a host as an opportunistic pathogen. Three free-living amoebae are well known human pathogens: N. fowleri, B. mandrillaris, and Acanthamoeba spp.

Granuloma: an area, with a high density of immune cells, which is formed in response to chronic inflammation. Granulomas predominantly consist of mature macrophages which try to encapsulate the pathogen from the body and facilitate its eradication.

Granulomatous amoebic encephalitis (GAE): an infection of the brain by either Acanthamoeba spp. or B. mandrillaris. It is characterized by slow disease progression and granuloma formation.

Human brain microvascular endothelial cells (HBMECs): cells that are the major component of the blood–brain barrier; they are often used in in vitro studies.

Immune status: the ability of the immune system to fight off microorganisms. Immunocompetent people can clear most microorganisms whereas immunocompromised people are less capable due to a reduction of the activation or the efficacy of the immune system. This can be the result of a

related as both are centramoeboids that are included in the Amoebozoa group, which was earlier considered to be a supergroup but is now often regarded as a member of the supergroup Amorphea [8]. In contrast, N. fowleri is a member of the Heterolobosea, part of the supergroup Discoba [3]. All three species can exist in trophozoite and cyst stages, but only N. fowleri can also transform into a flagellate stage (Figure 1). In all species, the trophozoite is the feeding stage that actively moves and replicates. All three species can transform into a cyst form when the environment is not suitable for continued feeding and growth (such as cold temperatures, or shortage of nutrients). Cysts are environmentally resistant and increase the chances of survival until better environmental conditions occur. Regarding the metabolism of the three FLA, Acanthamoeba spp. as well as N. fowleri have aerobically functioning mitochondria with Krebs cycle activity, an electron-transport chain and oxidative phosphorylation, and they need oxygen for normal functioning and growth [9–11]. The metabolism of B. mandrillaris has not yet been investigated, indicating the need for future studies.
Naegleria fowleri

Invasion of the brain occurs directly via the nose

The route of infection used by *N. fowleri*, and the clinical aspects, are briefly presented in Box 1. *N. fowleri* enters the human body via the nose and penetrates the olfactory mucosa, after which it moves to the brain and causes PAM (Figure 2). For successful infection, *N. fowleri* needs to adhere to the olfactory mucosa. In this process, α-D-mannose and α-L-fucose residues on the amoebae are involved. Secretory immunoglobulin (Ig) A and mucus prevent adhesion of *N. fowleri* to apical membrane epithelial cells and, after invasion, also inhibit basolaterally the adhesion to collagen (Figure 2A) [12–14]. However, *N. fowleri* counters this by secreting mucin-degrading proteins such as glycosidases [15]. Epithelial cells recognize *N. fowleri* through Toll-like receptor 4 (TLR4) and react with a proinflammatory response by producing interleukin-1-beta (IL-1β), IL-8, and tumor necrosis factor-alpha (TNF-α) (Figure 2B) [16,17]. In mice, this immune response will recruit leukocytes to the area, and neutrophils will bind to amoebae opsonized with IgA [12]. Mucus production, IgA secretion, and neutrophil influx are mechanisms that slow down or even clear the *N. fowleri* infection in the nose. However, if these mechanisms are absent, or if *N. fowleri* can evade these attacks, binding to the epithelial lining will occur and tissue invasion starts. *N. fowleri* will then disrupt the tight junctions connecting the epithelial cells to make its way through the epithelial lining without causing destruction (Figure 2B) [18]. By using this strategy, there will be no products originating from apoptotic or necrotic cells that attract and activate immune cells, which
**Box 1. N. fowleri – route of infection and clinical aspects**

*N. fowleri* is a world-wide-distributed free-living amoeba that lives in warm water and occasionally infects humans. Many infections with *N. fowleri* occur during swimming in water heated by the sun, or thermally polluted by industries [100]. Among the many identified species of *Naegleria*, *N. fowleri* is the only one that is a human pathogen, causing an acute encephalitis. Despite extensive studies, it is still unknown what makes *N. fowleri* pathogenic – in contrast to *N. gruberi* and other *Naegleria* species which are nonpathogenic [101]. *N. fowleri* enters the brain via the olfactory neuroepithelium. Infection by *N. fowleri* is most commonly associated with activities that result in water entering the nose, such as water recreation or ablution rituals [2–4]. Deep inhalation of infected water brings the trophozoites to the upper regions of the nose, where they attach and invade the olfactory epithelium (see Figure 2 in main text). Subsequently, the trophozoites migrate through the lamina propria and the cribriform plate towards the olfactory bulb [2–4]. This neuro-olfactory route circumvents the normal barriers that protect the CNS and provides access to the brain without much resistance [24]. However, once *N. fowleri* arrives in the brain, inflammation and tissue damage are extensive, leading to primary amoebic meningoencephalitis (PAM). *N. fowleri* trophozoites have only sporadically been observed outside the CNS.

