Title: Microglial immune response is impaired against the neurotropic fungus *Lomentospora prolificans*

Running title: Impaired microglial response against *L. prolificans*

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Summary

Lomentospora (Scedosporium) prolificans is an opportunistic pathogen capable of causing invasive infections in immunocompromised patients. The fungus is able to disseminate via the bloodstream finally arriving at the central nervous system (CNS) producing neurological symptoms and in many cases, patient death. In this context, microglial cells, which are the resident immune cells in the CNS, may play an important role in these infections. However, this aspect of anti-*L. prolificans* immunity has been poorly researched to date. Thus, the interactions and activity of microglial cells against *L. prolificans* were analyzed, and the results show that there was a remarkable impairment in their performance regarding phagocytosis, the development of oxidative burst, and in the production of pro-inflammatory cytokines, compared to macrophages. Interestingly, *L. prolificans* displays great growth also when challenged with immune cells, even when inside them. We also proved that microglial phagocytosis of the fungus is highly dependent on mannose receptor and especially, on dectin-1. Taken together these data provide evidence for an impaired microglial response against *L. prolificans* and contribute to understanding the pathobiology of its neurotropism.

Keywords: microglia, CNS infections, filamentous fungi, antifungal immunity.

Introduction

Fungal infections, especially those with invasive profiles, have become a significant cause of mortality among those population groups at risk, such as immunocompromised patients. While immune antifungal responses to relevant fungi, such as *Aspergillus* or *Candida* (Romani, 2004; Romani, 2011; Becker *et al.*, 2015), have been extensively studied, little knowledge on immunity to most other infectious fungi has been published, especially with respect to emerging or endemic pathogens whose pathobiology, physiology, or virulence show remarkable differences from these two important genera mentioned above (Brown *et al.*, 2012).

Of these emerging pathogens, *Lomentospora (Scedosporium) prolificans* (Lackner *et al.*, 2014) represents a great clinical challenge, above all due to its high inherent resistance to all available antifungal compounds (reviewed in Pellon *et al.*, 2017). Moreover, the ability to produce conidia inside host body fluids and tissues allows this fungus to spread easily via the bloodstream and in consequence, to colonize other organs separated from the point of entry. In the case where the host is deeply immunocompromised, *L. prolificans* tends to invade the Central Nervous System (CNS), causing serious neurological disorders and usually causes patient death (Cortez *et al.*, 2008; Rodriguez-Tudela *et al.*, 2009).

Innate immune responses to filamentous fungi comprise both cellular and molecular events. Among others, phagocytic cells are one of the first cell types activated, being able to detect, phagocytize, and kill fungal cells, while also participating in the development of more complex and specific responses (Brakhage *et al.*, 2010). While the adaptive humoral response to *L. prolificans* has been analyzed (Pellon *et al.*, 2014; Pellon *et al.*, 2016; Buldain *et al.*, 2016), to date few studies focusing on the interactions of phagocytic cells with this pathogen, or its molecular recognition, have been published. Interestingly, these papers demonstrated the ability

of human polymorphonuclear (PMN) and monocyte-derived macrophages (MDM) to successfully phagocytize *L. prolificans*, to promote hyphal damage in a reactive oxygen species (ROS)-dependent manner, and to induce the production of the relevant pro-inflammatory cytokines TNF-α and IL-6 (reviewed in Roilides *et al.*, 2009). In turn, the participation of pattern recognition receptors (PRR) in *L. prolificans* sensing has been poorly described, with only indirect evidence of the involvement of Toll-like receptors (Lamaris *et al.*, 2007) and dectin-1 (Lamaris *et al.*, 2008) being available.

Microglial cells are the resident immune cells in the CNS, and are the most important of the cell types that tackle invading microorganisms upon their arrival in the brain. These phagocytic cells are usually in a quiescent state, maintaining homeostasis in the brain (e.g. as scavenger cells removing dead cells or protein aggregates). However, microbial molecules (such as pathogen-associated molecular patterns or PAMPs) may activate them, dramatically changing their morphology and physiology and increasing their expression of PRRs, or other molecules, such as cytokines (Rock *et al.*, 2004). Although microglial function against other fungi related with CNS infections, such as *Cryptococcus neoformans* (Blasi *et al.*, 1995) or *Candida albicans* (Neglia *et al.*, 2006), have already been studied, little is known about their role during neurotropic infections caused by filamentous fungi, especially *L. prolificans*.

Therefore, our hypothesis is that microglial cells may be impaired during *L. prolificans* challenges. So, we evaluated the *in vitro* interactions between microglia and *L. prolificans*, analyzing the relevant parameters and comparing the data obtained with other immune cell types and to related microorganisms. In addition, the role of two PRRs during microglial phagocytosis of the fungus was determined, namely mannose receptor and dectin-1.

