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Toll-like receptor 4 and CD11b expressed on microglia coordinate eradication of *Candida albicans* cerebral mycosis

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DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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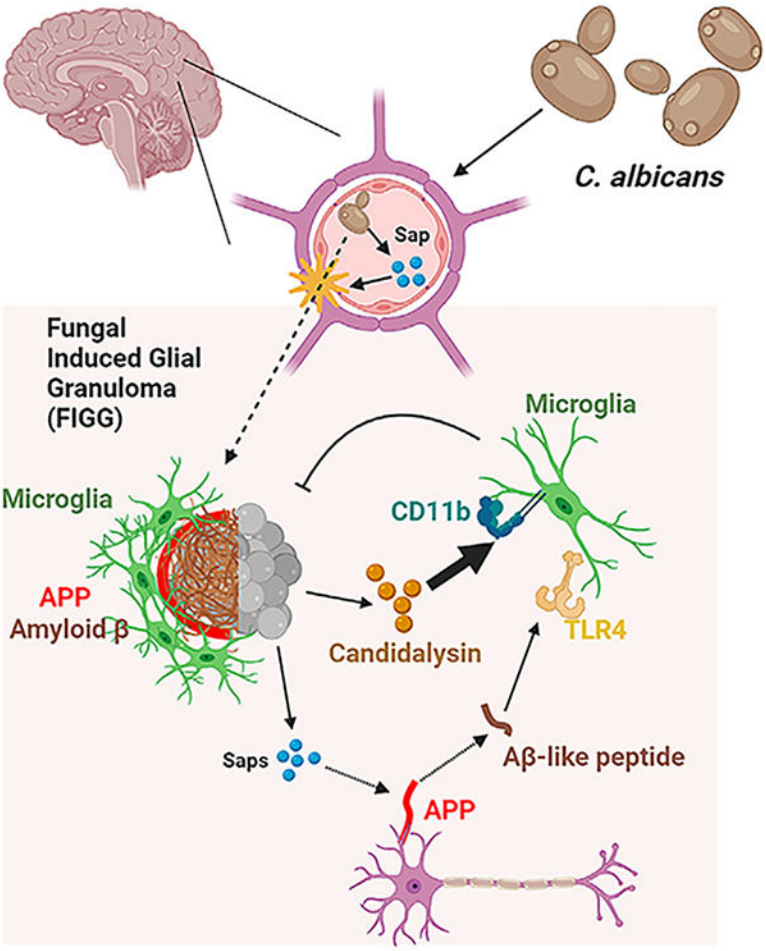
SUMMARY

The fungal pathogen *Candida albicans* is linked to chronic brain diseases such as Alzheimer's disease (AD), but the molecular basis of brain anti-*Candida* immunity remains unknown. We show that *C. albicans* enters the mouse brain from the blood and induces two neuroimmune sensing mechanisms involving secreted aspartic proteinases (Saps) and candidalysin. Saps disrupt tight junction proteins of the blood-brain barrier (BBB) to permit fungal brain invasion. Saps also hydrolyze amyloid precursor protein (APP) into amyloid β (Aβ)-like peptides that bind to Toll-like receptor 4 (TLR4) and promote fungal killing *in vitro* while candidalysin engages the integrin CD11b (Mac-1) on microglia. Recognition of Aβ-like peptides and candidalysin promotes fungal clearance from the brain, and disruption of candidalysin recognition through CD11b markedly prolongs *C. albicans* cerebral mycosis. Thus, *C. albicans* is cleared from the brain through innate immune mechanisms involving Saps, Aβ, candidalysin, and CD11b.

In brief

Microbes, including fungi, routinely infect the brain, but specific immune pathways are undefined. Wu et al. show that *Candida albicans* activates microglia through two mechanisms involving the production of amyloid β-like peptides that signal through TLR4 and candidalysin that activates CD11b, together promoting clearance of *C. albicans* from the brain.

Graphical Abstract



INTRODUCTION

Environmental and commensal fungi are increasingly recognized as key drivers of diverse chronic inflammatory disorders, including asthma, chronic rhinosinusitis (CRS), chronic pulmonary aspergillosis, eczema and other pruritic dermatoses, vaginosis, and colitis. Only a few hundred distinct fungal species are pathogenic for humans, with *Candida albicans* being one of the most common fungal pathogens causing diverse infections.

C. albicans, together with other closely related *Candida* species that are pathogenic for humans, is a commensal member of the human microbiota but produces often serious opportunistic mucosal and blood-borne infections, especially in the context of immune deficiencies.[1,2] Of particular concern is *C. albicans* cerebral mycosis, the most common form of fungal cerebritis,[3] which develops as a sequela of acute sepsis, especially in the perinatal period and as a complication of intensive care.[4] Sepsis from all causes is a risk factor for both in-hospital mortality and dementia arising as a complication of critical illness.[5–7]

C. albicans has also been detected in the brains of subjects with Alzheimer's disease (AD) and other chronic neurodegenerative disorders that lack obvious features of acute illness such as fever, rapid onset of altered mental status, headache, and leukocytosis.[8] Although this implies that *C. albicans* cerebritis fails to elicit sterilizing immunity in the brain leading to long-term persistence, we have previously shown that acute, low-grade *C. albicans* cerebritis is fully resolved in otherwise asymptomatic, healthy mice after 10 days.[9]

Fungi are recognized immunologically by receptors present especially on phagocytic cells (neutrophils, macrophages, dendritic cells) such as Dectin1–3, Toll-like receptors 2/4, galectin-3, and DC-SIGN, which engage the fungal ligands β-glucan, chitin, β-mannose, and N/O-mannose, respectively.[10] Such recognition promotes phagocytic, anti-microbial peptide, oxidative, inflammasome, and adaptive immune responses that efficiently resolve most acute infections. *C. albicans* is specifically recognized through detection of the secreted peptide toxin Candidalysin,[11] which, in the context of acute airway mycosis, is detected by the platelet-expressed receptor GP1bα to promote release of the Wnt pathway antagonist protein dickkopf-1 (Dkk) that drives anti-fungal Th2 and Th17 responses.[12] However, during acute, low-grade cerebral mycosis due to *C. albicans*, conventional macrophages, neutrophils, and lymphocytes are not detected in the mouse brain. Instead, the fungal cells become surrounded by highly activated microglial cells forming fungal-induced glial granulomas (FIGGs) that coordinate fungal killing through currently unknown mechanisms.[9] Additionally, it is unknown how *C. albicans* enters the brain, a necessary initial step leading to cerebral mycosis.

We show that secreted aspartic proteinases (Saps), especially Sap2, from *C. albicans* degrade endothelial tight junction proteins to permit cerebral invasion. However, once in the brain parenchyma, Saps cleave neuronally expressed amyloid precursor protein (APP) into amyloid beta (Aβ)-like peptides that bind Toll-like receptor 4 (TLR4) to activate microglial cells. We demonstrate further that candidalysin secreted by *C. albicans* is a second major activator of microglia through binding to the integrin CD11b and that failure to recognize candidalysin in mice by abolishing the CD11b-candidalysin cognate interaction results in prolonged brain infection. Thus, *C. albicans* Saps and candidalysin are central to the pathogenesis of cerebral mycosis due to *C. albicans*.

RESULTS

Sap2 is required for cerebral entry of *C. albicans*

The ability of *C. albicans* to rapidly gain entry into the brain parenchyma from the blood suggests that it utilizes a special mechanism to disrupt the blood-brain barrier (BBB) that otherwise restricts the passage of pathogens and macromolecules into the brain under physiological conditions.[13] As tight junction proteins comprise much of the integrity of the BBB, we initially explored whether proteolysis by *C. albicans* could account for loss of BBB integrity after intravenous injection. *C. albicans* possesses ten distinct aspartic class proteinases (Saps1–10) that have been shown to play critical roles in many infection models.[14–17] All *C. albicans* Saps, but especially Sap2, are potent proteinases that are capable of hydrolyzing most human proteins.[15]

To determine if one or more Saps are required for BBB disruption and cerebral entry of *C. albicans*, we challenged mice intravenously with increasing doses of viable *C. albicans* cells triply deficient in *SAP1–3* or *SAP4–6* as compared to the parental wild-type strain. Lack of *SAP4–6* resulted in markedly reduced fungal brain burdens as compared to control wild-type *C. albicans*, whereas lack of *SAP1–3* completely abolished the ability of *C. albicans* to enter the brain (Figure 1A). This suggested that Saps1–3 are especially critical for *C. albicans* cerebral entry.

Since Sap2 is the most abundant Sap in many pathological contexts and exhibits broad substrate specificity for mammalian proteins,[15] we conducted similar experiments with *C. albicans* selectively lacking Sap2. We discovered that Sap2 deficiency abrogated the ability of *C. albicans* to enter the mouse brain after intravenous injection, similar to Sap1–3 deficiency (Figure 1B). Whereas Sap deficiency did not affect fungal burdens in lung and liver, any disruption of *SAP* genes resulted in 16- to 36-fold greater fungal burdens in kidneys as compared with wild-type *C. albicans* at higher administered doses (Figures S1A–S1C). We further determined the relative importance of Saps1–3 for cerebral invasion by comparing brain fungal burdens from mice challenged with *C. albicans* deficient individually in Sap1, Sap2, or Sap3. We found that Sap1 or Sap3 deficiency resulted in a greater than 8-fold reduction in *C. albicans* brain burdens but that Sap2 deficiency abrogated the ability of *C. albicans* to enter the brain parenchyma (Figure S1D). These data demonstrate that multiple *C. albicans* Saps contribute to brain invasion but that Sap2 is of greatest importance.

