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CARD9$^+$ Microglia Protect against Fungal Invasion via IL-1β and CXCL1-mediated Neutrophil Recruitment

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Abstract

The C-type lectin receptor/Syk adaptor CARD9 facilitates protective antifungal immunity within the central nervous system (CNS), as human CARD9-deficiency causes fungal-specific CNS-targeted infection susceptibility. CARD9 is required for neutrophil recruitment to the fungal-infected CNS, which mediates fungal clearance. Here, we investigated host and pathogen factors that promote protective neutrophil recruitment during *Candida albicans* CNS invasion. IL-1β was essential for CNS antifungal immunity by driving CXCL1 production, which recruited CXCR2-expressing neutrophils. Neutrophil-recruiting IL-1β and CXCL1 production was induced in microglia by the fungal-secreted toxin Candidalysin, in a p38-cFos-dependent manner. Importantly, microglia relied on CARD9 for production of IL-1β, via both *Il1b* transcriptional regulation and inflammasome activation, and of CXCL1 in the fungal-infected CNS. Microglia-specific *Card9* deletion impaired IL-1β and CXCL1 production and neutrophil recruitment, and increased CNS fungal proliferation. Taken together, an intricate network of host-pathogen interactions promotes CNS antifungal immunity, which is impaired in human CARD9-deficiency leading to CNS fungal disease.

Introduction

The CNS is invaded by microorganisms during systemic infections, yet the mechanisms of CNS-specific anti-microbial immunity remain poorly-understood. This is particularly true for CNS fungal infections, which present unmet diagnostic and treatment challenges, leading to unacceptably high mortality (>50%)[1]. Fungal CNS invasion is enhanced by fungal-specific risk factors, including HIV infection, neutropenia, corticosteroid use, and Bruton's tyrosine kinase inhibition[1]. However, the most striking human risk factor for selective CNS fungal infection susceptibility is inherited deficiency of the C-type lectin receptor (CLR)–Syk adaptor CARD9.

CARD9 relays fungal-sensing signals downstream of the CLR superfamily of pattern recognition receptors, including Dectin-1, Dectin-2, Dectin-3 and Mincle. Syk kinase is recruited to phosphorylated ITAM sequences of CLRs or their signaling partner FcRγ to form the CARD9-BCL10-MALT1 signalosome, which activates downstream effectors including NFκB, NLRP3 inflammasome and MAPK signaling.

CARD9-deficient patients manifest fungal-specific infection susceptibility, predominantly in the CNS by *Candida albicans*[2, 3, 4]. We previously showed that CARD9-deficiency in humans and mice confers a fungal- and brain-specific defect in neutrophil recruitment, which is detrimental for control of CNS fungal invasion[5]. However, the CNS cellular and molecular cues that promote protective neutrophil recruitment during *C. albicans* invasion and their dependence on CARD9 *in vivo* remain unknown.

Herein, we systematically investigated host and pathogen factors that promote protective neutrophil influx into the *C. albicans*-infected CNS to better understand the pathogenesis of human CARD9-deficiency. We uncover an intricate pathway by which the *C. albicans*-secreted toxin Candidalysin engages microglia to produce IL-1β and CXCL1 for protective recruitment of CXCR2-expressing neutrophils. Importantly, microglial IL-1β and CXCL1 production depends on CARD9 and specific deletion of microglial CARD9 impairs neutrophil recruitment to the *C. albicans*-infected CNS. Collectively, our data unveil complex host-pathogen interactions that recruit protective neutrophils during fungal CNS invasion and reveal the mechanism that underlies CNS fungal susceptibility in CARD9-deficiency.

Results

CLR Functional Redundancy during Fungal CNS Invasion

As previously shown, CARD9 is essential for protective CNS immunity against *C. albicans*, principally through promoting early neutrophil recruitment (**Fig. 1a**). We first investigated the relative contribution of CARD9-coupled CLRs, which are expressed by brain-resident microglia (**Supplementary Fig. 1**), in mediating this protective neutrophil recruitment. We infected mice deficient in Dectin-1 (*Clec7a*$^{-/-}$), Dectin-2 (*Clec4n*$^{-/-}$), Dectin-3 (*Clec4d*$^{-/-}$) and Mincle (*Clec4e*$^{-/-}$) and measured brain neutrophil accumulation at 24 h post-infection (**Supplementary Fig. 2**). We chose this time-point since it is the peak of the neutrophil response in wild-type animals and neutrophil depletion at this time-point increases susceptibility to brain fungal invasion[5].

Animals individually deficient in CARD9-coupled CLRs recruited neutrophils to the infected brain normally (**Fig. 1b**). Despite this, we observed increases in fungal brain burdens at 72 h post-infection in mice deficient in Dectin-1 or Dectin-2 but not Dectin-3 or Mincle (**Fig. 1c**), suggesting that Dectin-1 and Dectin-2 employ neutrophil recruitment-independent mechanisms to protect against brain fungal proliferation. Indeed, brain-infiltrating neutrophils from Dectin-1 and Dectin-2-deficient animals exhibited reduced fungal phagocytosis (**Supplementary Fig. 3**), consistent with prior findings[6].

To activate CARD9-dependent signaling, phosphorylation occurs on the ITAM sequence within the intracellular tail of Dectin-1 or FcRγ, which Dectin-2, Dectin-3 and Mincle associate with. Therefore, we assessed whether deletion of all four CLRs affected the neutrophil response in

the infected brain. We used mice doubly-deficient in Dectin-1 and FcRγ (*Clec7a*$^{-/-}$*Fcerg1*$^{-/-}$) and found that loss of both Dectin-1 and the FcRγ-coupled CLRs phenocopied Card9-deficiency with significantly decreased neutrophil recruitment and corresponding increased brain fungal burdens (**Fig. 1b,c**). Taken together, CARD9-coupled CLRs functionally compensate to mediate neutrophil recruitment-dependent protection against *C. albicans* CNS invasion.

MALT1 is Required for Defense against CNS Candidiasis

The CARD9-MALT1-BCL10 signalosome is necessary for transducing fungal-sensing intracellular signals. Human deficiencies of MALT1 or BCL10 cause defective innate and adaptive immune responses, and many of these patients die in childhood from bacterial and viral infections[2]. Human MALT1-deficiency additionally manifests with mucosal candidiasis, suggesting that antifungal immunity is impaired in these patients. However, whether MALT1-deficiency also predisposes to brain-targeted candidiasis is unknown. To test this, we infected Malt1-deficient mice and assessed control of CNS *C. albicans* growth. *Malt1*$^{-/-}$ animals recruited neutrophils to the brain similarly to wild-type, however these animals exhibited uncontrolled brain fungal growth at 72 h post-infection (**Fig. 1d**). Therefore, MALT1 is critical for protective CNS immunity against *C. albicans*; however, the MALT1-dependent protective mechanisms operating in this tissue are independent of neutrophil recruitment, unlike CARD9.