Results

Microglial cells show impaired phagocytic capacity against Lomentospora prolificans

To assess the anti-*L. prolificans* activity of this cell type, we performed co-cultures of the fungus and immune cells. The BV-2 microglial cell line was able to phagocytize fungal cells successfully at all times assessed, showing a phagocytosis peak of 19.65±3.26% after 4 h of co-culture. Compared to these cells, the macrophage-like cell line J774A.1 exhibited a very significant increased ability to internalize *L. prolificans* conidia at all experimental times, with a phagocytosis minimum of 19.79±2.51% at 2 h, and a maximum of 48.87±7.40% at 8 h post-infection. Due to the large differences found between the two cell types, these results needed to be validated using primary cell cultures, primary microglia and bone marrow-derived macrophages (BMMs). Interestingly, the different phagocytic capacity observed between microglia and macrophages using cells lines was corroborated with the primary cell cultures, primary microglia showing even lower values when compared to BV-2 cells.. Remarkably, none of the cellular models used was able to completely phagocytize all *L. prolificans* conidia at any time assessed (Figure 1a) and, regarding the phagocytic index, we did not find significant differences between both BV-2 and J774A.1 (data not shown).

On the other hand, to prove that the inefficient phagocytic capacity of microglia was related to *L. prolificans* and not to the cellular model itself, we challenged BV-2 cells with other fungal species, namely the related pathogens *S. boydii* and *S. aurantiacum*, and the yeast *C. albicans*. In this way, it was observed that microglial phagocytosis was completely dependent on the microorganism, with *L. prolificans* being the least efficiently phagocytized (Figure 1b).

Moreover, we explored the role of opsonins during the phagocytosis of *L. prolificans* by BV-2 and J774A.1 cells. Interestingly, while no significant differences were found when normal or heat-inactivated FBS was used (Figure 1c), the presence of 10% normal mouse serum increased the phagocytic rates of both cell lines (Figure 1d), which was significantly reduced when heat-inactivated serum was used. However, although microglial capacity was enhanced in the presence of opsonins, the differences compared to J774A.1 cells were still very high, the latter almost reaching 100% of phagocytosis rate from the first time point.

Interactions with microglia do not reduce Lomentospora prolificans *growth*

Fungal germination rates were also analyzed during co-cultures with immune cells and, while *L. prolificans* did not change its germination rate in the co-culture with microglia compared to when cultured alone, a significant arrest in fungal germination was observed in the presence of BMMs (Figure 2a). Moreover, the fungus showed an increase in the hyphal branching percentage when it was in contact with BV-2 cells, which was not supported by experiments using primary microglial cultures. In contrast, J774A.1 cells were able to slightly delay hyphal branching, while BMMs almost stopped this fungal growth feature (Figure 2b)

Immune cell survival is dramatically affected by Lomentospora prolificans

Phagocytosis is one of the main stages in pathogen clearance by immune cells. However, some microorganisms, including fungal pathogens, are able to evade processes occurring after phagocytosis, such as microbial killing. In the co-culture models used in this study, we demonstrated with the application of fluorescent vital staining Calcein-AM, that *L. prolificans* is able to bring about cell death in both microglial models, evading antifungal activity. Microglial

cell survival significantly decreased in comparison with non-infected cultures, from 6 h in BV-2 and from 2 h in primary microglia. Conversely, cell survival of J774A.1 was maintained until 10 h post-infection and in BMMs until 6 h (Figure 3a).

To clearly observe fungus-immune cell interactions, immunofluorescence staining of fungal chitin with calcofluor white, and immune cell actin filaments with Phalloidin-FITC was carried out. Interestingly, it was observed that, as hours passed, engulfed conidia were germinated inside phagocytes, indicating that conidia are probably able to pierce cell membranes, this activity may contribute to the observed cell death induced by fungal cells (Figure 3b).

Lomentospora prolificans *survival during pH stress*

After *L. prolificans* conidia are phagocytized, extreme acidic environments may be found inside phagolysosomes. So, owing to the absence of nutrients in the media and the acid pH these conditions may resemble the environment that the fungus faces inside phagolysosomes (Slesiona *et al*., 2012), therefore the viability of fungal spores after 3 or 7 days of culture in PBS at several pH was determined. Interestingly, *L. prolificans* cells were able to maintain their viability over the whole of the pH range tested (Figure 4a). Despite losing up to 22.45% viability after 3 days and up to 29.38% after 7 days in neutral pH (both at pH 6), most of the *L. prolificans* cells were able to survive pH stress, keeping high viability levels both in basic and acidic conditions. Similarly, spotting assays showed that *L. prolificans* was able to survive and form colonies over the whole of the pH range tested (Figure 4b).