We conducted additional experiments to determine how Sap2 promotes egression of *C. albicans* from blood to brain. Pretreatment of mice with pertussis toxin (PT) to pharmacologically compromise the BBB[18] partially restored the ability of *sap2*Δ/Δ *C. albicans* to invade the brain after intravenous injection, suggesting that physical disruption of the BBB is a major mechanism by which *C. albicans* gain entry to the brain from blood (Figure 1C). To confirm this mechanism, we challenged wild-type mice with the parental strain or *sap2*Δ/Δ *C. albicans* and determined the relative ability of tetramethylrhodamine (TRITC)-dextran to enter the brain from the blood.[19] We observed significantly elevated median fluorescence intensity (MFI) of TRITC from brain post-infection with wild-type *C. albicans*, whereas the MFI from brain post-challenge with *sap2*Δ/Δ *C. albicans* was not different from control mice (Figure 1D).

Exposure of human cerebral cortex endothelial cells (HBEC-5i cells) to recombinant Sap2 resulted in significant shrinkage and rounding of cells as compared to vehicle pH control and an equivalent concentration of the endogenous proteinase trypsin (Figure S1E). Sap2 further degraded tight junction proteins such as occludin-1 as assessed by western blot analysis of Sap2-challenged HBEC-5i monolayers (Figure S1F). Finally, Sap2 markedly attenuated the transendothelial electrical resistance (TEER) of HBEC-5i monolayers to a greater extent than trypsin (Figure 1E). Together, these results indicate that Sap2 mediates cerebral entry of *C. albicans* in part by degrading tight junction proteins of BBB endothelial cells.

Saps cleave APP into Aβ -like peptides

We demonstrated previously that *C. albicans* cerebritis is accompanied by the production of Aβ-like peptides that form over the fungi present in the center of FIGGs and that activate microglial anti-fungal immunity.[9] Therefore, we next determined if Saps modify FIGG formation and the clearance of *C. albicans* from the brain. Since Aβ peptides are endogenously cleaved from recombinant human APP by β- and γ-secretases, which, like *C. albicans* Saps, are both aspartic proteinases,[20,21] we determined if *C. albicans* Saps are alone sufficient to cleave APP to generate similar Aβ-like peptides.

Exposure of neural crest-derived mouse Neuro2a (N2a) cells that normally express APP[22] to recombinant Saps (Sap1, -2, -3, -5, -9, and -10 at a 1:1:1:1:1:1 ratio) resulted in the accumulation of Aβ-like peptides in the supernatants that are similar to native Aβ40 and Aβ42 peptides (Figure 2A). We verified that the elevated Aβ-like peptide concentrations were due to protein cleavage by confirming that APP mRNA was unchanged in Saptreated N2a cells but that APP protein was decreased (Figures S2A and S2B). To eliminate the potential effect of endogenous secretases on N2a cells, we confirmed that recombinant Saps were alone sufficient to generate Aβ40-like and Aβ42-like peptides from APP at different working pH (Figure 2B). Furthermore, recombinant Saps also generated peptides of the appropriate approximate size (3.5–10 kDa; Aβ40/42 peptides are approximately 4.5 kDa in size) from APP by western blot (Figure 2C).

To verify that *C. albicans* Saps could generate Aβ-like peptides in the absence of endogenous secretases *in vivo*, we challenged β-secretase-deficient (*BACE1*$^{-/-}$) mice with *C. albicans*. *BACE1*$^{-/-}$ mice yielded significantly enhanced Aβ42-like peptide, but not Aβ40-like peptide, from the brain 4 days post-infection (Figure 2D). We further assessed FIGG formation from these mice using immunofluorescence microscopy of coronal brain sections of fungal-challenged *BACE1*$^{-/-}$ and APP$^{-/-}$ mice. We found similar Aβ-like peptide staining within FIGGs of *BACE1*$^{-/-}$ mice as previously described[9] but a complete absence of Aβ-like peptides in *APP*$^{-/-}$ mice (Figure 2E). Together, these observations indicate that Saps from *C. albicans* cleave APP into Aβ-like peptides *in vitro* and *in vivo* in the absence of endogenous secretase activity, although endogenous secretases may contribute to the production of Aβ40.

Aβ promotes anti-fungal activity of microglia via TLR4

We showed previously that Aβ peptides enhance the anti-fungal activity of microglial cells,[9] but the specific molecular mechanism remains unknown. To address this, we considered a potential role for TLR4, which is both a receptor for Aβ[23] and mediates antifungal responses due to fibrinogen cleavage products (FCPs; Millien et al.[24]). We used an *in vitro* fungistasis assay, in which Aβ peptides stimulate the microglial cell line BV-2 to kill *C. albicans* cells.[9] Addition of the TLR4 antagonist LPS-RS[25] to this assay abolished Aβ-dependent enhanced anti-fungal activity (Figure 3A). We also isolated the Aβ-like peptides of 3.5–10 kDa cleaved from rhAPP by rSaps in Figure 2C and assessed their activity in the same fungistasis assay. These Aβ-like peptides enhanced the antifungal activity of BV-2 cells in a TLR4-dependent manner like that of full-length synthetic Aβ peptides (Figure 3B).

We verified the importance of TLR4 *in vivo* by injecting 25,000 viable parental strain *C. albicans* cells into wild-type, *APP*$^{-/-}$, or *TLR4*$^{-/-}$ mice intravenously. As compared with wild-type mice, brain fungal burdens of *APP*$^{-/-}$ and *TLR4*$^{-/-}$ mice were significantly elevated at multiple time points, and the rates of fungal clearance from the brains of the mutant mice were also similar (Figure 3C). These findings suggested that *APP*$^{-/-}$Aβ and TLR4 function in the same anti-fungal signaling pathway and that Aβ peptides are ligands for TLR4. We confirmed the significant binding affinity between TLR4 and Aβ peptides using modified ELISAs that demonstrated that both Aβ40 and Aβ42, but not scrambled control peptide, bind to TLR4 and that such binding is blocked by LPS-RS (Figures 3D–3F). Together, our data demonstrate that Aβ-like peptides generated by *C. albicans* Saps are extremely similar, if not identical, to Aβ40/42 peptides, all of which equivalently enhance the anti-fungal activity of microglia via TLR4.

Candidalysin is critical for cerebral clearance of *C. albicans*

Although TLR4 enhances *C. albicans* clearance from the brain, TLR4 was ultimately not required for sterilizing immunity (Figure 3B). We therefore considered whether other signaling pathways promote *C. albicans* eradication from brain. Given the critical role played by the peptide toxin candidalysin (encoded by the gene *ECE1*) in driving immune responses in oral,[26–29] vaginal,[30,31] systemic,[32] and allergic airway disease,[12] as well as its ability to strongly stimulate microglia,[33] we determined the role of this essential virulence factor in cerebral mycosis by challenging wild-type mice with *ece1*Δ/Δ (candidalysin-deficient) and wild-type *C. albicans*. *Ece1*Δ/Δ cells entered the brain >2-fold more efficiently as compared with wild-type cells, but *ece1*Δ/Δ cells were less efficiently cleared, requiring up to 60 days, whereas wild-type *C. albicans* cells were cleared from the brain in 10–14 days (Figure 4A).[9]

Immunofluorescence staining of coronal sections of brain from mice 21 days post-infection with *ece1Δ/Δ C. albicans* revealed FIGGs in the cerebral cortex that were marked by enhanced expression of ionized calcium-binding adapter molecule 1 (IBA1) on microglial cells, enhanced expression of glial fibrillary acidic protein (GFAP) on activated astrocytes, and accumulation of Aβ-like peptides on fungal cells, which we showed previously[34] cluster in the centers of the FIGGs. (Figure 4B). Similar to wild-type *C. albicans*,[9] *ece1*Δ/Δ *C. albicans* also elicited upregulation of APP in whole brain (Figures S3A–S3B).

Fungistasis assays using wild-type or *ece1*Δ/Δ *C. albicans* added to BV-2 cells showed that BV-2 cells more efficiently inhibited wild-type *C. albicans* as compared to *ece1*Δ/Δ with and without activation with Aβ peptides (Figure 4C). BV-2 cells stimulated with synthetic candidalysin also produced the pro-inflammatory cytokines interleukin-1β (IL-1β), IL-β, tumor necrosis factor (TNF), and interferon β (IFN-β) (Figure 4D). These studies together indicated that candidalysin critically enhances innate microglial responses against *C. albicans*.

Candidalysin signals through CD11b to activate microglia

While candidalysin activates host cells through damage induced by membrane intercalation and pore formation,[29] we recently identified a specific host receptor (GP1bα) for

candidalysin on platelets that drives protective immunity during allergic airway disease.[12] As GP1bα is expressed primarily on platelets and megakaryocytes, we sought a distinct candidalysin receptor expressed on microglial cells, a major cellular constituent of the FIGG that efficiently kills *C. albicans in vitro*.[9] We initially focused on CD11b (CD18/CD11b; Mac-1), a heterodimeric integrin that is highly expressed on microglial cells,[35] because our previous studies demonstrated that CD11b is a receptor for fungal proteinase-cleaved fibrinogen, an immunologically relevant clotting protein that also mediates innate immunity against the filamentous fungus *Aspergillus niger*.[24]

CD11b$^{-/-}$ mice showed similarly delayed brain clearance of wild-type *C. albicans* as compared with wild-type mice challenged with *ece1*Δ/Δ *C. albicans* (Figure 5A). Both anti-CD11b antibody and the secreted hookworm product neutrophil inhibitory factor (NIF), which blocks Mac-1 via the CD11b I domain,[36] also inhibited the ability of synthetic candidalysin to enhance the killing of *C. albicans* by BV-2 cells (Figures 5B and 5C). Moreover, although BV-2 cells were overall markedly less able to inhibit the growth of *ece1*Δ/Δ *C. albicans*, candidalysin nonetheless enhanced the ability of BV-2 cells to kill the mutant fungus, an effect that was again abrogated by CD11b antagonists (Figure 5D). Moreover, we verified that CD11b antagonists do not influence baseline anti-fungal activity of BV-2 cells (Figure S4A). We confirmed the importance of CD11b for the *in vitro* killing of *C. albicans* using primary mouse brain microglial cells (Figure 5E), but, notably, primary brain astrocytes showed no ability to kill *C. albicans in vitro* (Figure 5F). Thus, the candidalysin-dependent activation of microglial antifungal responses required a cognate interaction with CD11b on microglia but not on astrocytes.