***Candida* Drives CNS Neutrophil Influx via IL-1β and CXCL1**

To determine the local cues that recruit protective neutrophils in the infected CNS, we examined key cytokine and chemokine circuits using gene-deficient mice. We began by

129 infecting IL-1 receptor (IL-1R)-deficient mice, because production of IL-1β by human peripheral
130 blood mononuclear cells upon fungal stimulation depends on CARD9[5, 7], and because IL-1R
131 was previously shown to promote neutrophil recruitment to fungal-infected mucosal tissues[8, 9],
132 and to the bacterial-infected brain[10]. Upon *C. albicans* challenge, IL-1R-deficient mice
133 phenocopied Card9-deficient mice, with a loss of early brain neutrophil recruitment and
134 accompanying increased fungal brain burdens (**Fig. 2a,b**). Consistent with this, loss of the IL-
135 1R signaling adaptor MyD88 caused similar defects in recruiting neutrophils and controlling
136 fungal proliferation in the infected brain (**Fig. 2a,b**).
137
138 We next assessed which IL-1R ligands were important for driving CNS protection by infecting
139 mice deficient in IL-1α, IL-1β or both. Mice lacking IL-1α had a small reduction in neutrophil
140 numbers and a slight increase in fungal brain burden at 24 h post-infection (**Fig. 2a,b**).
141 However, the lack of IL-1α appeared to be compensated by IL-1β, since *Il1a*$^{-/-}$ animals
142 recovered and controlled fungal brain infection similar to wild-type by 72 h post-infection (**Fig.**
143 **2b**). In keeping with the critical contribution of IL-1β, mice deficient in IL-1β, or both IL-1α/IL-
144 1β, exhibited significantly reduced neutrophil accumulation and were highly susceptible for
145 fungal brain invasion (**Fig. 2a,b**). Therefore, IL-1β is a critical mediator of neutrophil
146 recruitment to promote control of *C. albicans* brain infection.
147
148 Downstream of IL-1R, the production of local chemotactic mediators recruits immune cells to
149 infected tissues. Previously, we showed that the CNS-neutropenia observed in mouse and
150 human CARD9-deficiency is not caused by neutrophil-intrinsic chemotaxis defects[5], but rather
151 by insufficient local production of soluble chemotactic mediators. However, which among the

several chemoattractants and their receptors recruit(s) protective neutrophils to the *C. albicans*-infected CNS is unknown.

CARD9 was shown to drive production of the CXCR2 ligands CXCL1 and CXCL2 during inflammatory arthritis[11] and murine subcutaneous phaeohyphomycosis[12]. During systemic *C. albicans* infection, CCR1 drives renal neutrophil accumulation and immune-related kidney destruction[13], the leukotriene B4 (LTB_4) receptor LTB4R1 promotes detrimental pulmonary neutrophil accumulation[14], and CXCR1 mediates neutrophil-dependent fungal killing in the kidney[15]. However, the role of these receptors in CNS anti-*Candida* immunity is unknown, while CXCR2 and fMet-Leu-Phe (fMLP) receptor FPR1 have not been examined in anti-*Candida* defense.

To test the relative dependence on these major neutrophil-targeted chemoattractant receptors in protecting the fungal-infected brain, we infected mice deficient in CCR1, CXCR1, CXCR2, LTB4R1 or FPR1 and measured neutrophil recruitment and fungal brain burdens. We found no involvement of the CCL3–CCR1, CXCL5–CXCR1, LTB4–LTB4R1 or fMLP–FPR1 axes in controlling fungal brain infection, in line with normal early neutrophil recruitment in infected *Ccr1*$^{-/-}$, *Cxcr1*$^{-/-}$, *Ltb4r1*$^{-/-}$ and *Fpr1*$^{-/-}$ animals (**Fig. 2c,d and Supplementary Fig. 4**). In contrast, CXCR2-deficient mice had significantly reduced neutrophil accumulation and corresponding significantly increased fungal brain growth (**Fig. 2c,d**). These data demonstrate the importance of the CXCR2 axis in neutrophil-mediated protection against *C. albicans* brain infection.

Next, we wondered which CXCR2 ligand may recruit protective neutrophils to the fungal-infected brain. We infected *Cxcl1*$^{-/-}$ mice that lack expression of the potent neutrophil chemoattractant CXCL1. Notably, these animals had decreased neutrophil recruitment to the brain post-infection and exhibited a similar CNS invasion susceptibility phenotype to the *Cxcr2*$^{-/-}$ mice (**Fig. 2c,d**). Therefore, the CXCL1/CXCR2 chemokine axis is critical for protection against *C. albicans* brain invasion by recruiting protective neutrophils. Importantly, this data indicates that the reduced CXCL1 in the human CARD9-deficient *C. albicans*-infected CSF is biologically relevant and significant[5].

IL-1β Activates CXCL1 in the Fungal-Infected Brain

Since both IL-1β and CXCL1 were required for protection, we investigated whether their activation in the infected brain was simultaneous or sequential. We measured IL-1β and CXCL1 in brain homogenates at 24 h post-infection in animals lacking these inflammatory mediators using ELISA. We found no defect in IL-1β levels in CXCL1-deficient infected brains; however, we discovered a significant defect in CXCL1 production in the absence of IL-1β (**Fig. 3a**). To define the IL-1β-dependent brain cellular sources of CXCL1, we infected wild-type and IL-1β-deficient mice and used intracellular flow cytometry. CXCL1 and pro-IL-1β were produced by multiple myeloid phagocytes in the fungal-infected brain, including resident microglia, the most numerous immune cells in the brain, recruited Ly6C^{hi} monocytes which have been implicated in controlling *C. albicans* CNS invasion[16], and neutrophils themselves (**Fig. 3b**). Interestingly, *Il1b*$^{-/-}$ microglia recovered from *C albicans*-infected brains had a profound defect in CXCL1 production, exhibiting significant reductions under every *ex vivo* restimulation condition tested (**Fig. 3c**). Ly6C^{hi} monocytes isolated from *Il1b*$^{-/-}$ *C. albicans*-

198 infected brains produced less CXCL1 when restimulated *ex vivo* with LPS, with no differences
199 detected under non-stimulated or zymosan-stimulated conditions. Neutrophil production of
200 CXCL1 did not differ between the two mouse groups (**Fig. 3c**). Therefore, IL-1β is required for
201 subsequent CXCL1 production from resident microglia and recruited monocytes, which in turn
202 recruits CXCR2-expressing neutrophils to the fungal-infected brain (**Fig. 2c**).
203
204 **Candidalysin is a Fungal Avirulence Factor in the Brain**
205 Use of genetically-deficient mice allowed us to map the host pathway promoting protection
206 against *C. albicans* brain infection, in which IL-1β–IL-1R–MyD88 signaling activates CXCL1
207 production by resident microglia and recruited monocytes to mobilize neutrophils into the CNS.
208 To identify the pathogen-associated factors that induce this protective host pathway, we
209 infected animals with *C. albicans* strains lacking known virulence factors and assessed
210 neutrophil recruitment and IL-1β and CXCL1 production in the infected brain.
211
212 *C. albicans* hyphae are the predominant CNS-invasive morphology of *C. albicans*[5] and hyphal
213 formation is associated with important virulence traits such as toxin and protease production,
214 adhesion, invasion, and immune system activation[17]. Thus, we first asked whether neutrophil
215 recruitment was impaired during infection with the *hgc1*Δ/Δ *C. albicans* strain which cannot
216 filament *in vivo*[18]. Indeed, infection with hypha-deficient *hgc1*Δ/Δ *C. albicans* significantly
217 impaired neutrophil recruitment and enhanced fungal CNS tissue invasion relative to the
218 isogenic wild-type *C. albicans* strain (**Fig. 4a**). Thus, filamentation is strikingly not required for
219 *C. albicans* invasion of brain tissue, in contrast to other organs such as the kidney[18].
220

Candidalysin is a recently-described peptide toxin encoded by *ECE1* and expressed exclusively by *C. albicans* hyphae[19]. Candidalysin was shown to mediate epithelial cell damage via pore formation in the plasma cell membrane resulting in IL-1α release and pro-inflammatory cytokine production. Hence, Candidalysin-null mutants were highly attenuated in murine oropharyngeal and vulvovaginal candidiasis models[19, 20, 21]. Instead, we found that lack of Candidalysin promoted brain infection, and that this phenotype was specific to the Candidalysin peptide since mutant strains deficient in the entire gene (*ece1*Δ/Δ) or specifically in the Candidalysin-encoding portion of the gene (*ece1*Δ/Δ + *ECE1*$_{\Delta 184\text{-}279}$) were both hyper-virulent for *C. albicans* brain invasion (**Fig. 4b**).