PAM is a severe and fast-developing disease, as symptoms develop 5–7 days after contact and can rapidly progress, with patients usually dying 7–10 days after the appearance of the first symptoms [2–4]. Diagnosis is difficult, as PAM symptoms (fever, headache, nausea, lethargy, coma) overlap with symptoms from a bacterial or viral meningitis. PAM is a rare disease, as only 431 case descriptions have been published according to a recent review, although many more cases are probably not reported [2]. Furthermore, it is assumed that even more cases remain undiagnosed as *N. fowleri* is often not suspected or diagnostic tools are not available [102]. Antibodies against *N. fowleri* (IgA, IgM, and IgG) are present in a high number of children and adolescents [83], hospitalized patients [91], and healthy adults [103]. However, this high prevalence could also reflect exposure to *Naegleria lovaniensis*, a widely present nonpathogenic relative of *N. fowleri*, leading to cross-reactive antibodies recognizing *N. fowleri* antigens [91].

probably enables *N. fowleri* to invade without eliciting a strong immune response. Once past the epithelial lining, *N. fowleri* adheres to the extracellular matrix (ECM) by an integrin-like protein [19] and secretes a range of proteases [20–23] to break down the ECM (Figure 2C). In this way, *N. fowleri* penetrates the epithelium and swiftly migrates towards the central nervous system (CNS).

**Infection of the brain occurs very rapidly and attracts neutrophils**

Although replication of *N. fowleri* starts already after arrival in the olfactory bulb, this does not immediately result in brain inflammation or damage [24]. The first influx of immune cells is seen approximately 4 days postinfection and consists of eosinophils and neutrophils. A decline in eosinophils and recruitment of additional neutrophils follows, together with the influx of macrophages [24]. At a later stage, tissue damage occurs, consisting of extensive necrotic areas, hemorrhage, and formation of cellular debris [24].

Neutrophilic inflammation and concurrent damage are abundant in PAM, and the neutrophils then produce extracellular traps (NETs) and secrete myeloperoxidase (MPO) (Figure 2D) [25–28]. NETs consist of DNA fibers that immobilize *N. fowleri*, whereas MPO damages *N. fowleri* as well as the surrounding cells. *N. fowleri* reacts to MPO by overexpressing antioxidants such as glutathione peroxidase, superoxide dismutase, catalase, thioredoxin reductase, and peroxiredoxin, which neutralize the effects of MPO and thus improve survival of *N. fowleri* [28]. However, neutrophils can kill *N. fowleri* if they are opsonized with IgG and/or IgA, indicating that previous contact with *N. fowleri* promotes protection [27]. Furthermore, in vitro experiments indicate that *N. fowleri* induces a proinflammatory cytokine response in microglia and brain microvascular endothelial cells. This response leads to an increased expression of IL-6, IL-1β, and TNF-α in microglia (Figure 2E,H) [29–31], and of IL-6 and IL-1β in astrocytes (Figure 2G) [32]. This proinflammatory cytokine response boosts the recruitment of immune cells and subsequent inflammation. Macrophages are recruited, although the battle between *N. fowleri* and macrophages can go either way [24,25,33]. *N. fowleri* can damage macrophages, but *N. fowleri* can also be the victim, as direct cytolytic mechanisms of activated macrophages can lyse *N. fowleri* [34,35]. These direct cytolytic mechanisms include the production of nitric oxide and have been described as
N. fowleri can also produce nitric oxide and reactive oxygen species, which participate in damaging brain cells and T cells (Figure 2F) [36,37]. N. fowleri causes damage to brain cells by several cytopathic strategies, one of which involves specialized structures, called food cups, that are used to damage cells in a way similar to trogocytosis (Figure 2H) [35,38]. Furthermore, the aforementioned proteolytic secretions described to degrade ECM in the nose (Figure 2C) can degrade the brain ECM, which is essential for several vital functions [39]. However, brain damage during PAM is probably caused mainly by the over-reacting host immune response, as less damage is present in areas of the brain with trophozoites, but without immune cells [24]. Inflammation is regulated by the adaptive immune system, and indeed an in vivo study demonstrated that protection of mice immunized against N. fowleri occurred via an anti-inflammatory Th2-biased immune response [40]. In nonphagocytic events [34]. Conversely, N. fowleri can also produce nitric oxide and reactive oxygen species, which participate in damaging brain cells and T cells (Figure 2F) [36,37].
addition, in CD38 knockout mice lacking natural killer cells, T cells, and B cells, inflammation and mortality were delayed after infection with *N. fowleri* [24].