CD11b belongs to the 4-member CD11 family of structurally similar integrins (CD11a–d), of which CD11a and CD11c also have significant immunological roles and CD11c,[37] but not CD11a,[38] is expressed on mouse microglial cells. This raised the possibility that candidalysin could also bind to at least CD11c to promote anti-fungal immunity. To assess this, we performed pull-down assays using CHO cells transfected with human CD11a, CD11b, and CD11c using biotinylated candidalysin. These studies confirmed that candidalysin binds to CD11b but also, in this over-expression context, to CD11c; no binding to CD11a was detected (Figure 5G).

We also performed modified ELISAs, in which binding between the synthetic candidalysin and the human CD11b extracellular domain was determined using CD11b as the capture and candidalysin as the detecting agent or reciprocally with candidalysin as the capture and CD11b as the detecting agent (Figures 5H and 5I). Regardless of their configurations, these assays demonstrated specific affinity between CD11b and candidalysin in comparison to a scrambled peptide. Additionally, we used candidalysin as the capture reagent and His-tagged CD11b or CD11c as detecting reagent, confirming that candidalysin binds to CD11b but failing to demonstrate CD11c binding in this context (Figure 5J).

We also tested direct binding between candidalysin and CD11b through *ex vivo* experiments in which splenocytes from wild-type or *CD11b*$^{-/-}$ mice were incubated with biotinylated candidalysin or scrambled control followed by the fluorophore streptavidin-AF647. Flow cytometric analysis of these cells revealed that compared to CD11b-deficient splenocytes,

candidalysin, but not scrambled control peptide, bound to wild-type splenocytes with approximately 3-fold greater affinity, as assessed by the percentage of positive cells and the MFI (Figure 5K).

To resolve the conflicting binding data between candidalysin and CD11c, we determined the physiological importance of CD11c binding to candidalysin using fungistasis assays with BV-2 cells in which CD11c was blocked with three different CD11c-specific antibodies. Whereas a single anti-CD11b antibody again completely blocked candidalysin-dependent enhancement of fungistasis, none of three different anti-CD11c antibodies blocked this response (Figure S4B). Together, our results demonstrate that candidalysin binds to CD11b and, in some contexts, to CD11c, but only binding to CD11b induces microglial anti-fungal immunity.

Finally, we assessed the possibility that our data could be confounded by the *C. albicans* protein Pra1, which was previously shown to bind to CD11b.[39,40] We challenged wild-type mice intravenously (i.v.) with 25,000 viable cells of wild-type parental strain and pra1Δ/Δ *C. albicans* and assessed cerebral fungal burdens 4 days post-infection. We found no difference in the clearance of wild-type parental strain and pra1Δ/Δ *C. albicans* from the brain (Figure S5). These findings confirm that candidalysin, but not Pra1, is a critical CD11b ligand that initiates innate anti-*C. albicans* immunity.

DISCUSSION

Despite its recognition as one of the most important of human pathogens and critical insight gained into the mechanisms by which *C. albicans* causes disease through virulence factors such as Saps and candidalysin, the major mammalian immune mechanisms that control *C. albicans* infections remain incompletely understood. We have shown that *C. albicans* Saps mediate entry of bloodborne fungal cells into the mouse brain parenchyma. Following this pathologic event, brain microglia recognize Aβ peptides that are created by Sap-mediated cleavage of APP via TLR4 to activate anti-fungal responses. We have further shown that candidalysin secreted by *C. albicans* binds CD11b on microglial cells to also drive innate anti-fungal immunity. Mice unable to recognize *C. albicans* through CD11b demonstrated prolonged inability to clear the fungus from the brain. In addition to generic fungal detection pathways likely operating concomitantly that include the recognition of β-glucan by dectin 1 and mannan residues by TLR2/4, mannose receptors, dectin 2/3, galectin 3, and DC-SIGN,[41] *C. albicans*-specific Aβ-TLR4 and candidalysin-CD11b-based anti-fungal immune pathways were highly effective in resolving acute *C. albicans* cerebritis. Despite these robust anti-fungal pathways, *C. albicans* is capable of persisting in multiple organs, including the human brain,[3,42–44] potentially lifelong. Therefore, understanding how *C. albicans* persists *in vivo* despite highly effective sterilizing immunity operating at least in the non-senescent mouse brain will lead to improved preventative and therapeutic approaches against this important fungal pathogen.

Although Sap2 is indispensable for breaching the BBB by *C. albicans*, other Saps (i.e., Sap1, Sap3, and Sap4–6) assist in this process, indicating that synergistic action of multiple Saps leads to rapid entry of *C. albicans* into the mouse brain. Barrier translocation likely results

from the ability of Saps to degrade tight junction proteins such as E-cadherin in the oral cavity[45] and occludin-1 present in the BBB. Once inside the brain, Saps can target APP, a membrane protein expressed in many tissues and concentrated in neuronal synapses. Saps are therefore similar to β- and γ-secretases that mediate intrinsic APP cleavage, as both mouse and human enzymes are aspartic class proteinases that function optimally at acidic pH (pH 3.5–6).[15] *C. albicans* is known to generate acidic microniches during infection, which should facilitate optimal Sap activity that is required for BBB translocation and APP cleavage.

Saps are remarkably powerful proteinases that are capable of completely hydrolyzing many proteins,[15] but our findings indicate that APP is a remarkable exception wherein the Aβ region is resistant to Sap hydrolysis. Such resistance *in vivo* could in part be due to this region being partially contained within the neuronal membrane and thereby protected somewhat from proteolytic attack. However, resistance to proteolysis remains when recombinant APP that is not associated with membranes is incubated with Saps, indicating that resistance from proteolysis is an intrinsic feature of the Aβ region. Moreover, the ability of *C.albicans* to penetrate and disrupt host cell membranes indicates that full-length APP would be available for proteolysis due to *C. albicans in vivo*.[11]

The proteolytic release of immunostimulatory Aβ-like peptides could have evolved as a sentinel in the context of brain invasion by *C. albicans* and possibly other highly proteolytic organisms to alert the innate immune system via TLR4. This possibility gains additional traction given our previous demonstration that fibrinogen in the airway serves a critical immunostimulatory role, again via CD11b and TLR4, in the context of airway mycosis due to filamentous fungi.[24,46] Fibrinogen cannot serve the same function in the context of *C. albicans* airway or brain disease because it is completely degraded by *C. albicans* Saps, precluding release of immune-relevant peptides that are generated by the less potent mold proteinases.[47]

We cannot be certain whether Sap-generated Aβ-like peptides are identical to endogenously created Aβ peptides resulting from the sequential action of β- and γ-secretases.[20,21] However, the observations that *C. albicans*-dependent Aβ-like peptides (1) aggregate directly on fungal cells in the center of FIGGs, even in *BACE*$^{-/-}$ mice,[9] (2) are readily detected immunologically with antibodies made against Aβ peptides, and (3) bind to TLR4 and activate microglial anti-fungal activity in a manner like Aβ40 and Aβ42 peptides provides robust evidence that Aβ-like peptides are very similar, and likely identical, to canonical Aβ peptides. We propose that brain Aβ aggregates that characterize multiple *Candida*-associated neurodegenerative conditions including AD,[43] Parkinson's disease,[44] and others[48,49] may be the result of both fungal and intrinsic proteinases.

Candidalysin is a critical virulence factor of *C. albicans*, as underscored by the multiple mammalian receptors that have evolved to recognize either peptide toxin activity such as the epidermal growth factor receptor (EGFR),[50] or the toxin itself such as GP1bα,[12] and CD11b (this study). Direct engagement of GP1bα and CD11b by candidalysin promotes potent antifungal responses through the adaptive and innate immune systems, respectively. Together with our previous finding that anti-fungal immunity against the filamentous fungus

Aspergillus niger is coordinated through macrophage CD11b recognizing proteolytically activated fibrinogen,[51] our findings emphasize the importance of CD11b as an immune sensor capable of detecting multiple fungus-derived ligands to coordinate anti-fungal immunity.

Notwithstanding the effectiveness of the Aβ-TLR4- and candidalysin-CD11b-dependent immune mechanisms as shown here and the dominant role that *C. albicans* plays in determining effective human immune responses against diverse fungi,[52] the critical unresolved paradox of *C. albicans* infections is the ability of the fungus to persist *in vivo* despite the existence of robust sterilizing immune responses at sites such as the brain and elsewhere. *In vivo* persistence of *C. albicans* in the brain could result from immune senescence, as illustrated by CD11b deficiency; reactivation of infection from a dormant state or a privileged site that prevents immune detection; frequent reinfection given the ubiquity of *C. albicans* in human populations; and, likely, all three mechanisms. Clarification of these issues is therefore critical to understanding the long-term threat that *C. albicans* poses to human health and the potential role of this fungus in neurological disorders.

Limitations of the study

Most of the work presented in this study was performed using *in vitro* experiments and in mice, which imperfectly replicate human tissues, metabolism, and physiology. Therefore, the relevance of these finding to human infections due to *C. albicans* and related fungi and specifically to human degenerative brain diseases such as AD remains unknown. Experimentally, although *in vivo* experiments using *TLR4*$^{-/-}$ and *CD11b*$^{-/-}$ mice showed slower cerebral clearance of *C. albicans* in a manner like *APP*$^{-/-}$ mice and *ece1*Δ/Δ *C. albicans* given to wild-type mice, additional molecular pathways that potentially include other TLRs are potentially involved in this complex immune response and deserve additional study.