The increased ability of the Candidalysin-null mutants to proliferate within the brain directly correlated with the degree of neutrophil recruitment. We found a near absence of neutrophils in the brains of wild-type animals infected with Candidalysin-null strains and observed hyphal forms growing in the brain parenchyma with no neutrophilic reaction (**Fig. 4c**). In contrast, the Candidalysin-producing parental strain and the re-integrant control strain promoted neutrophil recruitment at 24 h post-infection, and these neutrophils clustered around invading hyphae (**Fig. 4c**). In line with the absence of neutrophils in the brains of mice infected with Candidalysin-null strains, IL-1β and CXCL1 were significantly reduced in brain homogenates from animals infected with these strains (**Fig. 4d**). Therefore, Candidalysin is a key fungal factor that activates the IL-1β–CXCL1 protective pathway *in vivo*. Notably, in contrast to its role in the mucosa, Candidalysin acts as an avirulence factor in the brain by instigating protective host CNS immunity, underscoring the tissue-specific opposing roles that a microbial factor may play during infection with the same pathogen[17].

We next wondered whether other *C. albicans* hyphae-associated secreted proteins also activate protective neutrophil responses in the brain. Secreted aspartyl proteases (Saps) are enzymes with extracellular proteolytic activity and are linked to virulence[22]. *C. albicans* Saps promote neutrophil recruitment during vulvovaginal candidiasis in mice[23, 24]. Expression of the *SAP4-6* subfamily is coordinately regulated with hyphal formation[22], therefore we tested whether these hyphal-associated Saps contributed towards virulence during brain invasion. Wild-type animals infected with the triple-deficient strain *sap4/5/6*Δ/Δ had comparable brain fungal burdens to animals infected with the complemented control strain (**Fig. 4e**). In line with this, we saw no difference in CNS neutrophil recruitment in these animals, indicating that *C. albicans* Saps exhibit tissue-specific roles in promoting neutrophil recruitment during infection[23, 24] (**Fig. 4e**). Therefore, protective CNS neutrophil recruitment is activated by Candidalysin, and not by other *C. albicans* hyphae-secreted enzymes.

Candidalysin Drives Microglial IL-1β and CXCL1 *in vivo*

Since Candidalysin activates host CNS immunity, we sought to define the Candidalysin-responsive CNS immune cells. We infected wild-type mice with either the parental strain of *C. albicans* (BWP17) or the Candidalysin-null strain (*ece1*Δ/Δ), and analyzed IL-1β and CXCL1 production using intracellular flow cytometry at 24 h post-infection (**Fig. 5**).

Although all brain phagocytes produced both IL-1β and CXCL1, microglia were the only population to exhibit dependence on Candidalysin, since microglia isolated from *ece1*Δ/Δ-infected brains produced significantly less IL-1β and CXCL1 *ex vivo* (**Fig. 5a,b**). In contrast, Ly6C^{hi} monocytes and neutrophils did not depend on Candidalysin for IL-1β and CXCL1

267 production, suggesting that other as-yet unidentified fungal factors activate this pathway in
268 these phagocytes. Together, our data show that Candidalysin acts on microglia to stimulate IL-
269 1β release, which in turn drives CXCL1 production that is required for protective neutrophil
270 CNS recruitment.
271
272 **Candidalysin Drives Differing Glial IL-1β–CXCL1 *ex vivo***
273 To gain mechanistic insights into how microglia respond to Candidalysin, we cultured the
274 microglia cell line BV-2[25] in the presence of synthetic Candidalysin and measured IL-1β and
275 CXCL1 in the supernatants by ELISA. In line with our *in vivo* work, we found time- and dose-
276 dependent IL-1β production by BV-2 cells in response to Candidalysin (**Fig. 6a**). However, we
277 did not detect CXCL1 from BV-2 cells stimulated under these conditions. We first considered
278 that this could be due to Candidalysin-induced damage that may prevent BV-2 cells from
279 producing CXCL1 after IL-1β secretion. Indeed, as shown for epithelial cells[19], Candidalysin
280 mediated dose-dependent cell damage to BV-2 microglia (**Fig. 6b**). Alternatively, additional
281 signals beyond IL-1β, derived from non-microglial CNS cells, might be required for microglial
282 CXCL1 induction, acting *in trans*. To test this hypothesis, we co-cultured BV-2 cells with
283 immortalized C8-D1A astrocytes in the presence of Candidalysin and measured IL-1β and
284 CXCL1 in the supernatants. We chose astrocytes since they are known to respond to IL-1β to
285 produce inflammatory mediators, including CXCL1, in other models of CNS inflammation[26, 27,
286 28]. We found that astrocytes responded to Candidalysin to produce CXCL1 (**Fig. 6c**), but not
287 IL-1β (data not shown), and that CXCL1 production significantly increased when astrocytes
288 and microglia were co-cultured (**Fig. 6c**). To confirm that microglia are a relevant cellular
289 source of CXCL1 detected during microglia-astrocyte co-culture, we performed intracellular

staining for CXCL1 and found that BV-2 microglia are significant producers of CXCL1 in response to Candidalysin, but only when astrocytes were present (**Fig. 6d**). Therefore, astrocytes provide additional signals to microglia that are needed for CXCL1 production in response to Candidalysin.

We next investigated the pathway activated by Candidalysin in BV-2 microglia to produce IL-1β. Candidalysin was previously shown in epithelial cells to activate c-Fos, in a p38-dependent manner, and the phosphatase MKP-1[20]. We thus asked whether the same pathways are activated by Candidalysin in BV-2 microglia. We found that Candidalysin sequentially and dose-dependently activated MKP-1 and c-Fos (**Fig. 6e**), and chemical inhibition of p38 or c-Fos significantly reduced IL-1β release by Candidalysin-stimulated BV-2 cells (**Fig. 6f**). Therefore, microglia produce IL-1β in response to Candidalysin via activation of p38 and c-Fos.