Oxidative and nitrosative bursts developed against Lomentospora prolificans

Reactive compounds, such as oxygen- or nitrogen-containing molecules, are key mediators of microbial killing after engulfment. Therefore, the production of ROS and reactive nitrogen species (RNS) by immune cells in response to *L. prolificans* infection were evaluated. Interestingly, ROS production levels in BV-2 cells were significantly lower compared to those produced by J774A.1, and were only slightly higher 2 h post-infection (Figure 5a). Regarding RNS release, both cell lines responded to fungal exposure by producing RNS without any significant differences between them (Figure 5b,c). Remarkably, no detectable levels of either ROS or RNS were observed in primary cultures of microglia (data not shown).

Release of pro-inflammatory cytokines by microglia and macrophages

Of all the molecules involved in the inflammatory response, TNF-α and IL-6 were measured in this study so as to evaluate the pro-inflammatory effect of *L. prolificans* cells on BV-2 and J774A.1 cells (Figure 6). Both cell types produced basal levels of TNF-α and IL-6 in the absence of any stimulation (Figure 6, black bars). However, when cells were exposed to *L. prolificans*, time-dependent rising concentrations of both cytokines were detected in the culture medium. In the case of BV-2 cells, significantly higher levels of TNF-α and IL-6 were produced 10 h after inoculation with conidia when compared to the control (Figure 6a,c). Regarding J774A.1 macrophage-like cells, TNF-α followed a similar time course of release to that of BV-2 cells (Figure 6b). However, IL-6 release was very fast, starting to be significantly higher 4 h post-infection (Figure 6d). More importantly, higher concentrations of released cytokines in the extracellular medium were achieved by J774A.1 cells for both TNF-α and IL-6 than those from BV-2 microglial cells.

Role of mannose receptor and dectin-1 on microglial phagocytosis of Lomentospora prolificans

Since PRR are essential for pathogen recognition, we evaluated the role of two C-type lectin receptors on the phagocytic process of *L. prolificans* by microglia, mannose receptor (MR) and dectin-1. To achieve this, the phagocytic capacity of these cells in the presence of blocking agents, mannan and laminarin, was analyzed. First, phagocytosis of *L. prolificans* significantly decreased (up to 71.31 ± 3.40%) during short incubation times (2 and 4 h of co-culture) when mannan was applied to block MR. This was also observed, but to a lesser extent, 6 h after inoculation with conidia (Figure 7a). Additionally, laminarin was used to block the participation of dectin-1, inducing in this case a higher inhibition than mannan at all times assessed, with the greatest inhibition occurring 2 h post-infection (84.76 ± 4.33%) (Figure 7b). More interestingly, when both blocking agents were used simultaneously on microglial cells only a slight additive effect was observed 6 h post-infection, the inhibition values reached being similar to those obtained using laminarin only (Figure 7c).

Discussion

Emerging fungal pathogens are becoming an important clinical concern, especially for patients suffering from underlying diseases. Among these pathogens, *L. prolificans* is of special interest owing to its resistance to a wide range of antifungal compounds and its tendency to develop systemic infections. After spreading through the bloodstream, *L. prolificans* is able to infect the CNS and produces either meningitis or abscesses. Here, the resident immune cells of the CNS, microglia, are the first to encounter the infection. However, the performance of these cells during a *L. prolificans* invasion is completely unknown. Therefore, in this study the interactions between microglia and fungal cells were analyzed to increase our understanding of this pathogenic microbe's infection mechanisms.

First, the phagocytosis dynamics of *L. prolificans* conidia by BV-2 microglial cells were analyzed, it was observed that they were able to take up fungal spores, with the highest percentage of phagocytosis recorded 4 h post-infection. In addition, these results were compared with other phagocytes, the macrophage-like cell line J774A.1 and with primary cultures of microglia to confirm whether the BV-2 cell line was acting successfully against the fungus. Interestingly, J774A.1 cells were able to phagocytize *L. prolificans* conidia at significantly higher rates, achieving phagocytic rates almost two and a half times higher than the BV-2 cells. This data is consistent with previously published results concerning human MDM (Gil-Lamaignere *et al.*, 2001). In accordance with the inefficient phagocytic capacity shown by BV-2 cells, primary microglial cultures exhibited even lower phagocytosis rates, while BMMs behave similarly to J774A.1, which further supported our hypothesis.