In addition to the candidalysin-CD11b interaction, our *in vitro* pull-down assay showed affinity of candidalysin for CD11c, though no interaction was observed in our comparative ELISA. This suggests that under some conditions, candidalysin might also bind to CD11c. Although we utilized 3 different antiCD11c antibodies in our fungistasis assay, none of which inhibited fungal killing, none of the antibodies have been shown to functionally block CD11c, precluding firm conclusions as to their neutralizing efficacy in this assay.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, David B. Corry, M.D. (dcorry@bcm.edu).

Materials availability—No new materials were generated in this study.

Data and code availability—All data are available in the main text or the supplementary materials.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice—8 week-old C57BL/6J male and female mice (wild type, *TLR4*$^{-/-}$, *APP*$^{-/-}$, *BACE1*$^{-/-}$, *CD11b*$^{-/-}$) were purchased from Jackson Laboratories (cat: 007227, 004133, 004714, 003991). Wildtype mice and genetic deletants were bred to create heterozygous mice that were then bred together to recreate homozygous deletants and wildtype littermates. All mice were bred and housed at the American Association for Accreditation of Laboratory Animal Care-accredited vivarium at Baylor College of Medicine under specific-pathogen-free conditions and in the same room to ensure similar microbiomes to the greatest extent possible. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and followed federal guidelines.

Cell lines and transfections—A CHO cell line stably expressing the human CD18 subunit (CHO-CD18) was established and used to develop additional mutants expressing human CD11a, CD11b, and CD11c (CHO-CD11a/b/c) for use in candidalysin binding experiments as described.[55] HBEC-5i cells (CRL-3245, ATCC, Manassas, VA) were cultured in complete DMEM/F12 medium. N2a cells (CCL-131, ATCC, Manassas, VA) were cultured in complete DMEM. BV-2 cells were obtained from ATCC, determined as mycoplasma and chlamydia free and maintained frozen in liquid nitrogen. Cells were thawed, expanded in DMEM medium, and expression of standard microglial surface markers (TREM2, CD68, and MCM5 by RT-qPCR) was confirmed.[9]

Fungi and reagents—For experiments in which only wild type fungus was used, wild type *C. albicans* was isolated as described previously[9] and validated as *C. albicans* through the San Antonio Center for Medical Mycology. *SAP* gene deletants, *ece1*Δ/Δ (*ECE1* gene encodes the Ece1 protein from which candidalysin is derived), *pra1*Δ/Δ and their parental wildtype strain (BWP17/CIp30) of *C. albicans* were generated as previously described.[29,54,56] *C. albicans* was propagated in YPD broth (BD, Sparks, 21152) overnight at room temperature and collected in pyrogen-free phosphate buffered saline (PBS; Corning cellgro, Mediatech, Manassus, VA), passed through 40 μm nylon mesh, and washed twice with PBS by centrifugation (10,000 × *g*, 5 min, 40°C). Fungal cells were then suspended in PBS and aliquots frozen in liquid nitrogen at 5×10^7/mL. Viability after freezing (>95%) was confirmed by comparing haemacytometer-derived cell counts to CFU as determined by plating serial dilutions on Sabouraud's agar. Thawed, >95% viable cells were washed once, counted, and suspended in normal saline at indicated concentrations for intravenous challenge.

METHOD DETAILS

Fungal cultivation—Sabouraud's agar (BD, Sparks, 21152) was dissolved in water at 50 g/L, and autoclaved for 30 min. Chloramphenicol (Sigma Aldrich, St. Louis) was added to the warm solution at 50 mg/L and the agar was sterilely poured into Petri dishes (VWR, Radnor, 19087) and cooled overnight. Plates were sealed and kept at 40°C until used for fungal growth.

Intravenous injection and brain dissemination assay—Viable cells (from 25,000 to 100,000) of *C. albicans* in 100 μL normal saline were injected through the tail veil using

a tuberculin syringe and 27 ga needle. At different days post infection starting from day 4, mice were euthanized with pentobarbital (Beuthanasia, Intervet Inc, Madison NJ) and exsanguinated by transecting the descending aorta followed by perfusing the brain with normal saline. Brains were removed after sterilely removing the calvarium, weighed, and put into 1 mL of sterile PBS. Brains were then homogenized, and spread directly onto Sabouraud's agar (one brain per plate). Plates were sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL) and incubated at 37°C for a maximum of 7 days. CFU were then enumerated.

Pertussis toxin treatment—Mice were i.p. pertussis toxin (P7208, Sigma Aldrich, St. Louis) twice at day 0 and day 2 at 200 ng per mouse.[57] Fungal infection was then introduced at day 4.

Blood brain barrier integrity test—Mice were challenge with 100,000 viable cells of *C. albicans* 4 days post infection, mice were injected with 200 μg of 70kDa TRITC-dextran (T1162, Sigma Aldrich, St. Louis) 2 h before sacrifice. Mice were euthanized, perfused and brains were isolated and deaggregated by pressing gently through 40μm cell strainers in 3 mL of RPMI medium. The homogenate was then centrifuged (1400 × *g*, 5 min, 4°C), and supernatants were collected. Median fluorescence intensity (MFI) of TRITC was then measured in the supernatant using a fluorescent ELISA reader.

Transepithelial/transendothelial electrical resistance (TEER)—Confluent HBEC-5i cells (CRL-3245, ATCC, Manassas, VA[58]) were cultured in complete DMEM/F12 medium (11320033, Thermo Fisher Scientific, Waltham MA) in 24 well plates containing transwell inserts (#3470, Corning, Kennebuck, ME). Upon reaching confluence, culture medium was changed to serum free DMEM/F12 with 10 mg/mL Sap2 (at pH = 6), Trypsin (pH = 7) or control at pH = 6. TEER was measured using EVOM2 (World Precision Instruments, Sarasota, FL 34240) at indicated time points.

Cytokine measurement—Brain homogenates were collected and then supernatants were used to quantify IL-1β, IL-6, TNF, IFN-β and Aβ proteins using standard ELISA after comparison to recombinant standard (Cytokines: ab210895, ab213749 and ab212073, Abcam, Cambridge MA; DY8234–05, R&D systems, Minneapolis, MN. Aβ capture antibody: #800701, detection antibody: #805504 or #805404, 1:100, Biolegend, San Diego, CA)

Production of recombinant secreted aspartic proteases—Recombinant *C. albicans* Sap proteases were produced as described previously.[53,59] Briefly, Saps were expressed in *Pichia pastoris* and subsequently purified from *P. pastoris* supernatants via anion-exchange chromatography on Q-Sepharose columns (Sap1, Sap2, Sap3, Sap9, and Sap10) or via cation-exchange chromatography on SP-Sepharose columns (Sap6). Sap5 was concentrated from *P. pastoris* supernatants via ultracentrifugation.

N2a cell treatment with secreted aspartic proteinases—N2a cells (CCL-131, ATCC, Manassas, VA) were acquired from culture using a cell scraper and seeded in 24-well plates overnight at 200,000 cells per well in complete DMEM. Culture medium was then

discarded and serum free DMEM (pH = 6) containing 0.2 mg/mL of recombinant Saps[53] (Sap1, 2, 3, 5, 9, 10, 1:1:1:1:1:1) were added, and cells were incubated at 37°C for 2h. Supernatants were then isolated and proteinase inhibitors (1:100, #78442, Thermo Fisher Scientific, Waltham MA) were added to stop proteinase activity, and Aβ concentration was quantified using ELISA. Treated N2a cells were isolated and digested with RIPA buffer or TRIZol. APP was semi-quantified using Western blot (4%–12% Nupage Bis-Tris gel, Thermo Fisher Scientific, Waltham MA) and quantitative PCR. (ab32136, 1:5000 Abcam, Cambridge MA; Mm01344172, Invitrogen, Carlsbad, CA).

APP cleavage with Saps—1 μg/mL of recombinant human APP (843201, Biolegend, San Diego, CA) was buffer exchanged to TBS (pH 3.5 or 6) using amicon centrifugal filters (UFC801024, 10kDa MWCO, Sigma Aldrich, St. Louis) and mixed with recombinant Saps[53] (Sap1, 2, 3, 5, 9, 10, 1:1:1:1:1:1) at a final concentration of 0.2 mg/mL for 2h at 37°C. Proteinase inhibitor (1:100, #78442, Thermo Fisher Scientific, Waltham MA) was then added to the mixture and Aβ-like peptide concentration was quantified using ELISA. The same hydrolysate was subjected to SDS-PAGE followed by western blotting for amyloid β peptides (NP0321BOX, 4%–12% Nupage Bis-Tris gel, Thermo Fisher Scientific, Waltham MA; 800701, Biolegend, San Diego, CA). Alternatively, after incubation of rhAPP and rSaps, for 2 h at 37°C, the mixture was passed through Amicon centrifugal filters (UFC501024, 10kDa MWCO, Sigma Aldrich, St. Louis) to collect Aβ-like peptides for fungistasis assay.

Histology and immunostaining—Mice were perfused with 4% PFA, and the brains were post-fixed in 4% PFA overnight at 4°C followed by cryoprotection in 40% sucrose. 30 μm coronal sections were cut with a microtome and stored at in cryoprotectant at −20°C. For each experiment, sections were collected randomly from at least three animals. Sections were mounted onto slides and were extensively washed in PBS, blocked with PBST containing 3% BSA and 2% donkey serum for 30 min, and then incubated in primary antibody diluted in blocking solution overnight at 4°C (anti-GFAP (1:1000, Millipore, catalog #MAB3402), anti-Iba1 (1:500, Wako, catalog #019–19741), anti-Aβ (1:250 Abcam, ab126649)). After washing, sections or coverslips were incubated in secondary antibody for 1h at room temperature and mounted in DAPI solution after final washing.