The Microglia IL-1β–CXCL1 Response Requires CARD9

CARD9-deficiency is the only known risk factor that uniquely predisposes to CNS candidiasis in the absence of iatrogenic intervention[2, 5]. We first examined whether CARD9-deficiency causes developmental defects in resident microglia, but found no defects in abundance or activation markers at steady state in *Card9*$^{-/-}$ microglia, which accumulated in similar numbers as wild-type microglia after fungal infection (**Supplementary Fig. 5**). Since *C. albicans* activates the microglial IL-1β–CXCL1 axis to regulate protective neutrophil CNS recruitment, we next analyzed the dependence on CARD9 for induction of this pathway in microglia post-infection *in vivo*. We hypothesized that CARD9 is required for these functions, as microglia

highly express CARD9 and we previously found reduced transcription of CXC chemokines by *Card9*$^{-/-}$ microglia harvested from the *C. albicans*-infected brain[5]. We infected *Card9*$^{+/+}$ and *Card9*$^{-/-}$ animals with wild-type Candidalysin-expressing *C. albicans*, isolated phagocytes from the brain and measured pro-IL-1β and CXCL1 production following *ex vivo* restimulation. We found significantly decreased frequencies of CXCL1^{+} and pro-IL-1β^{+} cells in the fungal-infected *Card9*$^{-/-}$ brain, and these decreases mapped to microglia (**Fig. 7a,b**).

Since production and secretion of mature IL-1β depends on pro-IL-1β expression and consecutive inflammasome-dependent processing, we asked whether microglia depend on CARD9 for pro-IL-1β transcription and/or inflammasome activation. We FACS-sorted microglia from wild-type and *Card9*$^{-/-}$ infected brains, and examined *Il1b* transcription by qRT-PCR, and levels of pro-IL-1β and cleaved and pro-caspase-1 by immunoblot. We found significantly decreased *Il1b* transcription in *Card9*$^{-/-}$ microglia, which we confirmed at the protein level (**Fig. 7c,d**). These data are in line with the reported CARD9-dependent pro-IL-1β transcription in bone marrow-derived dendritic cells post-viral infection[29]. We also found significantly reduced cleaved caspase-1 in *Card9*$^{-/-}$ microglia (**Fig. 7d**), indicating that Card9 also operates at the level of inflammasome activation for IL-1β production. Given that c-Fos mediated Candidalysin-induced IL-1β production by BV-2 cells (**Fig. 6e,f**), we measured c-Fos expression in WT and *Card9*$^{-/-}$ microglia by immunoblot and found significantly decreased c-Fos expression in *Card9*$^{-/-}$ microglia (**Fig. 7d**).

We next examined the NLRP3 inflammasome in FACS-sorted WT and *Card9*$^{-/-}$ microglia. We focused on NLRP3 because CARD9 was reported to negatively regulate NLRP3 activation

during macrophage *Salmonella* infection[30], and we recently showed that Candidalysin activates NLRP3 in bone marrow-derived macrophages[31]. We found significantly decreased NLRP3 protein expression in *Card9*$^{-/-}$ microglia (**Fig. 7e**). Of interest, *Nlrp3*$^{-/-}$ animals had significantly decreased neutrophil accumulation to the *C. albicans*-infected brain and increased fungal load post-infection, consistent with a potential role of Card9-dependent NLRP3-inflammasome activation for protective neutrophil influx in the fungal-infected CNS (**Fig. 7f**). Together, microglia require CARD9 for c-Fos activation and for production of mature IL-1β via *Il1b* transcriptional regulation and inflammasome activation, to activate the IL-1β–CXCL1 axis in response to fungal invasion.

Microglial CARD9 Deletion Causes CNS Fungal Invasion

We next directly examined the impact of genetic *Card9* deletion specifically within microglia by utilizing mice expressing tamoxifen-inducible Cre recombinase under the *Cx3cr1* promoter (*Cx3cr1*^{CreER})[32]. These mice has been used to genetically manipulate long-lived CX3CR1^{+} microglia while leaving short-lived CX3CR1^{+} monocytes and monocyte-derived macrophages unaffected. We bred *Cx3cr1*^{CreER} animals to Card9-floxed mice[11], tamoxifen-pulsed the progeny to activate Cre expression and waited 4-6 weeks to allow replenishment of short-lived non-microglia CX3CR1^{+} cells from the bone marrow, while long-lived microglia remained Card9-deficient (**Supplementary Fig. S6**). *C. albicans* infection of *Card9*$^{fl/fl}$*Cx3cr1*$^{CreER+/–}$ animals revealed a significant dependence on *Card9* expression by the long-lived CX3CR1^{+} cellular compartment for control of fungal brain growth (**Fig. 8a**), while fungal control in the kidney was unaffected in microglia-specific conditional *Card9*$^{-/-}$ mice (**Fig. 8a**).

To analyze whether the susceptibility to brain infection in *Card9*$^{fl/fl}$*Cx3cr1*$^{CreER+/-}$ mice was related to a neutrophil recruitment defect, we quantified neutrophils within the infected brains of *Card9*$^{fl/fl}$*Cx3cr1*$^{CreER+/-}$ mice and their Cre-negative littermates. We found that microglial deletion of *Card9* significantly reduced the protective early influx of neutrophils into the fungal-infected brain (**Fig. 8b**), which correlated with significantly decreased expression of microglial pro-IL1β and CXCL1 in the conditional *Card9*$^{-/-}$ mice (**Fig. 8c**). Together, our data shows that CARD9-expressing microglia orchestrate control of fungal brain invasion, in part by responding to fungus-secreted Candidalysin, to produce IL-1β-induced CXCL1, which recruits CXCR2-expressing neutrophils that are required for CNS fungal clearance (**Supplementary Fig. S7**).

Discussion

Herein, we demonstrate the critical contribution of CARD9-mediated IL-1β and CXCL1 in recruiting protective neutrophils to the fungal-infected CNS. We identify microglia as major producers of CARD9-dependent IL-1β and CXCL1 during *C. albicans* CNS invasion and the fungal-secreted toxin Candidalysin as a critical pathogen-derived factor activating this pathway. Our study offers novel insights into the network of host and fungal factors that protect against CNS fungal invasion and unveil the mechanism of CNS fungal susceptibility in inherited CARD9-deficiency.

Systemic candidiasis is a leading cause of nosocomial bloodstream infection with mortality >50% despite therapy[33]. Neutropenia is the major predisposing factor for systemic candidiasis and *Candida* CNS invasion in particular[5, 34]. Moreover, CNS invasion is prevalent during systemic candidiasis in low-birth weight neonates and also occurs as an iatrogenic complication post-neurosurgical procedures[35, 36]. Strikingly, CARD9-deficiency is a primary immunodeficiency disorder (PID) characterized by heightened susceptibility to fungal infections of which CNS candidiasis is a hallmark[2, 3]. CARD9-deficiency is the only known PID that causes fungal-specific infection susceptibility without other infectious or non-infectious manifestations, and the only PID that causes fungal disease in which CNS is a primary target tissue[17]. We previously demonstrated that *Candida* CNS disease in CARD9-deficiency is caused by a fungal- and brain-specific defect in neutrophil recruitment[5]. CNS neutropenia is now confirmed in several CARD9-deficient patients with CNS candidiasis[7, 37, 38]. Nonetheless,

how CARD9 mediates protective neutrophil trafficking into the fungal-infected CNS remained unclear.

Our analysis of mice deficient in several CLR, cytokine and chemokine circuits uncovered a) the functional redundancy among CLRs, which may suggest the presence of yet-undiscovered CARD9-coupled receptors for tissue-specific protection against fungal invasion; b) the indispensable role of the CARD9 partner MALT1 in controlling CNS fungal invasion independent of neutrophil recruitment, which implies that *MALT1*$^{-/-}$ patients may be at risk for CNS fungal disease; and c) the critical contribution of IL-1β–CXCL1-mediated neutrophil recruitment for control of CNS fungal invasion. CXCR2 was known to mediate neutrophil trafficking during viral infection, parasitic meningitis[39] and fungal pneumonia[40], and herein we reveal its importance for recruiting neutrophils during *C. albicans* CNS infection, principally through binding CXCL1. In contrast, CCR1, CXCR1 and LTB4R1 are dispensable despite them regulating neutrophil recruitment and function in other *C. albicans*-infected tissues[13, 14, 15]. These studies further underscore the organ- and context-specific dependence on chemotactic molecules for protective host immunity.