However, these results were very surprising since the values obtained were very low in comparison to those reported with the same cell line but with other fungi, ranging from 35 to

almost 100% for *Candida* spp. (Neglia *et al.*, 2006; Orsi *et al.*, 2010), and 40-50% for *Cr. neoformans* (Blasi *et al.*, 1992; Blasi *et al.*, 1995) after only 2 h of phagocytosis. In spite of the fact that experimental differences between studies should be taken into account (experimental times, multiplicity-of-infection (MOI) used, etc.), phagocytic processes may differ depending on several biological and physical features. In fact, it is a well-known fact that phagocytosis is highly dependent on the species (Luther *et al.*, 2006). Consequently, experiments using the related species *Scedosporium boydii* and *S. aurantiacum*, and the high-prevalent yeast *C. albicans* were performed. Interestingly, it was observed that the different microbes were engulfed at varying rates, *L. prolificans* and *C. albicans* being the least and most efficiently phagocytized, respectively. This data proves that the impaired phagocytic capacity of microglia was in fact related to *L. prolificans* pathobiology and not to the cellular model itself.

In addition, *L. prolificans* growth was examined by measuring its germination rate and the proportion of branched hyphae produced. These parameters are relevant since germination has been related to virulence during infective processes in filamentous fungi (Brand, 2012). In this case, only BMMs were able to delay fungal germination, with *L. prolificans* being germinated both inside and outside phagocytes, confirmed by immunofluorescence**.** In fact, germination became so excessive that no reliable data could be obtained after 8 h of co-culture. This could contribute to the induction of immune cell death by *L. prolificans* over very short experimental times, especially in primary cultures, something that was less pronounced in J774A.1 cells. These findings contrast significantly with the results previously reported by Gil-Lamaignere and co-workers who observed around 80% inhibition of germination after 7 h of co-culture, but in this case human MDM was used (Gil-Lamaignere *et al.*, 2001). More interestingly, hyphal

branching rates were almost inhibited by BMMs and were found to be higher in the presence of BV-2 cells, which can be considered as a more virulent phenotype in the presence of microglia. Unfortunately, the branching rate data obtained from the BV-2 cell line was not confirmed in primary cultures, indicating that this phenotype may not be expressed by *L. prolificans* during CNS infections.

After phagocytosis, immune cells may contribute to fungal clearance by both killing invading cells and/or promoting pro-inflammatory signals to attract other immune cells (Brakhage *et al.*, 2010). In this sense, phagolysosome acidification, or production of ROS and RNS are important mechanisms during fungal killing by macrophages. So, the first step was to measure *L. prolificans* survival at different pH values, either in liquid or solid media. Interestingly, fungal cells were able to survive these stressful environmental conditions, maintaining high cell viability (more than 70% in liquid medium or forming colonies on agar) over the whole pH range, including very acidic conditions such as pH 3. This characteristic may allow fungal cells to persist inside phagolysosomes until they start to germinate and subsequently escape from phagocytes by piercing cell membranes. These two strategies, persistence and escape, have already been described in different *Aspergillus* species (Slesiona *et al.*, 2012), but it seems *L. prolificans* is able to develop both.

Moreover, we quantified the production of both ROS and RNS by immune cells when exposed to fungal cells. While RNS release was not very high in comparison with non-infected cells, and was similar for both the microglia and macrophage cellular models, ROS production was highly induced in the latter, being consistent with previously reported data using human PMNs and MDMs (Gil-Lamaignere *et al.*, 2001). More interestingly, ROS production and phagocytosis dynamics matched each other in J774A.1 cells, suggesting that they are coupled in order to

eliminate the fungal threat. In this sense, *Scedosporium*/*Lomentospora* species rely on a repertoire of detoxifying enzymes related to oxidative or nitrosative stress, such as catalase or superoxide dismutase (Pellon *et al.*, 2014; Staerck *et al.*, 2017), which may be related to fungal survival inside phagolysosomes. Therefore, this suggests that although macrophages and microglia develop oxidative and nitrosative bursts, the fungus may eliminate these toxic molecules efficiently and so overcome immune cell activity.

In addition, the release of TNF-α and IL-6 was determined, the results show that even though both cell types produced these pro-inflammatory molecules, macrophage-like cells released higher cytokine quantities and in the case of IL-6, over shorter experimental times, which may contribute to an improved recruitment of other immune cells to the site of infection. Although microglial pro-inflammatory responses are highly regulated in order to avoid neuronal tissue damage (Aloisi, 2001), we propose that our data may be related to their inefficiency against *L. prolificans* since high cytokine production has been reported when these cells are challenged by other microbes (Lee *et al.*, 2017).