Imaging—Fluorescent immunostained brain sections were imaged using the EVOS FL Auto system to locate sites of infection, and then were imaged using a Leica laser confocal microscope.

Primary microglia and astrocyte isolation—Euthanized mice were perfused with ice-cold PBS for 10 min after which brains were divided into approximately 5 mm portions and incubated in digestion buffer (2 mg/ml collagenase (#LS004177, Worthington), 0.04 mg/ml DNAse (#10104159001, Sigma Aldrich, St. Louis) 1, 20% FBS in HBSS) for 1 h at 37°C after which they were deaggregated by pressing through a 40mm nylon mesh.[12,60] 15 mL of complete medium DMEM/F12 (11320033, ThermoFisher scientific, Waltham MA) was added to cells and then centrifuged at 400 × g for 5 min at room temperature. For microglia isolation, the cell pellet was resuspended in 4mL of 37% stock isotonic Percoll

(SIP) (P1644, Sigma Aldrich, St. Louis). 4mL of 70% SIP was underlaid and 1mL of HBSS was overlaid. Tubes were then centrifuged at 300 × g for 40 min, room temperature without centrifuge braking. Microglia were collected from the 37–70% interphase and washed with complete DMEM/F12.[61] For astrocyte isolation, the cell pellet was resuspended in 4mL of 35% SIP. 4mL of 50% SIP was underlaid and 1mL of HBSS was overlaid. Tubes were then centrifuged at 2000 × g for 20 min, room temperature with no braking. Astrocytes were collected from the 35–50% interphase and washed with complete DMEM/F12.[62] Cell purity was confirmed to be >98% by flow cytometry.

***In vitro* fungistasis assay—**BV-2 cells were cultured in 24-well flat tissue culture plates at 12,000 cells per well in complete medium (DMEM with 10% heat inactivated fetal bovine serum, glutamine and Penicillin-Streptomycin). Immediately prior to stimulation, complete medium was replaced with serum free DMEM. Cells were then pre-treated with or without LPS-RS (1 μg/mL, tlrl-prslps, Invivogen, San Diego, CA) for 2.5 h and stimulated with 1 μM endotoxin-free Aβ peptides, scrambled peptide control (mouse Aβ: A-1007-1, A-1008-1, A-1004-1; human Aβ: A-1156-1, A-1166-1. rPeptide, Wadkinsville, GA) or Aβ-like peptides for 3.5 h. Wildtype or *ece1Δ/Δ C. albicans* were added to each well at 200 viable cells per well in a 37°C/5% CO2 incubator. Fungal germination events (FGEs) were counted after 16 h. Percent of fungal growth inhibition was calculated as the (# FGE in wells containing no cells – #FGE in wells containing cells/# FGE in wells containing no cells) × 100. Alternatively, seeded BV-2 cells (12,000 cells/well), primary microglia, or primary astrocytes (100,000 cells/well) were pre-treated with anti-CD11b (1 μg/mL, 101247, Biolegend, San Diego, CA) or neutrophil inhibitory factor (NIF) (1 μg/mL, 5845-NF, R&D systems, Minneapolis, MN) for 1 h at 37°C before stimulation with synthetic candidalysin (5 μM) (Figure 5B).

BV-2-candidalysin co-culture for ELISA—BV-2 cells were obtained from ATCC, determined as mycoplasma and chlamydia free and maintained frozen in liquid nitrogen. Cells were thawed, expanded in DMEM medium, and expression of standard microglial surface markers (TREM2, CD68, and MCM5 by RT-qPCR) was confirmed.[9] Cells were then seeded in 1 mL of DMEM (serum free, Gendepot, Barker TX) in 24 well plates at 100,000 cells per well for 6 h. Synthetic candidalysin (10 μM) was added to each well and incubated for 16 h at 37°C. Supernatants were then collected for ELISA as described above.

Candidalysin—Synthetic, biotinylated and Alexafluor 647-labeled candidalysin or scrambled peptide control were obtained commercially and were >98% pure and endotoxin free (Peptide Protein Research Ltd, SO32 1QD, UK, or Genscript 08854, NJ, USA). Candidalysin sequence: SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK. Scrambled control (SC) peptide sequence: IFKIIISKIQIVMGLNGIPIKVA GSQNIGMI. Peptide biotinylation was performed on the N terminus.

Aβ-TLR4 binding assay—96-well plates (9018, Corning, Kennebuck, ME) were coated with 5 mg/mL Aβ 1–40, 1–42 or scrambled peptide control in carbonate buffer (pH = 9.0) overnight at 4°C. Plates were blocked with i-Block (2%) for 2 h at 37°C and incubated with a 1/2 serial dilution of Histagged TLR4 (3146-TM, R&D systems, Minneapolis, MN)

starting from 50 nM for 2 h at 37°C. After washing, plates were blocked with LPS-RS (500nM, tlrl-prslps, Invivogen, San Diego, CA) at incubated with biotinylated anti-His antibody (5 μg/mL, BAM050, R&D systems, Minneapolis, MN) for 2 h at 37°C followed by SAv-HRP (1:250, 51–9002813, BD Biosciences, San Jose, CA). The plate was further developed using TMB substrate solution (N301, Thermofisher scientific, Waltham MA) and detected at the absorbance wavelength of 450 nm. Alternatively, plates were coated with 5 μg/mL TLR4 or BSA control and a 2/3 serial dilution of biotinylated Aβ 1–40 or 1–42 starting from 50 nM was performed followed by addition of SAv-HRP and TMB.

CD11-candidalysin binding assays—96-well plates (9018, Corning, Kennebuck, ME) were coated with 5 μg/mL candidalysin or scrambled peptide control in carbonate buffer (pH = 9.0) overnight at 4°C. Plates were blocked with i-Block (2%) for 2 h at 37°C and incubated with a 2/3 serial dilution of Histagged CD11b (7959-AM, R&D systems, Minneapolis, MN) or CD11c (7987-AX, R&D systems, Minneapolis, MN), starting at 50nM for 2 h at 37°C. After washing, plates were incubated with biotinylated anti-His antibody (5 μg/mL, BAM050, R&D systems, Minneapolis, MN) for 2 h at 37C followed by SAv-HRP (1:250, 51–9002813, BD Biosciences, San Jose, CA). Plates were further developed using TMB substrate solution (N301, ThermoFisher scientific, Waltham MA) and detected at 450 nm. Alternatively, plates were coated with 5 μg/mL CD11b and a 2/3 serial dilution of biotinylated candidalysin or scrambled peptide control was performed starting at 5nM followed by addition of SAv-HRP and TMB.

CD11 pull-down assays—CHO cells (CHO-CD11a/CD18, CHO-CD11b/CD18 and CHO-CD11c/CD18) were lysed with Pierce IP Lysis Buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol, 87787, ThermoFisher Scientific, USA) and the NaCl concentration was adjusted to 900 mM. Protease and phosphatase inhibitors (P8340/P5726, Sigma-Aldrich, USA) were added to prevent protein degradation. Immunoprecipitation was carried out as following: 500 μg of lysates was pre-cleared with Streptavidin Magnetic Beads (S1420S, New England BioLabs, USA) for 1–2 h at room temperature on a rotator. Pre-cleared lysates (prey) were incubated with biotinylated-candidalysin (bait) or biotinylated-serine (control) (20 μM) for 1–2 h at room temperature on a rotator. The mixtures were loaded onto pre-washed streptavidin beads (100 μL) and incubated overnight on a rotator at 4°C. The next day, beads were washed with 0.5–1 mL of IP buffer 4 times, resuspended in 1X LSB (1M DTT, 4x SDS, PBS) and heated for 7 min at 95°C to elute the bait-prey complexes from the beads. The samples were loaded onto SDS-PAGE gels to detect CD11a (E5S9K) (26703S, Cell Signaling Technology, UK), CD11b/ITGAM (D6X1N) (49420S, Cell Signaling Technology) and CD11c (D3V1E) XP(R) (45581S, Cell Signaling Technology) via Western blotting.

Splenocyte binding to candidalysin—Splenocytes were isolated from the spleens of wildtype or *CD11*$^{-/-}$ mice by passing through 40μm cell strainers as described before, and incubated with biotinylated candidalysin or scrambled peptide control (10 μM) for 1 h at room temperature. Cells were then washed with PBS and centrifuged (400 × g, 5 min, 4°C), and streptavidin-AF647 (405237, Biolegend, San Diego, CA) was added after which binding to splenocytes was assessed by flow cytometry.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are presented as means ± standard errors of the means. Significant differences relative to appropriate controls are expressed by p values of <0.05, as measured two tailed Student's t-test, one-way ANOVA followed by Tukey's test or Dunnett's test for multiple comparison as appropriate to the dataset. Data normality was confirmed using the Shapiro-Wilk test. All experiments were performed in duplicate or triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *C. albicans* Saps cleave APP into Aβ-like peptides that activate microglia through TLR4
- *C. albicans*-derived candidalysin activates microglia through a second pathway via CD11b
- Both mechanisms promote anti-*Candida* immunity and clearance of fungal cells from brain