*Acanthamoeba* spp.

**Invasion of the brain occurs via lungs and skin**

The route of infection used by *Acanthamoeba* spp., and the clinical aspects, are briefly presented in Box 2. *Acanthamoeba* spp. can access the brain through two different routes of infection (Figure 3), via the lungs and via the skin. Therefore it has to be able to attach to multiple cell types. *Acanthamoeba* spp. can also invade the corneal surface of the eye and destroy monolayers of skin keratinocytes, which can result in severe keratitis in otherwise healthy persons [3,41]. Adhesion studies focused mostly on attachment to corneal epithelial cells to mimic *Acanthamoeba* keratitis, revealing the involvement of amannose-binding protein [42], but it is unknown whether this transmembrane protein on the surface of the amoebae is also important in *Acanthamoeba* adhesion to skin or lung or epithelial cells. In mice, adherence and invasion of the epithelium occur without apparent damage to epithelial cells [43,44]. While earlier in vitro studies described cytopathic effects of *Acanthamoeba* spp. on epithelial cells, more recent studies showed that *Acanthamoeba* trophozoites can degrade claudin 2 in vitro, impairing tight-junction function and allowing invasion without damaging the cells (Figure 3A) [43,45]. During the invasion, *Acanthamoeba* trophozoites produce a range of proteases that break down collagen and elastin (major components of the ECM) and hemoglobin [46,47].

The hematological route requires *Acanthamoeba* spp. to survive in blood, which is challenging, and different outcomes have been reported when the amoebae were exposed to human serum (Figure 3B). *Acanthamoeba* trophozoites can be lysed by complement activation via the alternative pathway [48] and they can be killed by neutrophils and macrophages in the presence of serum [49]. However, in other studies, a small *Acanthamoeba* subpopulation survived in undiluted serum [50] or a substantial population in diluted serum [51]. Furthermore, *Acanthamoeba* spp. degrade components of the complement system, which will support survival in blood [52]. *Acanthamoeba* spp. can also phagocytose and degrade erythrocytes [53]. Altogether, *Acanthamoeba* spp. may survive in blood if conditions are favorable, for instance, when the

**Box 2. *Acanthamoeba* spp. – route of infection and clinical aspects**

*Acanthamoeba* spp. are opportunistic parasites of humans. The exposures leading to acanthamoebiasis are generally unknown but are thought to be soil or water exposures [104]. *Acanthamoeba* spp. can enter via the lower respiratory tract or via lesions in the skin, and, in immunocompromised persons, can then invade the CNS via the bloodstream – or infection can result in skin lesions with or without CNS involvement. Apart from infections via the lungs or broken skin, *Acanthamoeba* spp. can also infect the eye; this can result in severe keratitis in otherwise healthy persons. Because of the slow development of granulomatous amoebic encephalitis (GAE), the exact route of *Acanthamoeba* spp. to the brain in humans is not always clear, but the lungs and skin are thought to be the most important points of entry [104]. The amoebae will then reach the brain via hematogenous spreading [104]. A recent murine model, evaluating an *Acanthamoeba* skin infection, identified the presence of amoebae in the brain only after the infected skin had been chronically irradiated by UV-B light [58]. This corresponds to the idea that a breach in the skin is required for *Acanthamoeba* to access the bloodstream from the skin.