Finally, the role of two PRR during the phagocytic process of *L. prolificans* by microglia, MR and dectin-1, were evaluated. These receptors have been described previously as very important during the recognition of fungal PAMPs, and are involved in the recognition of *N*-linked mannans and β-glucans, respectively (Netea *et al.*, 2008; Gresnigt *et al.*, 2012). Interestingly, we showed that both PRR were mediating *L. prolificans* conidia uptake by microglial BV-2 cells. To begin with, it was observed that individual blocking of either MR or dectin-1 successfully inhibited phagocytosis of *L. prolificans* over short incubation times (2 and 4 h of co-culture), but to a lesser extent 6 h after inoculation with conidia. Since inhibitors were removed before *L. prolificans* inoculation, immune cells may be adapting to the ineffective process over time, i.e.

284 expressing more receptor molecules or recognizing the pathogen by other mechanisms.
285 However, this hypothesis needs further research if it is to be proved. It is also remarkable that the
286 double inhibition of MR and dectin-1 did not result in a great synergistic response, achieving
287 similar phagocytosis levels as when only dectin-1 was inhibited. This finding on the role of these
288 receptors is quite important since, although dectin-1 has been indirectly related to the molecular
289 recognition of *Scedosporium* spp., no direct evidence has been reported to date (Roilides *et al.*,
290 2009). It should be noted that the blocking agents used in this study, although widely used for
291 this purpose, might interact with other receptors. Therefore, further analyses should be
292 performed in the future to determine the precise role of MR and dectin-1 in the phagocytosis of
293 *L. prolificans*. It is also worth highlighting the fact that complete phagocytosis inhibition was not
294 achieved under any experimental condition studied, suggesting that other receptors have to be
295 involved in *L. prolificans* recognition.
296 In conclusion, it has been shown that microglial cells bring about an impaired phagocytic
297 capacity in *L. prolificans*, as well as lower levels of pro-inflammatory cytokine release and ROS
298 production, compared to other phagocytes. Interestingly, the phagocytic process is highly
299 mediated by the MR and dectin-1, although the role of other pattern recognition receptors should
300 be studied further. With respect to the fungus, it was observed that it is able to maintain its
301 germination and hyphal branching rates, and to survive over a wide range of environmental pH.
302 Therefore, taking into account our results we propose that microglia may be inefficient for the
303 clearance of *L. prolificans* from the brain, and in fact may contribute to its neurotropism.
304

Experimental procedures

Ethics statement

All procedures and experiments involving animals, including their housing and care, were carried out according to the guidelines of the European Union Council (Directive 2010/63/EU) and Spanish Government regulations (RD 53/2013), and with the approval of the ethics committee of the University of the Basque Country (UPV/EHU) and the CIC bioGUNE.

Fungal cells

For this study, we used the *L. prolificans* strain CECT 20842, *S. boydii* UPV 93–251, *S. aurantiacum* CBS 116910, and *C. albicans* CECT 13062. Fungal cells were cryopreserved at -80°C and strains were reconstituted monthly on Potato dextrose agar (PDA) (Pronadisa, Madrid, Spain). To prepare conidia, 7-day old fungal cultures on PDA plates were washed twice with sterile saline (0.9% [w/v] NaCl), and cell suspensions were filtered through gauze to remove hyphae and other debris. In the case of *C. albicans* yeast, 1-day old cultures on PDA were processed as mentioned above. Cell densities were calculated using a Bürker counting chamber.

Cell lines

Two murine cell lines were used to evaluate their interactions with *L. prolificans*, the microglial cell line BV-2 (Blasi *et al.*, 1990) and the peritoneal macrophage-like cell line J774A.1 (Ralph *et al.*, 1975), both of which retained most of the morphological, phenotypical, and functional properties described for freshly isolated cells. Cell lines were maintained and propagated in a humidified atmosphere (95% relative humidity) and 5% CO_2, at 37°C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 200 mM L-Glutamine and 10% heat-inactivated

FBS (Complete Medium; CM), and a mixture of antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25 µg/ml amphotericin B). All culture media components were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Isolation and culture of primary cells