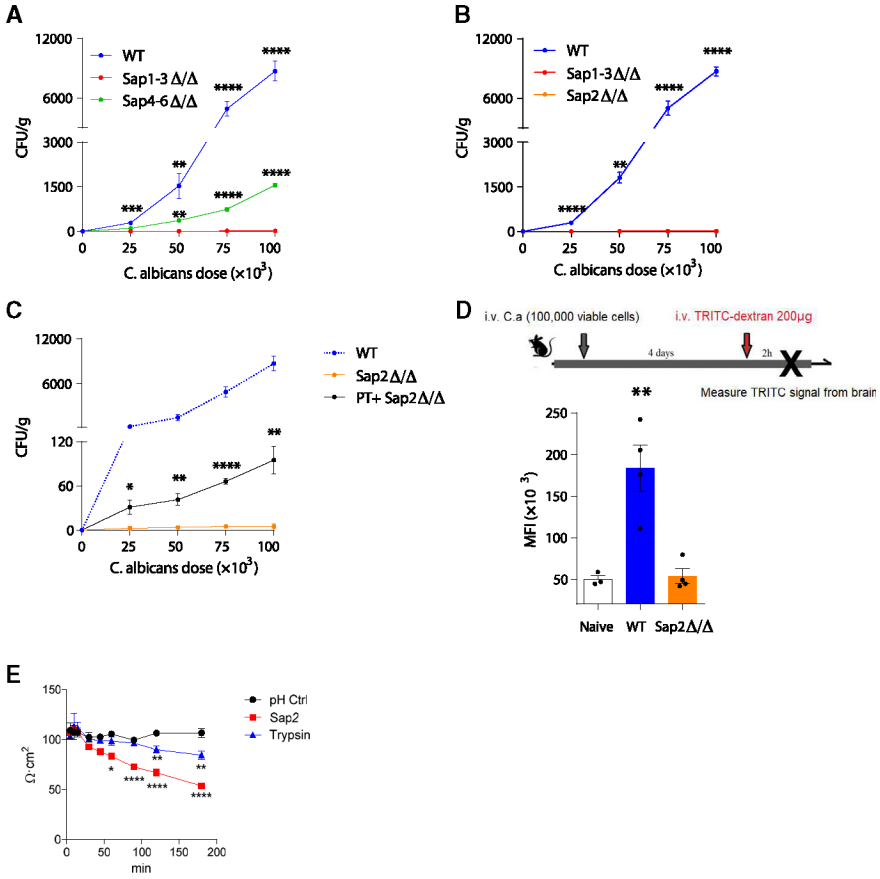

Figure 1. Saps mediate *C. albicans* cerebral invasion

Wild-type mice were challenged i.v. with wild-type parental strain or Sap-deficient *C. albicans* at indicated doses.

(A and B) Cerebral fungal burdens were assessed 4 days post-infection as colony-forming units (CFU) expressed per gram of brain post-challenge with (A) *sap1–3*Δ/Δ, *sap4–6*Δ/Δ and (B) *sap2*Δ/Δ *C. albicans*.

(C) Brain fungal burdens post-i.v. challenge with *sap2*Δ/Δ *C. albicans* from mice pretreated with pertussis toxin (PT) or vehicle.

(D) Mice were challenged i.v. with 100,000 viable cells of wild-type and *sap2*Δ/Δ *C. albicans*. After 2 h, TRITC-dextran were injected i.v., and 2 h later, brains were perfused and homogenized, and the MFI of TRITC was quantitated.

(E) Transendothelial electrical resistance (TEER) of monolayers of HBEC-5i cells was quantitated over time after Sap2 incubation.

n ≥ 4, mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 using one-way ANOVA followed by Tukey's test for multiple comparison. Data are representative of two independent experiments.

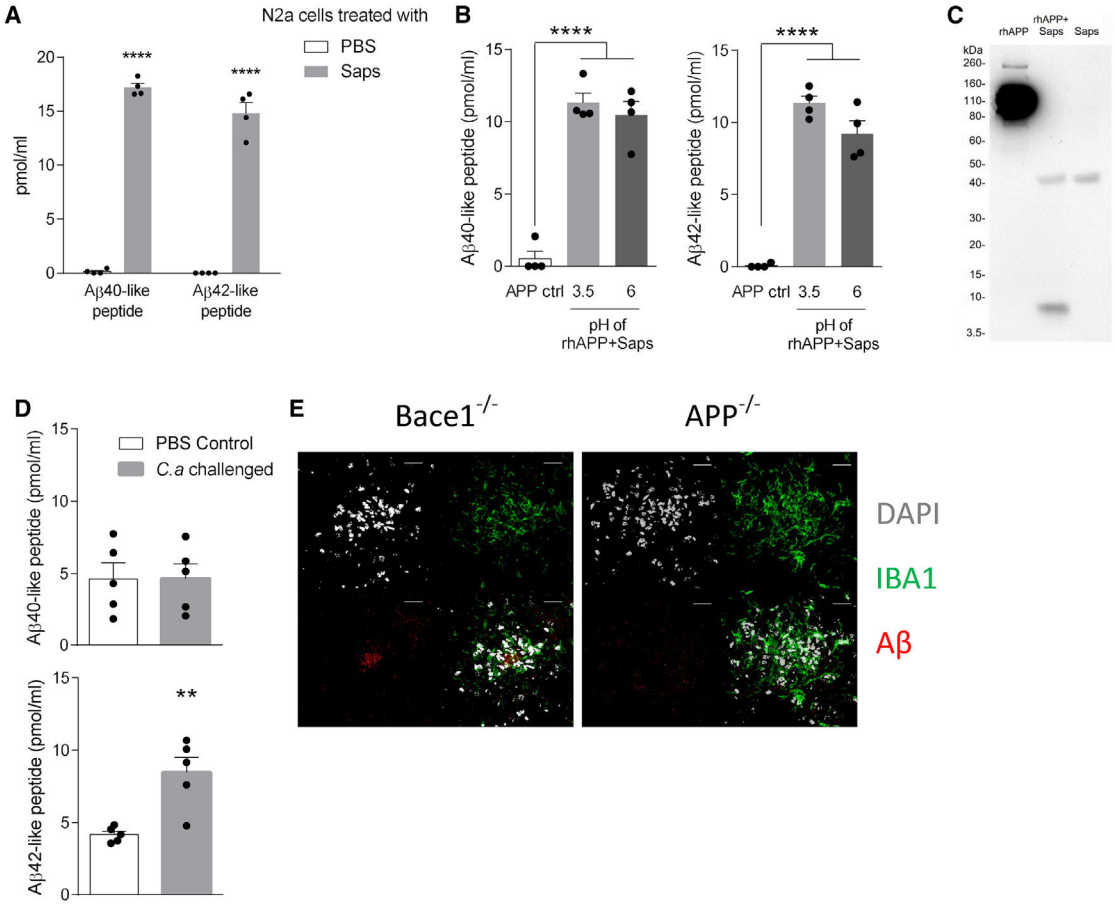

Figure 2. Saps hydrolyze APP to generate Aβ peptides

(A) N2a cells were incubated with indicated amounts of recombinant Saps, after which Aβ40- and Aβ42-like peptides were quantitated from supernatants by ELISA.

(B) Recombinant human APP was incubated with recombinant Saps at pH 3.5 or 6, and Aβ40- and Aβ42-like peptides were quantitated by ELISA.

(C) Recombinant human APP was incubated with a mixture of recombinant Saps, and Aβ-like peptides were visualized by western blotting (SDS-PAGE) for Aβ peptides.

(D) *BACE1*$^{-/-}$ mice were challenged with *C. albicans* and Aβ40- or Aβ42-like peptides were quantitated from brain homogenates by ELISA.

(E) Immunofluorescence staining of FIGGs revealing IBA1-expressing microglial cells, Aβ, and DAPI^{+} nuclei from *C. albicans*-challenged *BACE1*$^{-/-}$ or *APP*$^{-/-}$ mice.

n ≥ 4, mean ± SEM, **p < 0.01 and ****p < 0.0001 using one-way ANOVA followed by Dunnett's test for multiple comparison (B) or two-tailed Student's t test (A and D). Data are representative of three independent experiments.