*Acanthamoeba* GAE symptoms consist of fever, headache, nausea, seizures, lethargy, and coma. *Acanthamoeba* GAE is often a slow and chronic disease, although quick progression sometimes occurs [5,106]. The time from *Acanthamoeba* contact to the start of symptoms is often unclear as it is difficult to trace back the initial contact due to its ubiquitous presence and multiple possible routes of infection. Furthermore, a thorough study of the epidemiological features and clinical characteristics of *Acanthamoeba* spp. GAE is still lacking. Once symptoms have started, it can take one to two months until death occurs [3]. At least 83 human cases have been reported, although many more cases have probably occurred as *Acanthamoeba* GAE is seldom suspected and diagnostic tools might not be available [5]. Antibodies against *Acanthamoeba* (IgG, IgG, and IgG) are universally present in the healthy population, which is in line with the ubiquitous environmental presence of *Acanthamoeba* spp. [89]. Although *Acanthamoeba* GAE usually develops in immunocompromised patients, it can occur in immunocompetent individuals as well [6].
Once Acanthamoeba trophozoites have spread in the blood, the next barrier to breach is the blood–brain barrier (BBB). Several factors are important in this process, contact-dependent as well as contact-independent factors (Figure 3C). Acanthamoeba spp. produce proteases that degrade tight-junction proteins of human brain microvascular endothelial cells (HBMECs), providing access to the brain through movement between the cells [47]. Acanthamoeba spp. can also induce programmed cell death in HBMECs in vitro [54]. The extent to which Acanthamoeba trophozoites use either their BBB-damaging capacities or their strategy for stealthy brain invasion by moving in between cells is unknown.

Infection of the brain is chronic and includes granuloma formation
Acanthamoeba infection is associated with the recruitment to the brain of a variety of immune cells: T cells, macrophages, dendritic cells, neutrophils, B cells, and natural killer cells [55]. Granulomas are formed in reaction to Acanthamoeba spp. in the brain [25].
Several in vitro studies characterized the interactions of *Acanthamoeba* spp. with macrophages, microglia, T cells, Schwann cells, and neuroblastoma cells. The interactions of microglia and *Acanthamoeba* spp. have been studied extensively and this showed that the outcome of the combination of T cells, microglia, and *Acanthamoeba* is dependent on IFN-γ release by T cells, which induces IL-6 and TNF-α release by microglia and the destruction of *A. castellanii* (Figure 3D) [56]. The proinflammatory response by microglia results in the production of IL-1α, IL-1β, and TNF-α [57,58]. Interestingly, the outcome can be different for distinct *Acanthamoeba* species, as *A. castellanii* is killed by microglia whereas *A. culbertsoni* kills the microglia [57–59]. Extracellular vesicles and peptidases are postulated as mechanisms by which *Acanthamoeba* trophozoites kill glioma cells (Figure 3E) [59,60]. *Acanthamoeba culbertsoni* produces a specific cytotoxic pore-forming protein that lyses neuroblastoma cells (Figure 3F) [61]. Furthermore, *Acanthamoeba* spp. can induce apoptosis in neuroblastoma cells via caspases and Bax-proteins [62]. In vitro attachment of *A. culbertsoni* to Schwann cells results in necrosis and autophagy [63].

In vitro interactions between macrophages and various *Acanthamoeba* spp. are different. *A. culbertsoni* destroys macrophages, whereas *A. polyphaga* and *A. castellanii* show limited destruction, although this balance depends on activation of the macrophages, as activated macrophages damage or even phagocytose these amoebae [64]. TLRs are involved in the activation of macrophages, and it was shown that TLR4 is important in the recognition of, and response to, *Acanthamoeba* spp. [65]. Macrophages release a mixture of cytokines in response to *Acanthamoeba* spp., which consists of TNF-α, IL-6, IL-10, and IL-12, mediated through MyD88 and PAR1 (Figure 3G) [65–67]. TNF-α, IL-6, and IL-12 are regarded as proinflammatory cytokines, but the significant production of the anti-inflammatory cytokine IL-10 suggests a mixed-type immune response which might allow immune evasion of *Acanthamoeba* and limit the inflammatory response [66]. Furthermore, pathogenic *Acanthamoeba* strains induce a mixed-type immune response, whereas nonpathogenic *Acanthamoeba* strains induce mainly a proinflammatory response [67,68], which could result in rapid elimination of nonpathogenic *Acanthamoeba* spp.

*Acanthamoeba* spp. can partially evade the inflammatory response by transforming into a cyst form as intact *Acanthamoeba* cysts do not attract macrophages or neutrophils in vitro [69]. However, in mice challenged with formalin-fixed cysts, anti-*Acanthamoeba* IgG production and T cell proliferation occur, showing that cysts are immunogenic and antigenic [70]. Furthermore, cysts can be phagocytosed by macrophages and are killed by neutrophils through the secretion of MPO [69].