We showed that IL-1β is required for CXCL1 induction, in line with earlier work which showed IL-1β–induced CXCL1 production controlling neutrophil accumulation during bacterial peritonitis and autoimmune, traumatic or bacterial neuroinflammation[10, 41, 42]. Importantly, microglia are the primary myeloid cellular source of IL-1β-dependent CXCL1 production *in vivo*. During oral candidiasis, IL-1R is also required for neutrophil accumulation to the oral mucosa[8], as we have shown for systemic infection in the brain. However, further attesting to

413 the presence of tissue-specific anti-*Candida* immune response cues, the IL-1R-dependent
414 response in the oral epithelium is largely controlled by IL-1α released by damaged
415 keratinocytes[8], whereas we found that IL-1α plays a modest role in the control of brain fungal
416 invasion. In fact, IL-1α release by epithelial cells, in both the oral and vaginal mucosal barriers,
417 is driven by exposure to the fungal secreted toxin Candidalysin[19, 20, 21].
418
419 Candidalysin enables the establishment of *C. albicans* mucosal infections, since Candidalysin-
420 deficient strains are avirulent in these models[19, 21]. In contrast, we found that Candidalysin-
421 deficient strains are hyper-virulent for the brain, associated with decreased IL-1β and CXCL1
422 production and impaired neutrophil recruitment. These results indicate that Candidalysin is not
423 only a classical virulence factor, but also an immune modulator, which exerts context-specific
424 effects on the immune system. We propose that this dual function of Candidalysin is the result
425 of a co-evolutionary event; the fungus developed an efficient toxin to damage host
426 membranes, and, in response, the host evolved a sensitive Candidalysin detection system to
427 defend against this common mucosal pathogen. Whether Candidalysin is recognized by a
428 specific microglial innate receptor to mediate the protective IL-1β–CXCL1 axis is unclear, since
429 the toxin mediates cellular damage which could also activate glial cells. Therefore, identifying
430 how host epithelial and immune cells recognize Candidalysin merits investigation.
431
432 We found that Candidalysin selectively activates microglia for IL-1β and CXCL1 production, a
433 self-renewing macrophage population that contributes towards neuroinflammation in
434 neurodegenerative disorders and promotes pathogen and dead cell clearance within the
435 CNS[43]. Interestingly, downstream of Candidalysin-induced microglial IL-1β secretion, which

436 occurs via c-Fos activation, we show that additional signals derived from astrocytes acting *in*
437 *trans* are required for microglia to secrete CXCL1. Whether direct microglia-astrocyte contact
438 is required or astrocyte-derived soluble factors acting on microglia are needed remains
439 unknown. Indeed, microglia are known to interact with astrocytes to drive or suppress
440 inflammation[44, 45]. Therefore, the molecular factors that drive microglia-astrocyte cross-talk
441 within the fungal-infected brain warrant further investigation.
442
443 Lastly, we examined the dependence on CARD9 for the microglia-mediated, IL-1β–CXCL1-
444 dependent pathway that recruits protective neutrophils, using fully Card9-deficient and
445 conditional microglia-specific *Card9*$^{-/-}$ mice. We show that CARD9 is critical for c-Fos
446 activation and for the production of both IL-1β and CXCL1 by microglia in the infected CNS
447 operating at the levels of both transcriptional pro-IL-1β regulation and inflammasome activation
448 for IL-1β generation, with NLRP3 being at least partly involved, as shown with *Microsporum*
449 infection[46]. Together, these data shed light into the pathogenesis of inherited CARD9-
450 deficiency by outlining a pathway of CARD9-dependent microglial production of sequential IL-
451 1β and CXCL1 that recruits protective neutrophils into the fungal-infected CNS.
452
453 Future studies should examine how Card9 promotes microglial innate functions beyond
454 orchestrating neutrophil recruitment such as fungal uptake and killing. Of note, the phenotype
455 of the microglia-specific conditional knockout mice is less severe than that of *Card9*$^{-/-}$ mice,
456 which may reflect the important role of astrocytes, which express Card9 post-*Candida*
457 infection[13], in priming microglial CXCL1 production. Future work should also examine the
458 potentially differential tissue-specific dependence on Card9 for neutrophil recruitment by non-

CNS tissue-resident macrophages, such as Kupffer cells, as previously shown for macrophages and dendritic cells[47], which will help further understand the CNS-specificity of fungal disease in CARD9-deficiency. Beyond understanding the pathogenesis of inherited CARD9-deficiency, our findings have important implications for recognizing the potential fungal infection risk in patients who are increasingly receiving Syk inhibitors for the treatment of autoimmune and malignant diseases[48, 49]. Surveillance of Syk inhibitor-treated patients and research in conditional Syk-deficient mice will help determine their CNS fungal disease risk.

In summary, we present evidence of an intricate host immune pathway that protects the CNS from invading fungi. This work uncovers the complex interactions occurring between the host and the most common human fungal pathogen within the CNS, and sheds novel mechanistic light into the pathogenesis of inherited CARD9-deficiency.

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Authors' Contributions

R.A.D, B.H, J.R.N, T.M.H and M.S.L designed the study. R.A.D, M.S, V.O, B.Z, and I.M.D performed the experiments. B.C.S, A.C.B, K.D.M-B, S.A.L, Y.I, S.G.F, G.D.B, B.H, J.R.N and T.M.H provided key reagents/mouse lines and intellectual input into the experimental design regarding their use. R.A.D. and M.S.L assembled and wrote the manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding authors upon request.

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Methods

Mice

Animals (males and females) were used at 8-12 weeks of age and were maintained in individually ventilated cages under specific pathogen-free conditions at the 14BS facility at the National Institutes of Health (Bethesda, MD, USA), the Memorial Sloan Kettering Cancer Center Comparative Medicine Shared Resources (New York, NY, USA), or the Medical Research Facility at the University of Aberdeen (UK). The following strains (and their respective WT controls/littermates) were obtained from the NIAID Taconic contract; *Cxcr2*$^{-/-}$, *Il1r*$^{-/-}$, *Ltb4r1*$^{-/-}$, *Fpr1*$^{-/-}$. All other strains and their respective controls/littermates were bred in-house at the NIH (*Clec7a*$^{-/-}$, *Clec4n*$^{-/-}$, *Clec4e*$^{-/-}$, *Myd88*$^{-/-}$, *Ccr1*$^{-/-}$, *Cxcr1*$^{-/-}$, *Cxcl1*$^{-/-}$, *Il1a*$^{-/-}$, *Il1b*$^{-/-}$, *Il1a*$^{-/-}$*Il1b*$^{-/-}$, *Nlrp3*$^{-/-}$, *Card9*$^{fl/fl}$*Cx3CR1*$^{CreER+/-}$), Memorial Sloan-Kettering Cancer Center (*Clec7a*$^{-/-}$*Fcer1g*$^{-/-}$), University of Aberdeen (*Clec4d*$^{-/-}$), or USUHS (*Malt1*$^{-/-}$)[51]. Mice homozygous for the *Card9*^{tm1a} allele were purchased from the Wellcome Trust Sanger Institute (EUCOMM Project No. 44813), and these animals were bred with the FLPer deleter strain (Jackson Laboratories) to remove the FRT-flanked knock-out first cassette, generating *Card9*^{tm1c} homozygous mice (referred to as *Card9*$^{fl/fl}$ in this manuscript)[52,53]. Homozygous *Card9*$^{fl/fl}$ animals were bred with heterozygous *Cx3cr1*^{CreER} transgenic animals (Jackson Laboratories) to generate *Card9*$^{fl/fl}$*Cx3cr1*$^{CreER+/-}$ mice and littermate controls. Soon after weaning (~5-6 weeks old), *Card9*$^{fl/fl}$*Cx3cr1*$^{CreER+/-}$ mice and their controls were treated with two 10mg doses of tamoxifen (Sigma) administered in corn oil by oral gavage, given 48 h apart. After 4-6 weeks, these animals were infected and analyzed as outlined in the Figure legends.