Primary microglia were derived from mixed glial cultures obtained from the cerebral cortex of neonate Sprague-Dawley rats (P0-P2) as previously described (McCarthy and de Vellis, 1980), with modifications. In brief, extracted brain cortices were placed in 10 ml of Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}, supplemented with 400 µl of 0.05% trypsin (Gibco, Madrid, Spain) and 40 µl of 0.4% DNAse (Sigma-Aldrich), and incubated for 15 min at 37°C to enzymatically dissociate the tissue. To stop the reaction, Iscove's Modified Dulbecco's Medium supplemented with 10% of FBS was added, and tissue was centrifuged for 5 min at 300 g. Supernatants were discarded, and pellets were resuspended in the aforementioned medium to mechanically dissociate them by using needles of decreasing diameter (21G and 23G). Cell suspension was centrifuged at 400 g for 5 min, resuspended in medium, and seeded in culture flasks pre-coated with poly-D-lysine (Sigma-Aldrich). After two weeks in culture, flasks were shaken at 37°C at a speed of 300-400 rpm for 1 h, with supernatants containing microglia placed on Petri dishes, and left at 37°C for 48 h to let them adhere. Then, the supernatant was eliminated, removing non-adherent cells to obtain 95-99% microglia pure cultures. Primary microglia cultures were maintained in CM with antibiotics and antimycotics.

Bone marrow-derived macrophages (BMMs) were generated from adult rats as described (Barrett *et al.*, 2015). Briefly, bone marrow cells were flushed out from clean femurs and tibias into CM, filtered through a 70 µm-nylon mesh (Thermo Fisher Scientific, Rockford, IL, USA),

and centrifuged at 400 g for 5 min. Red blood cells were removed using ACK lysis buffer, and the remaining cells were incubated in 100 mm x 15 mm Petri dishes for 8 days in CM supplemented with 30 ng/ml of rat M-CSF (PeproTech, London, United Kingdom).

Fungal-immune cell co-cultures, phagocytosis assay, and germination dynamic

To evaluate the interactions between immune cells and *L. prolificans*, cell lines and BMMs were scrapped, counted under the microscope using a Bürker counting chamber, and stained with Trypan blue to determine their viability. Cell line passages below 80% of viability were discarded. Cells were placed on 24-well plates at a density of $5x10^4$ cells per well containing 12 mm-diameter cover slips and 400 µl of CM only supplemented with penicillin and streptomycin. After an overnight incubation, immune cells were co-cultured with *L. prolificans* conidia at a MOI of 1 (one conidium per mammalian cell). At the end of each incubation time, cover slips were extracted from wells, and placed in another plate containing cold PBS to stop phagocytic activity. Then, cover slips were stained with Giemsa (Sigma-Aldrich) and at least 300 immune cells were counted per well to determine the percentage of them that were completely engulfing conidia. In addition, *L. prolificans* germination and branching rates were determined, the latter being defined as the proportion of fungal cells with more than one hyphal tip. Control wells with no immune cells were also counted to measure fungal growth parameters.

In the case of primary microglia cultures, dishes containing cells were washed with HBSS, without Ca^{2+} and Mg^{2+}, and treated with trypsin at 37°C for 5 min. Once the cells were detached, cold CM was added to stop the reaction, and the cell suspension was centrifuged. Harvested cells were resuspended in CM with only antibiotics and then, the cell number and viability was determined as mentioned above. To minimize the number of sacrificed rats, experiments with

primary microglia were performed plating 10^4 cells per well in 200 µl of CM with only penicillin and streptomycin using 96-well plates. In the case of experiments performed using primary microglia, counts to determine phagocytosis, germination, and branching rates were performed directly on the 96-well plates.

Finally, to determine the role of opsonins, we performed the phagocytosis experiments using CM supplemented with 10% mouse serum or FBS either normal or heat-inactivated. To obtain mouse serum, four 8-12 week-old C57Bl/6 (B6) were euthanized and blood extracted by heart puncture, centrifuged after coagulation, and serum samples pooled. Opsonins were inactivated by heating samples at 56°C for 30 min when needed.

Immunofluorescence

To better visualize the interactions between fungal and immune cells, immunofluorescence staining was performed. After each co-culture time, cover slips were washed three times with PBS, and cells fixed with 3% paraformaldehyde for 20 min. Then, the cells were washed three times with PBS, and cell membranes permeabilized with 0.1% Triton X-100 in PBS for 4 min. Excess Triton X-100 was washed away with PBS three times and then Phalloidin-Atto 488 1/200 and calcofluor white 40 µg/ml (both from Sigma-Aldrich) in PBS was added to stain actin filaments and fungal chitin, respectively. Finally, after 35 min in the dark, unlabeled compounds were washed as mentioned above and then cover slips were placed using Fluoromount Aqueous Mounting Medium (Sigma-Aldrich). Fungus-immune cell interactions were visualized under a microscope Nikon Eclipse Ni (Nikon, Tokyo, Japan). All incubations were performed at room temperature.