The T cell response might be important in *Acanthamoeba* infections as there are differences in T cell response between immunocompetent mice and methylprednisolone-induced immunocompromised mice after nasal *Acanthamoeba* infection. In immunocompetent mice, selective Th1, Th2, and Th17 responses are induced, whereas in immunocompromised mice, *Acanthamoeba* spp. induce a robust Th1-mediated immunity without the participation of Th17 [71]. In humans, the type of T cell response also seems to be important, as proinflammatory T cell clones directed against *Acanthamoeba* spp. are found in healthy individuals [72].

*Balamuthia mandrillaris*

Invasion of the brain occurs via lungs and skin

The route of infection used by *B. mandrillaris*, and the clinical aspects, are briefly presented in Box 3. A schematic overview of the host–pathogen interactions used by *B. mandrillaris* can be seen in Figure 4. *B. mandrillaris* can destroy and feed on mammalian cells in vitro by invading the cells with its pseudopods first, before fully entering and consuming the cytoplasm (Figure 4A).
The nucleus is consumed later, whereafter the amoeba invades another cell. It is hypothesized that the intracellular location of *B. mandrillaris* facilitates evasion of the immune system [73]. *B. mandrillaris* kills human skin fibroblasts, but not keratinocyte cells, indicating that *B. mandrillaris* cannot breach the intact epidermis, and it has to invade via broken or ulcerated skin [41]. Once past the epidermis, *in vitro* studies showed that *B. mandrillaris* can kill mastocytoma cells efficiently, mainly in a contact-dependent manner [74]. *Balamuthia* is known to ingest bits and pieces of host tissue and to produce enzymes that degrade the tissue [4]. *B. mandrillaris* is thought to enter the brain via the bloodstream. The next barrier is the BBB, to which *B. mandrillaris* binds via a galactose-binding protein (Figure 4B) [75]. *B. mandrillaris* secretes proteases and an ecto-ATPase, but most of the cellular damage is caused via contact-dependent mechanisms [74,76]. The secreted proteases are hypothesized to facilitate *B. mandrillaris* infection by degrading ECM proteins (Figure 4C) [77].

Infection of the brain is chronic and includes granuloma formation

Once in the brain, trophozoites aggregate around blood vessels and induce a broad immune response in mice and humans [25,78]. Numerous lymphocytes and macrophages, as well as some eosinophils and multinucleated giant cells, are seen in the brains of mice infected with *B. mandrillaris* [79]. The immune response is important to survive a *B. mandrillaris* infection. The immune response is absent in brains of severe combined immunodeficiency (SCID) mice, which are deficient in B and T lymphocytes and thus lack immunoglobulins and cell-mediated immunity. Of SCID mice, 70% died after intranasal inoculation, compared to 10% of normal mice [79]. This difference is also observed in human cases, as histopathological examination of brains with a *B. mandrillaris* encephalitis revealed a broad spectrum, ranging from acute neutrophilic inflammation to granulomatous inflammation, possibly reflecting the immune status of the affected patient [25].

The T cell response is thought to be essential, as SCID mice survived infection if they received spleen cells from wild-type mice, but died if the CD4+ T cells were removed from these spleen cells [80]. Furthermore, wild-type mice that were depleted of CD4+ T cells were susceptible to
infection [80]. These T cells are thus thought to play an essential role in curbing a *B. mandrillaris* encephalitis by the recruitment and activation of macrophages and granulocytes.

**Comparison of the three amoeba species**

There are evident differences and similarities between the brain infections caused by *N. fowleri*, *Acanthamoeba* spp., and *B. mandrillaris*. Differences include the environmental presence of the amoebae, the route of infection, the pathogenicity mechanisms, the immune response that is induced, and the immune status of the host. Could these factors explain the differences between the three diseases caused by these amoebae? Conversely, it should also be realized that *Naegleria*, a member of the Heterolobosea, is evolutionarily very distant from *Acanthamoeba* and *Balamuthia*, which are both members of the Amoebozoa. It is unknown to what extent these evolutionary aspects might explain the differences and similarities between the three brain infections. In this respect it is tempting to speculate that an evolutionary background exists which could explain why both of these two Amoebozoa induce a chronic granulomatous brain infection while *N. fowleri* induces a rapid neutrophilic infection.