All experimentation conformed to conditions approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases.

Candidiasis Model and Fungal Burden Determination

Candida albicans strains used in this study were SC5314, BWP17, *ece1*Δ/Δ, *ece1*Δ/Δ+*ECE1*, *ece1*Δ/Δ+*ECE1*$_{\Delta 184\text{-}279}$ [19], CAI4+CIp10 and *sap4/5/6*Δ/Δ, and *hgc1*Δ/Δ and *hgc1*Δ/Δ + *HGC1*[18]. Yeast was serially passaged three times in YPD broth, grown at 30°C with shaking for 18-24 h at each passage. Yeast cells were washed in PBS, counted, and injected intravenously via the lateral tail vein. Animals were infected with 1.3×10^5 colony forming units (CFU) for analysis at 24 h post-infection, or 7×10^4 CFU for analysis at 72 h post-infection, unless otherwise stated in the corresponding Figure legends. For analysis of brain fungal burdens, animals were euthanized and brains weighed, homogenized in PBS, and serially diluted before plating onto YPD agar supplemented with Penicillin/Streptomycin (Invitrogen). Colonies were counted after incubation at 37°C for 24-48 h.

Analysis of Brain Neutrophil Recruitment by FACS

Leukocytes were isolated from brains using previously described methods[54], resuspended in PBS and stained with Live/Dead fluorescent dye (Invitrogen) for 10 min on ice. Cells were then stained with fluorophore-conjugated antibodies in the presence of anti-CD16/32 and 0.5% BSA for 30 min on ice. Samples were washed in PBS/0.5% BSA/0.01% sodium azide and acquired using the BD Fortessa instrument equipped with BD FACS Diva software (BD Biosciences). FlowJo (TreeStar) was used for the final analysis. Anti-mouse antibodies used in this study

were: CD45 (30-F11), CD11b (M1/70), both from eBiosciences, and Ly6G (1A8), Ly6C (AL-21), both from BD Biosciences.

Histology

Brains were removed from infected mice at the indicated time points and fixed in 10% formalin for 24 h before embedding in paraffin wax. Tissue sections were stained with periodic acid-Schiff (PAS).

Measurement of Cytokines and Chemokines in Brain Homogenates

Infected brains were isolated at 24 h post-infection and homogenized in 1 mL PBS supplemented with 0.05% Tween20 and protease inhibitor cocktail (Roche). Homogenized brains were centrifuged twice to remove debris and resulting supernatants snap-frozen on dry ice and stored at –80 °C prior to analysis. IL-1β and CXCL1 concentrations in the homogenates was determined by ELISA (R&D Systems), following the manufacturers' instructions.

***Ex Vivo* Restimulations and Intracellular FACS Analysis**

Animals were infected with 2×10^5 CFU of the indicated *C. albicans* strains intravenously, and brain leukocytes isolated 24 h later. For these experiments, brains were first digested in RPMI supplemented with 0.8 mg/mL Dispase (Gibco), 0.2mg/mL Collagenase Type 4 (Worthington), and 0.1 mg/mL DNAse (Roche) at 37 °C for 30 min, then pipetted vigorously to create a homogenous suspension. These suspensions were centrifuged (1500 rpm, 5 min, 4 °C), pellets resuspended in 5 mL 40% Percoll (GE Healthcare) and centrifuged again at 1700 rpm

748 for 20 min at 4 °C to remove myelin. Cell pellets were washed in RPMI supplemented with
749 10% heat-inactivated fetal bovine serum and Penicillin/Streptomycin (Invitrogen), and added to
750 FACS tubes for stimulations. Cells were incubated for 4 h at 37 °C in the presence of 5 μg/mL
751 Brefeldin A (Sigma) and, where indicated, 1 μg/mL LPS (Sigma) or 62.5 μg/mL depleted
752 zymosan (Sigma). After stimulation, cells were washed in PBS and stained for surface markers
753 as above. Fixation/permeabilization was performed with the eBioscience Foxp3 staining kit,
754 and staining for CXCL1 (IC4532R, from R&D Systems) or pro-IL-1β (NJTEN3; from
755 eBioscience) performed overnight at 4 °C. Samples were washed once in PBS/0.5%
756 BSA/0.01% sodium azide prior to acquisition using the BD Fortessa instrument equipped with
757 BD FACS Diva software (BD Biosciences). FlowJo (TreeStar) was used for the final analysis.
758 $CXCL1^+$ and pro-IL-$1\beta^+$ cells were determined by employing similar staining and gating in
759 animals deficient in these mediators (*Cxcl1*$^{-/-}$, *Il1b*$^{-/-}$) as negative controls.
760
761 **Cell Culture and Candidalysin Stimulations**
762 BV-2 cells were kindly provided by F. Crews (University of North Carolina School of Medicine).
763 C8-D1A astrocytes were kindly provided by D. McGavern (NINDS, NIH). BV-2 were
764 maintained at 37 °C, 5% CO_2 in RPMI supplemented with L-glutamine and HEPES (pH 7.0 –
765 7.4; Corning), 10% heat-inactivated fetal bovine serum and Penicillin/Streptomycin
766 (Invitrogen). DMEM media was used as the base media for C8-D1A culture, with the same
767 supplements as listed above and cultured as for BV-2. For BV-2 single culture experiments,
768 cells were lifted using cell scrapers and seeded into 24 well-plates at 5×10^5 cells/well (BV-2)
769 and left to adhere for 2 h at 37°C with either: 50 ng/mL LPS (Sigma), T-5224 (APExBIO)
770 and/or SB203580 (Adipogen); see Figure legends for details of each experiment. After 2 h,