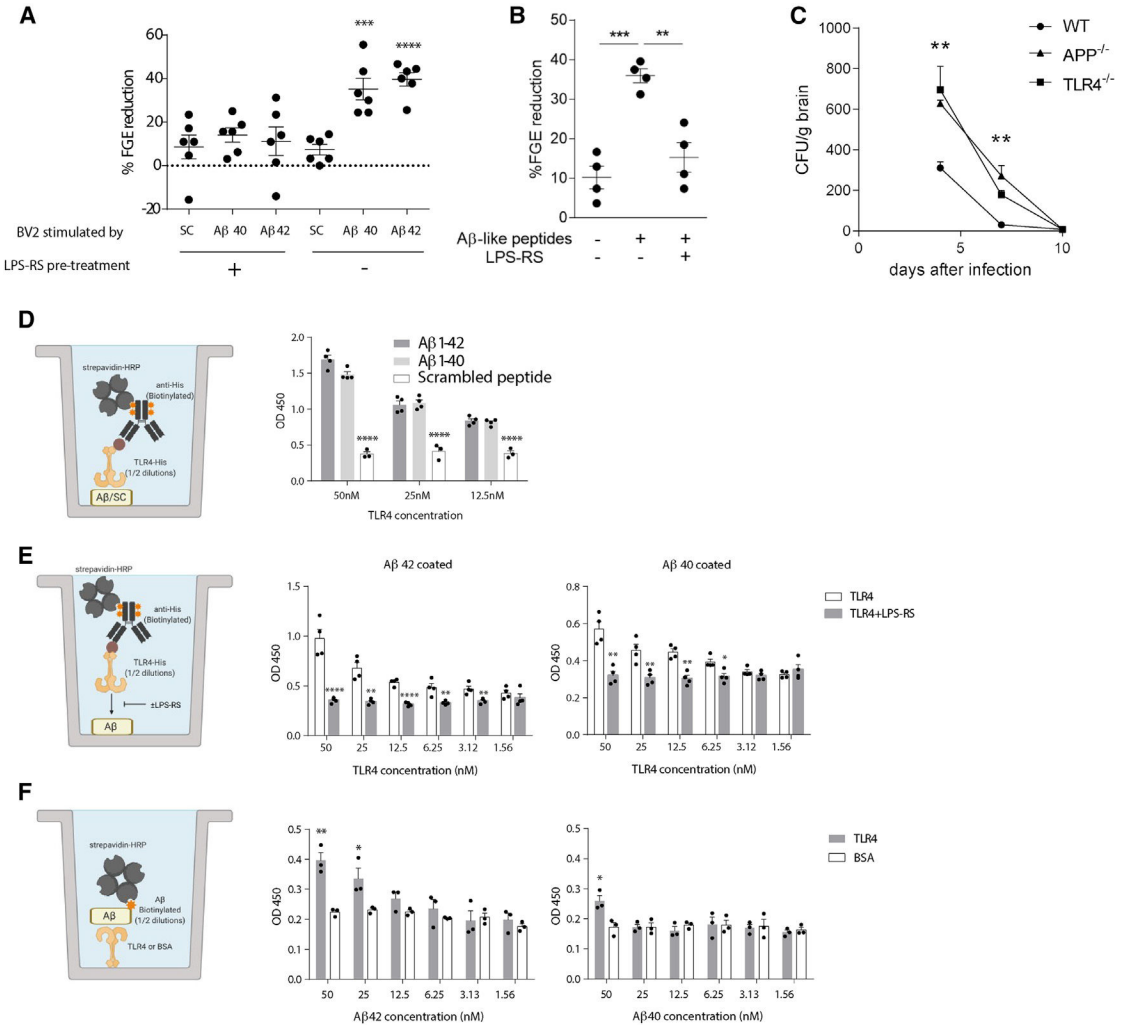

Figure 3. Aβ peptides enhance the anti-fungal activity of BV-2 cells via TLR4

(A and B) Fungistasis assays in which BV-2 cells were pretreated with LPS-RS before activation by (A) Aβ peptides or scrambled control (SC) peptide or (B) Aβ-like peptides derived from the Sap-dependent cleavage of APP and addition of viable *C. albicans*.

(C) Brain fungal burdens of wild-type (WT), *TLR4*$^{-/-}$, and *APP*$^{-/-}$ mice assessed between days 4 and 10 after i.v. injection of 25,000 viable WT *C. albicans* cells.

(D–F) Schematic diagrams of modified ELISAs assessing binding between Aβ peptides and TLR4 with capture and detecting reagent configurations with and without addition of LPS-RS and aggregate data as indicated.

n ≥ 4. Mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 using one-way ANOVA followed by Dunnett's test for multiple comparison or two-tailed Student's t test. Data are representative of two independent experiments.

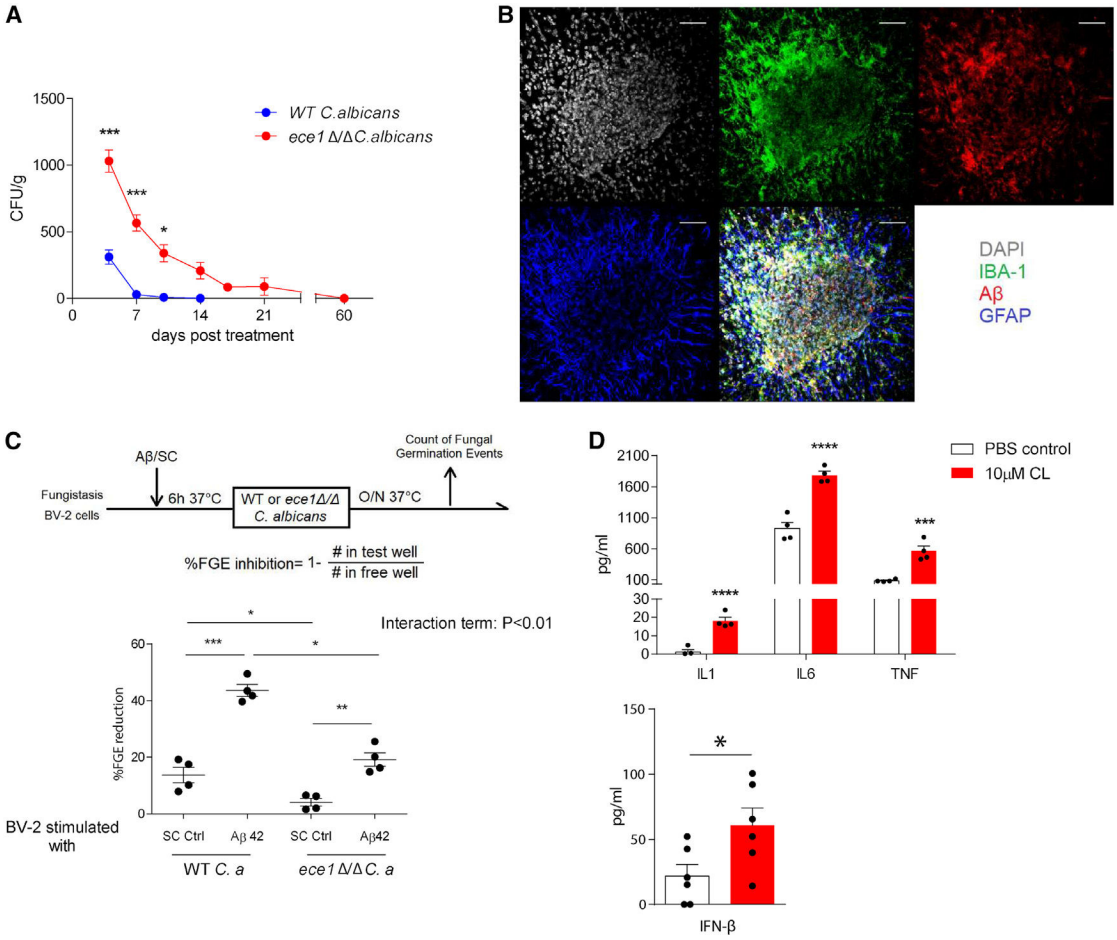

Figure 4. Candidalysin enhances cerebral clearance of *C. albicans*

(A) WT mice were challenged i.v. with 25,000 viable cells of WT or *ece1*Δ/Δ *C. albicans*, and cerebral clearance was assessed over 60 days.

(B) Immunofluorescence staining of FIGGs for DAPI, IBA-1, Aβ, and GFAP 21 days post-challenge.

(C) Schematic diagram of fungistasis assay and aggregate data of BV-2 cells challenged with WT or *ece1*Δ/Δ *C. albicans* after stimulation with either SC or Aβ42 peptides.

(D) BV-2 cells were incubated with synthetic candidalysin (10 μM) overnight, and secretion of IL-1, IL-6, TNF, and IFN-β was assessed by ELISA.

n ≥ 4, mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 using two-way ANOVA (C) or two tailed Student's t test (D–F). Data are representative of two independent experiments.

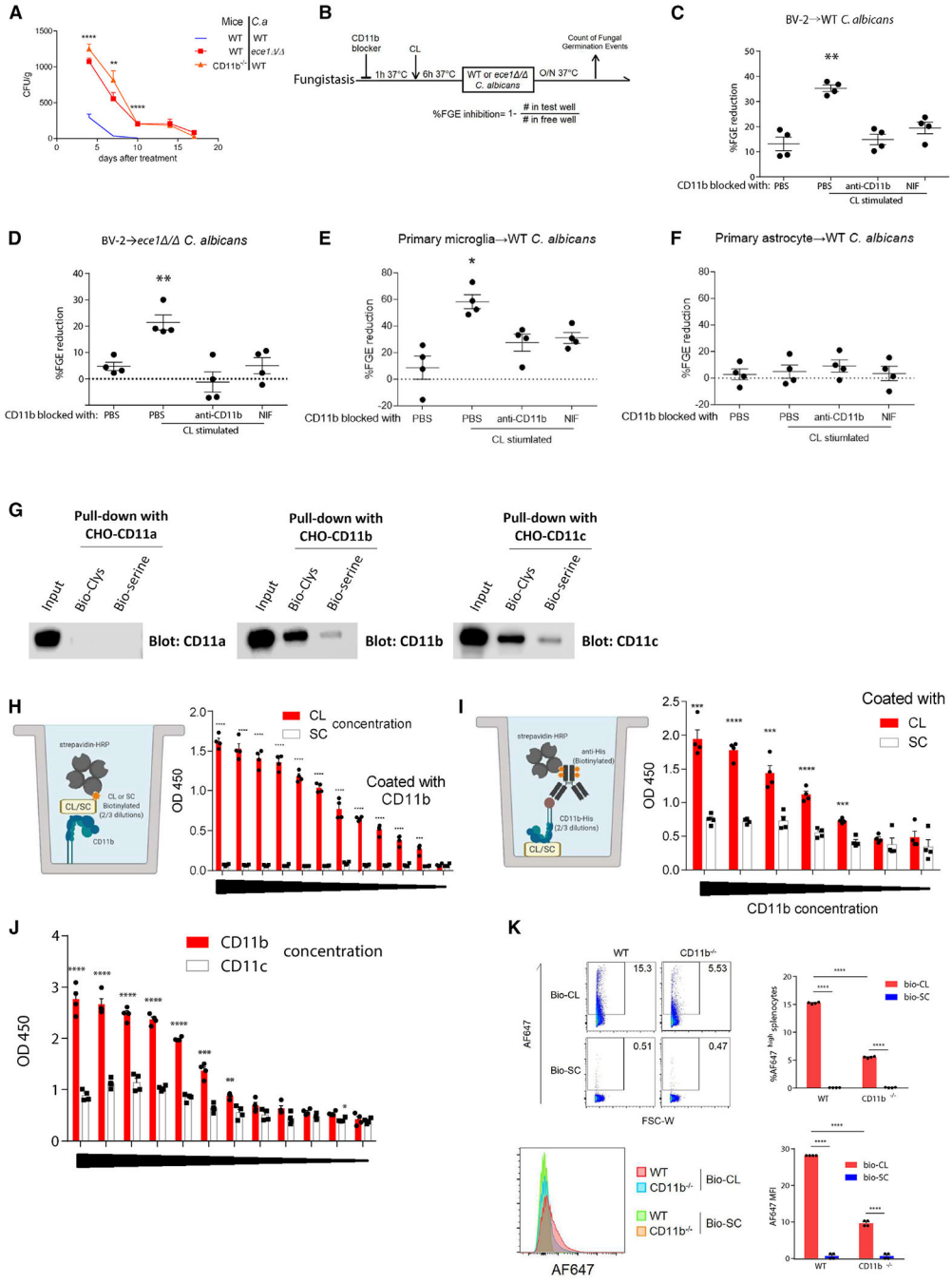

Figure 5. Candidalysin activates microglial anti-fungal responses through CD11b

(A) WT or *CD11b*$^{-/-}$ mice were challenged i.v. with 25,000 viable cells of WT or *ece1*Δ/Δ *C. albicans*, and cerebral clearance was assessed over 20 days.