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Figure 4. Host–pathogen interactions during *Balamuthia mandrillaris* infection. *B. mandrillaris* (Bm) can enter the human body via the skin [SC (skin cells)] or the lower respiratory tract. Bm can induce cytopathic effects on epithelial cells (EP) by penetration of finger-like projections (oval A). After the epithelial layer, Bm can efficiently kill mastocytoma cells (MC). After infection via the skin and lung, Bm reaches endothelial cells (EN) and enters the bloodstream. The blood–brain barrier is breached using cytopathic effects such as a galactose-binding protein (GBP) and ATPase (oval B). EN react by the production of the proinflammatory cytokine IL-6 (oval B). Bm can bind to, and break down, the extracellular matrix (ECM) (oval C). In the brain, cyst forms are sometimes found (C), alongside neutrophils (NE), T cells (T), and macrophages (M). Killed cells are indicated with gray color, proinflammatory cytokines are indicated in green. The icons in this figure are adaptations from icons in the Servier Medical Art collection (https://smart.servier.com).
Environmental presence

Differences exist in the environmental presence of the three FLA, as *Acanthamoeba* spp. are found more often in natural bodies of water [81,82], swimming pools [83], water-treatment plants [82], and drinking water [84] compared to *N. fowleri* and *B. mandrillaris*. It is intriguing that *Acanthamoeba* spp. are the most abundant pathogenic FLA yet cause fewer brain infections than *N. fowleri* and *B. mandrillaris*. This ubiquitous presence of *Acanthamoeba* spp. correlates with the high seropositivity rate among the general population [85,86]. The seropositivity rate for *B. mandrillaris*, which is far less widespread, is low in high-income countries in moderate climates and higher in rural Africa [87,88]. The seropositivity rate of *N. fowleri* was reported to be very high in the 1980s but more recent data are not available [89–91].

The efficiency of brain infection by FLA seems very low, considering the rather limited incidence of such infections, despite the widespread presence of them in the environment [92–94]. Using serological methods, attempts have been made to estimate the human exposure to FLA. These studies showed that specific antibodies can be demonstrated in PAM and GAE patients, and that cross-reactivity between FLA infections is limited [87,93,95]. In addition, these studies showed that the sera of healthy volunteers demonstrate some reactivity and if the reactivity in the sera of healthy volunteers against the three FLA are compared, it seems that most reactivity is found against *Balamuthia* and *Acanthamoeba*, which could suggest that contact with those FLA occurs more frequently than with *N. fowleri* [89,93,95]. However, it is questionable whether an observed low reactivity is specific and really reflects previous exposure or a past infection. Reliable determination of the exposure rate in healthy humans cannot be determined reliably by serological methods, and therefore the exposure:infection ratio is still unknown.

The concentration of the different amoebae in the environment also varies, and this results in exposure to different numbers of amoebae. *N. fowleri* is thermophilic and can be present in up to hundreds of amoebae per liter of water when conditions are favorable, such as in geothermal baths and in the cooling water of power plants [96]. *Acanthamoeba* spp. and *B. mandrillaris* are found not only in watery environments but also in soil and dust. Their presence in the environment was mostly determined using qualitative molecular tools, allowing no direct comparison with the presence of *N. fowleri* [82,97]. A comprehensive study on the numbers of FLA present in their respective environments would be valuable.

Routes of infection

The route of infection taken by the FLA is likely to impact the disease progression. For infections of *Acanthamoeba* spp. and *B. mandrillaris*, the route is often unknown, but these amoebae rarely, if ever, use the neuro-olfactory route, the one and only route of *N. fowleri*. As the neuro-olfactory route results in fast access to the brain, the adaptive immune response is probably insufficient, which might promote rapid disease progression. The hematogenous routes of infection used by *Acanthamoeba* spp. and *B. mandrillaris* are lengthier and, obviously, involve the bloodstream, which normally results in a strong immune response that combats the amoebae. The amoebae counter the actions of the immune system, and the result is a more chronic disease course that is similar to other immune-evading infectious diseases. Many animal studies of *Acanthamoeba* spp. and *B. mandrillaris* used intranasal infection, with a recent exception describing successful *Acanthamoeba* brain infection originating from irradiated skin [98]. Studies on the lung or skin infection routes in mice are most likely more applicable to the human situation. Furthermore, proper determination of the relative importance of the different infective routes in humans by *Acanthamoeba* spp. and *B. mandrillaris* could be valuable, as this could lead to more targeted preventative measures.
Biology and pathogenicity mechanisms