771 recombinant Candidalysin peptide (Peptide Protein Research) was added to the cells at the
772 indicated concentrations and incubation at 37 °C continued. For co-culture experiments, 3 ×
773 10^5 C8-D1A were added to each well of a 24 well-plate and incubated overnight at 37 °C. BV-2
774 cells and Candidalysin were added as described above. In both types of experiments,
775 supernatants or cells were collected at the indicated time points after Candidalysin addition
776 and analyzed for IL-1β and CXCL1 by ELISA (R&D Systems), CXCL1 staining by intracellular
777 flow cytometry, or by immunoblot.
778
779 **Immunoblot Analysis**
780 Whole cell lysates were suspended in RIPA buffer containing protease and phosphatase
781 inhibitors (Thermo Scientific). Lysates were separated in SDS-PAGE and transferred to a
782 nitrocellulose membrane, 0.2 μm (Bio-Rad Laboratories). The membrane was incubated with
783 the following primary antibodies: phospho-MPK1/MPK2 polyclonal [Ser296, Ser318] (Thermo
784 Scientific) and c-Fos (Cell Signaling), IL-1β [3A6] (Cell Signaling), Caspase-1 p20 [Casper-1]
785 (Adipogen Life Sciences) (Thermo Scientific), NLRP3 [D4D8T] (Cell Signaling). Normalization
786 was performed by probing the membrane with β-Actin antibody (Cell Signaling).
787 Chemiluminescence detection was performed with Clarity™ Western ECL Substrate (Bio-Rad
788 Laboratories), using the ChemiDoc™ MP Imaging System (Bio-Rad).
789
790 **FACS/MACS Sorting of Microglia**
791 Wild-type animals were infected with 1.3 × 10^5 CFU SC5314 and euthanized at 24 h post-
792 infection. Brains were digested as above and leukocytes stained with sterile antibodies[55].
793 Ly6C^{hi} monocytes (CD45^{hi} CD11b^{+} Ly6C^{hi} Ly6G^{-}) and microglia (CD45^{lo} CD11b^{+} Ly6G^{-} Ly6C^{-})

were FACS-sorted into sterile sorting buffer (HBSS supplemented with 2 mM EDTA, 10 % FCS, 100 U/mL penicillin, 100 μg/mL streptomycin) using a FACS Aria instrument for downstream qRT-PCR and immunoblot analyses. Purity of cells were greater than >95%, on average. In some experiments (qRT-PCR of CLRs in brain-resident microglia; **Supplementary Fig. 1**), microglia were instead sorted by magnetic separation using anti-CD11b microbeads (Miltenyi). Cells were then centrifuged (1500 rpm 5 min, 4 °C) and resuspended in Trizol for RNA purification or RIPA buffer for downstream immunoblot analysis. Depending on the experiment, up to 5 animals were pooled for individual sorts, or individual mice were analyzed separately (see Figure legends for details).

Generation of cDNA and qRT-PCR

RNA was extracted from sorted brain myeloid cells (defined using the gating strategy shown in **Supplementary Fig. 2**) using Trizol (Invitrogen) and the RNeasy kit (Qiagen) per the manufacturer's protocol. Purified RNA was used as a template for cDNA generation using the qScript cDNA SuperMix kit (Quanta Biosciences) with oligodT and random primers. Quantitative PCR was performed by TaqMan detection (PerfeCTa qPCR FastMix ROX; Quanta BioSciences) with the 7900HT Fast Real-Time PCR System (Applied Biosystems). All qPCR assays were performed in duplicate and the relative gene expression of each gene was determined after normalization with GAPDH transcript levels using the ΔΔCT method. TaqMan primers/probes (*Clec7a, Clec4n, Clec4d, Clec4e, Il1b, Card9, Gapdh*) were predesigned by Applied Biosystems.

Statistics

Statistical analyses were performed using GraphPad Prism 7.0 software. Details of individual tests are included in the figure legends. In general, data was tested for normal distribution by Kolmogorov-Smirnov normality test and analyzed accordingly by unpaired t-tests or Mann Whitney U-test. In cases where multiple data sets were analyzed, two-way ANOVA was used with Bonferroni correction. In all cases, *P* values <0.05 were considered significant.

Methods References:

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Figure Legends

Fig. 1: CARD9-coupled C-type lectin receptors functionally compensate for one another for protective neutrophil recruitment to the fungal-infected brain. a, *Card9*$^{-/-}$ mice (n=4 animals) and their wild-type controls (n=4 animals) were intravenously infected with *C. albicans* SC5314 and analyzed for neutrophil counts by flow cytometry at 24 h post-infection (left; dose: 1.3×10^5 CFU) and fungal growth within the brain at 72 h post-infection (right; dose: 7×10^4 CFU). **b,** Animals of the indicated genotype (WT n = 9 animals, *Clec7a*$^{-/-}$ n = 6 animals; WT n = 12 animals, *Clec4n*$^{-/-}$ n = 10 animals; WT n = 6 animals, *Clec4d*$^{-/-}$ n = 6 animals; WT n = 6 animals, *Clec4e*$^{-/-}$ n = 8 animals; WT n = 10 animals, *Clec7a*$^{-/-}$*Fcer1g*$^{-/-}$ n = 9 animals) were intravenously infected with *C. albicans* SC5314 (2×10^5 CFU for *Clec4d*$^{-/-}$ and *Clec7a*$^{-/-}$ *Fcer1g*$^{-/-}$ and their controls; 1.3×10^5 all others) and analyzed for neutrophil counts by flow cytometry at 24 h post-infection and **c,** fungal burdens in the brain at 24 and 72 h post-infection (WT n = 9 animals, *Clec7a*$^{-/-}$ n = 6 animals; WT n = 10 animals, *Clec4n*$^{-/-}$ n = 10 animals; WT n = 10 animals, *Clec4d*$^{-/-}$ n = 10 animals; WT n = 6 animals, *Clec4e*$^{-/-}$ n = 4 animals; WT n = 10 animals, *Clec7a*$^{-/-}$*Fcer1g*$^{-/-}$ n = 9 animals). **d,** *Malt1*$^{-/-}$ mice and their littermate controls were infected as above and analyzed for fungal burdens in the brain (right; WT n = 7 animals, *Malt1*$^{-/-}$ n = 5 animals) and neutrophil recruitment to the brain at 24 h post-infection (left; WT n = 5 animals, *Malt1*$^{-/-}$ n = 5 animals). In all cases, 'wild type' refers to appropriate matched control animals for each knock-out line for gender, age and genetic background. Individual points represent different mice. Data is pooled from 2 independent experiments and is shown as mean +/- SEM, and analyzed by unpaired two-tailed t-test (panel **a** [left], **b**) or two-tailed Mann Whitney U-test (panel **a** [right], **c**, **d**). *P<0.05, **P<0.01, ***P<0.005, ****P<0.001.

Fig. 2: IL-1β and CXCL1 are critical for protective neutrophil recruitment to the fungal-infected brain. a,b, Animals deficient in elements of the IL-1R signaling pathway or **c,d**, chemokine receptors and their ligands, were infected and analyzed for neutrophil recruitment at 24 h post-infection (**a,c**) and control of fungal brain infection (**b,d**) as in Fig. 1. 'Wild type' refers to appropriate matched control animals for each knock-out line for gender, age and genetic background. Individual points represent different mice; (**a**) WT n = 8 animals, *Il1r*$^{-/-}$ n = 8 animals; WT n = 7 animals, *Myd88*$^{-/-}$ n = 6 animals; WT n = 12 animals, *Il1a*$^{-/-}$ n = 12 animals; WT n = 6 animals, *Il1b*$^{-/-}$ n = 6 animals; WT n = 8 animals, *Il1a*$^{-/-}$*Il1b*$^{-/-}$ n = 6 animals. (**b**) WT n = 8 animals, *Il1r*$^{-/-}$ n = 7 animals; WT n = 7 animals, *Myd88*$^{-/-}$ n = 6 animals; WT n = 3-8 animals, *Il1a*$^{-/-}$ n = 4-8 animals; WT n = 6 animals, *Il1b*$^{-/-}$ n = 6 animals; WT n = 6 animals, *Il1a*$^{-/-}$*Il1b*$^{-/-}$ n = 6 animals. (**c**) WT n = 6 animals, *Ccr1*$^{-/-}$ n = 6 animals; WT n = 11 animals, *Cxcr1*$^{-/-}$ n = 11 animals; WT n = 14 animals, *Cxcr2*$^{-/-}$ n = 16 animals; WT n = 8 animals, *Cxcl1*$^{-/-}$ n = 7 animals. (**d**) WT n = 6-7 animals, *Ccr1*$^{-/-}$ n = 6-7 animals; WT n = 7-10 animals, *Cxcr1*$^{-/-}$ n = 7-10 animals; WT n = 8-10 animals, *Cxcr2*$^{-/-}$ n = 7-10 animals; WT n = 6-8 animals, *Cxcl1*$^{-/-}$ n = 5-7 animals. Data is pooled from 2-3 independent experiments and shown as mean +/- SEM, analyzed by unpaired two-tailed t-test (panel **a, c**) or two-tailed Mann Whitney U-test (panel **b, d**). **P*<0.05, ***P*<0.01, ****P*<0.005, *****P*<0.001.