Measurement of immune cell survival

Cellular damage induced by *L. prolificans* to immune cells was measured by Calcein-AM (Life Technologies, CA, USA). After each experimental period, cells were washed three times with sterile pre-warmed PBS, and Calcein-AM added at a final concentration of 1 μM in fresh CM. The cells were then incubated at 37°C for 30 min, and washed three times with PBS, the fluorescence emitted was measured by a spectrophotometer, Synergy HT (Biotek, MA, USA), the excitation and emission wavelengths being set at 485 nm and 527 nm, respectively. Arbitrary fluorescence units were used to calculate the percentage of cell survival in comparison with non-infected cells at each point in time.

Quantification of ROS, RNS, and pro-inflammatory cytokines

To determine the response of immune cells to *L. prolificans* infection, the production of ROS, RNS, and two pro-inflammatory cytokines, TNF-α and IL-6, were evaluated. Intracellular ROS levels were measured using a fluorescent probe, CM-H_2DCFDA (Life Technologies), as a general oxidative stress indicator. After each incubation period, wells were washed three times with pre-warmed PBS, and the probe was added diluted in PBS at a final concentration of 5 μM at 37°C for 20 min. Then the probe was removed and pre-warmed PBS was added to each well at 37°C for another 20 min. Finally, the fluorescence was detected spectrophotometrically using excitation and emission wavelengths of 492 nm and 517 nm respectively. As mentioned above, arbitrary fluorescence units were compared to those obtained in control wells, with the results expressed as percentage of control.

Supernatants of cells cultured alone or with *L. prolificans* were used to measure RNS and pro-inflammatory cytokines. On the one hand, RNS levels were measured by the Griess method.

Briefly, 150 µl of culture supernatant from each point in time and condition were placed in 96-well plates, and mixed with 20 µl of Griess reagent: equal volumes of 1% (w/v) Sulfanilamide in 5% (v/v) H_3PO_4, and 0.1% (w/v) N-(1-Naphthyl)-ethylenediamine dihydrochloride. Then samples were homogenized by adding 130 µl of distilled H_2O, and plates incubated for 30 min. The absorbance of the samples was measured at 548 nm, and nitrite concentration values inferred from a standard curve using sodium nitrite. On the other hand, TNF-α and IL-6 levels in supernatants were determined by sandwich ELISA according to the manufacturer's instructions (Life Technologies).

Fungal survival under pH stress

Conidiospores of *L. prolificans* were incubated at 37°C and 200 rpm for three or seven days in PBS with adjusted pH ranging from 3 to 8. At the end of each experimental incubation time, serial dilutions were plated in triplicate on PDA plates to test fungal viability. After 24 h of incubation at 37°C, the number of colony forming units (CFU) was recorded. Survival at pH 7 was set as 100%, and the relative survival in the rest of the pH range compared to pH 7 was determined. In addition, spotting assays were performed to evaluate the effect of pH on solid medium. Two µl of 1/10 serial dilutions were spotted onto PBS/2% glucose/0.5% yeast extract agar plates previously adjusted to different pH values. After two days at 37°C the plates were examined and photographed so that any fungal growth could be analyzed.

Receptor blocking assays

To determine the role of PRRs on microglial phagocytosis of *L. prolificans*, mannan from *Saccharomyces cerevisiae* (Sigma-Aldrich) and laminarin from *Laminaria digitata* (Sigma-

443 Aldrich) were used to block MR and dectin-1, respectively. Prior to the addition of *L. prolificans*
444 conidia, BV-2 cells were treated with the blocking reagents (15 min with mannan, or 10 min with
445 laminarin), as previously described (Slesiona *et al.*, 2012). After this incubation time, media
446 containing the inhibitors were removed, and cells washed once with CM only with antibiotics,
447 then the conidia were added.
448
449 *Statistics*
450 Statistical analyses were performed using the GraphPad Prism 7 software (GraphPad Software
451 Inc., CA, USA). At least three biological replicates were performed to measure each parameter in
452 each experimental condition, any statistically significant differences were analyzed as required.
453

Acknowledgments

This work has been supported by grants (GIU15/36, UFI11/25 and PPG17/41) from the UPV/EHU, and SAF2016-75292-R from the Spanish Ministry of Economy, Industry and Competitiveness (MINECO). IB and AA are recipients of predoctoral grants from the Basque Government (GV/EJ). We also thank the Member of the Chartered Institute of Linguists Nº 022913 for improving the English in the manuscript.

Transparency declarations

The authors of this manuscript declare no conflicts of interest.