The difference in course of the disease could be due to inherent biological characteristics of the amoebae, such as speed of movement, reproduction rate, and pathogenicity mechanisms. Most studies investigating these factors are in vitro studies, limiting the clinical translation of the results. An important factor within these in vitro studies is the temperature at which the experiments are performed. Generally, Acanthamoeba spp. grow best at 25°C, with the ability to grow at higher temperatures, where thermotolerance correlates with pathogenicity of the amoebae [99]. In numerous in vitro studies, Acanthamoeba cultures are maintained at 25°C or 30°C and experiments are not performed at 37°C. Despite these differences in cultivation, multiplication time and speed of movement are not distinctly different between the three amoebae in a nutrient-rich medium, although this condition is hardly comparable to the human body. Furthermore, all three free-living amoebae have several pathogenicity mechanisms, of which the mechanisms of N. fowleri and Acanthamoeba spp. have been studied in most detail, but mainly in vitro. All three amoebae use contact-independent and contact-dependent mechanisms to damage human cells or ECM, comprising protease secretion and phagocytic strategies. For instance, N. fowleri and Acanthamoeba spp. both invade tissues by migrating in between cells, as they break down the intercellular junctions and secrete a wide range of proteases, facilitating migration through the extracellular matrix. However, the extent to which these pathogenicity mechanisms are active and critical in vivo will decide the course of infection. Therefore, the pathogenicity mechanisms of the amoebae have to be studied in more detail, especially in studies describing the different amoebic species side by side in similar, preferably in vivo, conditions.

Immune response

A balanced immune response is paramount to prevent infection but also to prevent the immune system from damaging host tissue (Box 4). The human immune response is quite different in the

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Box 4. The YIN and YANG of the innate immune response

The powerful innate immune response has to tune its potential to the danger of the threat to prevent collateral damage.

**YANG, the sunny side**

Fast and robust cytotoxic responses are essential to combat and neutralize invading microorganisms [110]. The effector cells of the innate immune response are phagocytes that can engage with the pathogens either intracellularly, after phagocytosis, or extracellularly by releasing toxic compounds in the close vicinity of the invaders. These killing mechanisms rely mainly on the production of reactive oxygen species produced by a membrane-bound NADPH-oxidase and degradation of cytotoxic proteins by the fusing of granules containing these proteins with the membranes of the phagolysosome (intracellular killing) or the plasma membrane (extracellular killing) [111]. Intracellular killing is important for targets that are smaller than immune cells and can be phagocytosed. Neutrophils and, to a lesser extent, monocytes, mainly utilize this route. Larger targets, such as multicellular parasites, are killed extracellularly as they are too big to be phagocytosed. Eosinophils are specialized for attacking large targets [112], although neutrophils can contribute through the release of first granules and then their DNA which, in turn, forms neutrophil extracellular traps (NETs) [113].

**YIN, the shady side**

Robust innate immune responses run the risk of hyperactivation, that is, more activation than is necessary for the killing of the target. Under these conditions, the surrounding tissues are damaged as well [114]. This can even lead to a situation in which damage caused by the innate immune response outweighs the importance of the killing of the invading microorganism(s). This situation can lead to acute inflammatory responses with massive tissue damage, such as seen during sepsis caused by meningococci [115]. In addition, chronic activation can lead to continuous damage to the host tissue which is seen in a multitude of chronic inflammatory diseases such as asthma, inflammatory bowel disease, and autoimmune diseases. This tissue damage can even help microorganisms to enter tissues normally protected by adequate barriers.