(B–D) Schematic diagram of fungistasis assay (B) and aggregate data of candidalysin-activated BV-2 cells challenged with either WT or *ece1*Δ/Δ *C. albicans* with and without anti-CD11b or NIF blockade of CD11b (C and D).

(E and F) Fungistasis assay of candidalysin-activated (E) primary microglia or (F) primary astrocytes from WT mice with WT *C. albicans* with and without anti-CD11b or NIF blockade of CD11b.

(G) Pull-down assays using CD11-expressing CHO cells. CHO-CD11a/b/c cell lysates (prey) were incubated with biotinylated candidalysin (Bio-Clys; bait) or biotinylated serine (Bio-serine; control), and the mixtures were loaded onto prewashed streptavidin beads. Bait-prey complexes were eluted from the beads and loaded onto SDS-PAGE gels to detect CD11a, CD11b, and CD11c via western blotting.

(H–J) Schematic diagrams and aggregate data depicting *in vitro* assays in which the dose-dependent binding of plate-bound (H) CD11b extracellular domain or (I) candidalysin/SC peptide to the other reagent or (J) CD11b or CD11c extracellular domains binding to plate-bound candidalysin were determined colorimetrically (OD, optical density).

(K) Flow cytometric analysis of splenocytes from WT or *CD11b*$^{-/-}$ mice incubated with AF647-conjugated CL (10 μM).

n = 4, mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 using one-way ANOVA followed by Tukey's test for multiple comparison (A–F), two tailed Student's t test (H–J), or two-way ANOVA (K). Data are representative of two or three independent experiments.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-amyloid beta 17–24	Biolegend	Cat# 800701 RRID: AB_2564633
Biotin anti-β-Amyloid, 1–42	Biolegend	Cat# 805504 RRID: AB_2564688
Biotin anti-β-Amyloid, 1–40	Biolegend	Cat# 805404 RRID: AB_2564974
anti-GFAP antibody	Millipore	Cat# MAB3402 RRID: AB_94844
anti-Iba1 antibody	Wako	Cat# 019–19741 RRID: AB_839504
Anti-amyloid beta	abcam	Cat# ab126649 RRID: AB_2818982
anti-CD11b	Biolegend	Cat# 101247 RRID: AB_2813917
anti-His tag	R&D systems	Cat# BAM050 RRID: AB_356845
Anti-CD11a	Cell signaling	Cat# 26703S
anti-CD11b	Cell signaling	Cat# 49420S RRID: AB_2799357
anti-CD11c	Cell signaling	Cat# 45581S RRID: AB_2799286
Anti-Amyoid precursor protein	abcam	Cat#: ab32136 RRID: AB_2289606
Chemicals, peptides, and recombinant proteins		
YPD Broth	BD	DF0428-17-5
Sabouraud's agar	BD	DF0109-08-2
Chloramphenicol	Sigma Aldrich	C0378–100G
Recombinant Candidalysin (>98% purity): SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK	Peptide Protein Research Ltd Genscript	N/A
Recombinant Scrambled control (SC) peptide (>98% purity): IFKIIISKIQIVMGLNGIPIKVAGSQNIGMI	Peptide Protein Research Ltd Genscript	N/A
Recombinant Biotinylated Scrambled control (SC) peptide (>98% purity): (Bio)-IFKIIISKIQIVMGLNGIPIKVAGSQNIGMI	Genscript	N/A
Recombinant Biotinylated Candidalysin (>98% purity): (Bio)-SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK	Genscript	N/A
Secreted aspartic proteinases (Saps)	Dr. Bernhard Hube (Schild et al.)[53]	N/A
pertussis toxin	Sigma aldrich	P7208
TRITC-dextran	Sigma aldrich	T1162
DMEM/F12 medium	Thermofisher	11320033
RPMI medium	Thermofisher	11875093
proteinase inhibitors	Thermofisher	#78442
recombinant human APP	Biolegend	843201
collagenase	Worthington	LS004177

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Percoll	Sigma Aldrich	P1644
His-tagged CD11b	R&D	7959-AM
His-tagged CD11c	R&D	7987-AX
His-tagged TLR4	R&D	3146-TM
SAv-HRP	BD Biosciences	51–9002813
TMB substrate	Thermofisher	N301
Pierce IP Lysis Buffer	Thermofisher	87787
Streptavidin Magnetic Beads	New England BioLabs	S1420S
streptavidin-AF647	Biolegend	405237
Neutrophil inhibitory factor	R&D	5845-NF
LPS-RS	Invivogen	tlrl-prslps
Experimental models: Cell lines		
CHO cells	Dr. Bernhard Hube Dr. Julian Naglik	N/A
BV-2	Dr. Hui Zheng, ATCC (Wu et al.)[9]	N/A
N2a cells	ATCC	CCL-131
HBEC-5i	ATCC	CRL-3245
Experimental models: Organisms/strains		
Mice: C57BL/6J	The Jackson Laboratory	Stock number: 000664
Mice: APP$^{-/-}$	The Jackson Laboratory	Stock number: 004133
Mice: TLR4$^{-/-}$	The Jackson Laboratory	Stock number: 007227
Mice: BACE1$^{-/-}$	The Jackson Laboratory	Stock number: 004714
Mice: CD11b$^{-/-}$	The Jackson Laboratory	Stock number: 007227
C. albicans: WT (clinical isolate)	Wu et al.[9]	N/A
C. albicans: *SAP1–3 Δ/Δ*. Sap1, Sap2, Sap3 triple gene deletant sap1Δ:hisG/sap1Δ:hisG sap2Δ:hisG/sap2Δ:hisG sap3Δ:hisG/sap3Δ:hisG rps1:URA3	Dr. Bernhard Hube (Hube et al.)[54]; Moyes et al.[11]	N/A
C. albicans: *SAP4–6Δ/Δ*Sap4, Sap5, Sap6 triple gene deletant sap1Δ:hisG/sap1Δ:hisG sap2Δ:hisG/sap2Δ:hisG sap3Δ:hisG/sap3Δ:hisG rps1:URA3	Dr. Bernhard Hube (Hube et al.)[54]	N/A
C. albicans: *SAP1Δ/Δ*Sap1 deletant sap1Δ:hisG/sap1Δ:hisG rps1:URA3	Dr. Bernhard Hube (Hube et al.)[54]; Moyes et al.[11]	N/A
C. albicans: *SAP2Δ/Δ*.Sap2 deletant sap2Δ:hisG/sap2Δ:hisG rps1:URA3	Dr. Bernhard Hube (Hube et al.)[54]Moyes et al.[11]	N/A
C. albicans: *SAP3Δ/Δ*.Sap3 deletant sap3Δ:hisG/sap3Δ:hisG rps1:URA3	Dr. Bernhard Hube (Hube et al.)[54]; Moyes et al.[11];	N/A
C. albicans: Candidalysin gene deletant ece1:HIS1/ ece1:ARG4; RPS1/ rps1:URA3	Dr. Bernhard Hube Dr. Julian Naglik (Moyes et al.)[11]	N/A
C. albicans: *PRA1Δ/Δ*.Pra1 deletant ura3: imm434/ura3: imm434 his:hisG/his1:hisG *pra1::HIS1/pra1::ARG4*+Clp10	Dr. Bernhard Hube (Hube et al.)[54]; Moyes et al.[11]	N/A
C. albicans: BWP17/CIp30 Parental wildtype isogenic strain Rps1: (HIS1 ARG4 URA3)	Dr. Bernhard Hube Dr. Julian Naglik (Hube et al.)[54]; Moyes et al.[11]	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
FlowJo software (version 10.0.7)	LLC	https://www.flowjo.com
GraphPad PRISM v.6.0.1	GraphPad software	https://www.graphpad.com
Other		
Amicon ultra-4 centrifugal filter unit	Millipore Sigma	UFC8010
NuPAGE 4–12% Bis-Tris Protein Gels	Invitrogen	NP0335BOX
MES SDS Running Buffer	Invitrogen	NP0002
Precision Plus Protein Kaleidoscope Prestained Protein Standards	Bio-rad	#1610375
96-well plates	Corning	9018
Amicon filter unit	Sigma Aldrich	UFC801096
ELISA Antibody Pair: mouse IL-1β	Abcam	Cat# ab210895
ELISA Antibody Pair: mouse IL-6	Abcam	Cat# ab213749
ELISA Antibody Pair: mouse TNF	Abcam	Cat# ab212073
ELISA Antibody Pair: mouse IFN-β	R&D systems	Cat# DY8234–05