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Figure captions

Figure 1. Analysis of *Lomentospora prolificans* phagocytosis dynamics and comparison with other fungi. (a) Percentage of cells engulfing *L. prolificans* conidia at several post-infection (p.i.) times during co-cultures of *L. prolificans* with BV-2, primary microglia (PM), J774A.1, and bone marrow-derived macrophages (BMM). Results are shown as mean ± S.E.M., n ≥ 3. *p < 0.05, ***p < 0.001 compared to BV-2 cells. (b) Percentage of phagocytosis of BV-2 cells challenged with either *L. prolificans*, *Scedosporium boydii*, *S. aurantiacum* or *Candida albicans*. Results are shown as mean ± S.E.M., n = 3. One-way ANOVA was used, followed by Holm-Sidak post hoc test; *ns*, not significant, **p < 0.01, ***p < 0.001 compared to BV-2 challenged with *L. prolificans*. Phagocytosis rates were also measured in the presence of 10% fetal bovine serum (FBS; c) or mouse serum (MS; d) either normal or heat-inactivated (HI). Results are shown as mean ± S.E.M., n = 3. One-way ANOVA was used, followed by Holm-Sidak post hoc test; *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 2. Study of *Lomentospora prolificans* (*Lp*) germination and hyphal branching rates. Fungal growth dynamics were also monitored by analyzing germination (a) and hyphal branching (b) rates. Results are shown as mean ± S.E.M., n ≥ 3. One-way ANOVA was used, followed by Holm-Sidak post hoc test; ***p < 0.001 compared to *L. prolificans* alone.

Figure 3. Cell damage induced by *Lomentospora prolificans* cells on phagocytic cells. (a) Microglial cell, both BV-2 cell line and primary microglia (PM), and macrophage, J774A.1 cell line and bone marrow-derived macrophages (BMM), survival was measured by Calcein-AM in

the presence of fungal cells at different post-infection (p.i.) times. Results are shown as mean ± S.E.M., n ≥ 5, of the percentage of survival in comparison with non-infected cells, representing 100% of survival (dotted line). One-way ANOVA was used, followed by Holm-Sidak post hoc test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ in comparison with non-infected cultures. (b) Representative micrographs showing phagocyte (green)-*L. prolificans* (blue) interactions. White arrows depict points where fungal hyphae seem to pierce phagocytic cell membranes. Scale bar = 25 µm.

Figure 4. Survival of *Lomentospora prolificans* conidia at different pH. (a) Fungal survival at different pH was determined after 3 and 7 days of culture. Results are shown as mean ± S.E.M., n = 3, of the percentage of conidial survival compared to pH 7 (set as 100% of survival). One-way ANOVA was used, followed by Holm-Sidak post hoc test; *$p < 0.05$ compared to pH 7 for 3 days. (b) Spotting assays were performed using decimal dilutions of conidia (from 10^4 to 1) in order to observe the ability of *L. prolificans* to grow in a solid medium at different pH.

Figure 5. Release of ROS and RNS by phagocytic cells. (a) Intracellular ROS production induced by *Lomentospora prolificans* challenge was monitored at different post-infection (p.i.) times. Results are shown as mean ± S.E.M. of percentage respect to non-infected cells, n = 4. One-way ANOVA was used, followed by Holm-Sidak post hoc test; **$p < 0.01$, ***$p < 0.001$. RNS release was measured from non-infected (black bars) or fungus-infected (white bars) BV-2 (b) and J774A.1 (c) cells. Results are shown as mean ± S.E.M., n = 4. One-way ANOVA was used, followed by Holm-Sidak post hoc test; *$p < 0.05$, ***$p < 0.001$ compared to non-infected cultures.

Figure 6. Pro-inflammatory cytokines production by immune cells after stimulation with *Lomentospora prolificans*. Tumour necrosis factor-α and Interleukin-6 release by BV-2 (a,c) and J774A.1 (b,d) cells were measured when co-cultured with the fungus at different post-infection (p.i.) times. Results are shown as mean ± S.E.M., n = 4. One-way ANOVA was used, followed by Holm-Sidak post hoc test; $*p < 0.05$, $***p < 0.001$ compared to non-infected cultures.

Figure 7. Role of mannose receptor (MR) and dectin-1 (Dc-1) during *Lomentospora prolificans* phagocytosis by microglia. (a) Mannan was used as an inhibitor of MR, with phagocytosis being studied several post-infection (p.i.) times. (b) Similarly, laminarin was used for blocking Dc-1. (c) Simultaneous blocking of MR and Dc-1 was also performed to study *L. prolificans* phagocytosis. Results are shown as mean ± S.E.M., n ≥ 3. One-way ANOVA was used, followed by Holm-Sidak post hoc test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to co-cultures without blocking agent (black bars).