A healthy immune response is balanced, such that sufficient activity leads to the containment of the invading microorganism without causing tissue damage. Therefore, in infectious diseases, as well as in inflammatory diseases, treatment should be focused on restoring this balance – preventing the collateral damage that can facilitate a porte-d’entrée for more or different pathogens. In conclusion, the innate immune response is necessary to engage with free-living amoebae but this response should be contained to prevent tissue damage that potentially facilitates the infection.
different forms of encephalitis caused by the three amoeba species. *N. fowleri* PAM patients show an acute neutrophilic inflammation whereas *Acanthamoeba* GAE results in granulomatous inflammation of the brain. Brain tissue of patients with *B. mandrillaris* GAE showed either acute neutrophilic inflammation or granulomatous inflammation. Differences in immune response were also observed in mice, as intranasal infection of immunocompetent mice with *N. fowleri* or *Acanthamoeba* spp. resulted in extensive brain tissue damage and inflammation 96 h after infection with *N. fowleri*, whereas brains of the same breed of mice infected with *Acanthamoeba* spp. showed very limited inflammation at that same time after infection [24,44]. A possible explanation can be the cytokine response after infection by the different amoebae, as several cytokines are produced in response to pathogenic *Acanthamoeba* spp. but not in reaction to *N. fowleri*. One of these cytokines, IL-10, is considered to be a key regulator of the innate immune response and suppresses inflammation and macrophage activity. IL-12, IL-4, and IL-17 were also produced in reaction to *Acanthamoeba* spp. but not in response to *N. fowleri*. These cytokines are involved in T cell regulation, promoting the differentiation of CD4+ T cells into Th1, Th2, and Th17, respectively. This differentiation is impaired in immunocompromised mice infected with *Acanthamoeba* spp., indicating the importance of the T cell lineages. Furthermore, CD4+ T cells are essential for mice to survive a *B. mandrillaris* infection [80]. However, the immune response could also be contraproductive for the host as brain damage in an *N. fowleri* infection corresponded to the intensity of the host immune response in vivo [24]. The extent to which the immune response is responsible for the clinical course in *Acanthamoeba* spp. or *B. mandrillaris* GAE is unknown. Further research should focus on the identification of key features of the immune response to the three different amoebae in order to build a comprehensive map of the disease pathophysiology.

**Host immune status**

Another major difference between the three different amoebic brain infections is the immune status of the affected patients. *N. fowleri* affects healthy and relatively young individuals whereas *Acanthamoeba* spp. specifically target immunocompromised patients. *B. mandrillaris* can infect immunocompetent individuals, but immunocompromised individuals are affected at a far higher rate. This could be a result of several factors, one of which is the route of infection. *N. fowleri* solely uses the olfactory route and does not survive well in the bloodstream. *Acanthamoeba* spp. and *B. mandrillaris* usually reach the brain through the bloodstream, which is a hostile environment for pathogens. In immunocompetent people, the immune system is better able to kill *Acanthamoeba* spp. and *B. mandrillaris* that are present in the blood, eradicating the infection before the brain is reached. In immunocompromised patients, this barrier is easier to breach, possibly leading to higher rates of infection in this group.

**Concluding remarks**

Brain infections by free-living amoebae pose a small, but intimidating, threat to society. Understanding how these amoebae operate is the basis of the development of strategies to prevent and treat these infections. Several different subjects still require attention (see Outstanding questions). As these infections are under-reported, it is imperative to estimate the current burden of amoebic encephalitis and to know whether the infections are indeed increasing or just better registered. The striking difference in clinical course between PAM and GAE can only be explained by multiple factors. The route of infection, as well as the immune response, probably play a key role. *N. fowleri* circumvents the immune system by travelling via the olfactory nerve to the CNS. Upon arrival, the immune response is fierce, resulting in the influx of neutrophils with subsequent damage to brain tissue. This is in contrast to the route taken by *Acanthamoeba* spp. and *B. mandrillaris*, which first have to deal with the immune system in the blood. Therefore, in this case, a strategy evolved to remain relatively undetected, resulting in a more chronic disease.

**Outstanding questions**

- What is the actual burden of amoebic encephalitis currently?
- Is exposure of humans to an infectious dose of *N. fowleri*, *Acanthamoeba* spp., and *B. mandrillaris* increasing?
- What factors of these amoebae are crucial in the type and extent of the immune response?
- Do genetic factors of the host influence the susceptibility to a brain infection?
- Do genetic factors of the amoebae influence their pathogenicity?
course and a slowly building immune response. In the end, however, each of the three amoebic brain infections leads to death in almost all cases.

In recent years, good progress has been made in understanding host–parasite interactions but challenging questions remain to be answered. Future studies using different strains of mice could solve questions on the genetic factors of the host that influence the susceptibility for a brain infection. Preferably, these experiments should be performed with distinct amoeba strains to identify genetic factors that influence their pathogenicity. This will also shed some light on the limited incidence of FLA brain infections despite the wide spread of these amoebae in the environment.

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Declarations of interests
The authors declare no competing interests.

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