Fig. 3: Production of CXCL1 is dependent on IL-1β in the fungal-infected brain. a, Wild type (n = 6/7 animals), *Cxcl1*$^{-/-}$ (n = 5 animals) and *Il1b*$^{-/-}$ (n = 6 animals) animals were infected as in Fig. 1 and brains isolated at 24 h post-infection and analyzed for CXCL1 or IL-1β production by ELISA. Data is pooled from 2 independent experiments and analyzed by

889 unpaired two-tailed t-test. **b**, The relative proportions of myeloid cell populations (gated within
890 live CD45$^+$ singlets) in the uninfected (n = 6 animals) and 24 h infected WT (n = 6 animals)
891 brain (left), and the relative proportion of myeloid cell populations producing CXCL1 (n = 3
892 animals) or pro-IL-1β (n = 9 animals) in the 24 h infected brain (right). For the latter, total
893 CD45$^+$CXCL1(or IL-1β)$^+$ cells were first gated and then cell types defined within this initial gate
894 using lineage markers (see below), using samples from the unstimulated condition. Data is
895 shown as the mean +/- SEM. **c,** Wild type (n = 3 animals) and *Il1b*$^{-/-}$ mice (n = 4 animals) were
896 infected with 2 × 10^5 *C. albicans* and brain cells analyzed for CXCL1 production by intracellular
897 flow cytometry 24 h later. Brain cells were restimulated *ex vivo* with 62.5 µg/mL depleted
898 zymosan or 1 µg/mL LPS for 4 h in the presence of 5 µg/mL Brefeldin A. Representative plots
899 from the LPS-stimulated condition are gated on microglia (top; CD45^{int} Ly6G$^-$ CD11b$^+$), Ly6C^{hi}
900 monocytes (middle; CD45^{hi} Ly6C^{hi} Ly6G$^-$ CD11b$^+$) and neutrophils (bottom; CD45^{hi} Ly6C^{int}
901 Ly6G^{hi} CD11b$^+$), showing corresponding *Cxcl1*$^{-/-}$ cells as gating controls. In all panels, 'wild
902 type' refers to appropriate matched control animals for each knock-out line for gender, age and
903 genetic background. Individual points represent different mice. Data shown as mean +/- SEM,
904 and analyzed by unpaired two-tailed t-test. *P<0.05, **P<0.01.
905
906 **Fig. 4: Candidalysin is a specific hyphal-secreted factor promoting neutrophil**
907 **recruitment and control of fungal growth in the brain. a,b,c,e,** Animals were infected with 2
908 × 10^5 CFU of the indicated *C. albicans* strains (parental strains, closed symbols; deficient
909 mutants, open symbols) and analyzed as in Fig. 1 for fungal burdens (*hgc1*Δ/Δ n = 8 animals,
910 *hgc1*Δ/Δ + *HGC1* n = 7 animals; BWP17 n = 10 animals, *ece1*Δ/Δ n = 11 animals, *ece1*Δ/Δ +
911 *ECE1* n = 10 animals, *ece1*Δ/Δ + *ECE1*$_{Δ184-279}$ n = 11 animals; CAI4 + CIp10 n = 6 animals,

*sap4/5/6*Δ/Δ + Clp10 n = 6 animals) and neutrophil recruitment (*hgc1*Δ/Δ n = 10 animals, *hgc1*Δ/Δ + *HGC1* n = 10 animals; BWP17 n = 7 animals, *ece1*Δ/Δ n = 7 animals, *ece1*Δ/Δ + *ECE1* n = 7 animals, *ece1*Δ/Δ + $ECE1_{\Delta 184\text{-}279}$ n = 11 animals; CAI4 + Clp10 n = 6 animals, *sap4/5/6*Δ/Δ + Clp10 n = 7 animals). Histology shown in **(c)** is from 24 h post-infection, stained with PAS. Scale bar is 50 μm. **d,** Whole brain homogenates from animals infected with indicated strains were isolated at 24 h post-infection and analyzed for IL-1β and CXCL1 using ELISA (BWP17 n = 8-10 animals, *ece1*Δ/Δ n = 8-10 animals, *ece1*Δ/Δ + *ECE1* n = 6-11 animals, *ece1*Δ/Δ + $ECE1_{\Delta 184\text{-}279}$ n = 7-11 animals). Individual points represent different mice. Data is pooled from 2-4 independent experiments and shown as mean +/- SEM, analyzed by unpaired two-tailed t-test, or two-tailed Mann Whitney U-test (panel **a**, left). **P*<0.05, ***P*<0.01, ****P*<0.005; ns = not significant.

Fig. 5: Microglia produce IL-1β and CXCL1 in a Candidalysin-dependent manner. Animals were infected with wild-type *C. albicans* (BWP17; closed bars) or a Candidalysin-null strain (*ece1*Δ/Δ; open bars), and brain cells isolated 24 h later. Brain leukocytes were restimulated as in Fig. 3, and intracellular staining for **a,** IL-1β (unstimulated, n = 8 animals; zymosan, n = 4 animals; LPS n = 6 animals) and **b,** CXCL1 (unstimulated, n = 12 animals; zymosan, n = 6 animals; LPS n = 9 animals) was analyzed by flow cytometry. Box-and-whisker plots show the minimum/maximum values (whiskers), the 25^{th}/75^{th} percentiles and the median. Data is pooled from 2-4 independent experiments and analyzed by unpaired two-tailed t-tests. **P*<0.05, ***P*<0.01. Representative staining is shown for LPS-stimulated microglia (gated as in Fig. 3) from wild-type mice infected with indicated strains, or BWP17-infected cytokine-deficient mutants as control.

Fig. 6: Candidalysin activates IL-1β production from microglia via p38-cFos signaling and promotes CXCL1 production from microglia through interactions with astrocytes. BV-2 microglia were seeded into 24-well plates at 5 × 10^5 per well and left to adhere for 2 h in the presence of 50 n g/mL LPS (for priming) before the addition of purified Candidalysin at the indicated concentrations. Cell culture supernatants were analyzed for **a,** IL-1β production or **b,** LDH release after 24 h of stimulation. Data is shown with the mean +/- SEM, individual points represent individual culture wells (n = 4). **c-d,** In some experiments, BV-2 cells were co-cultured with 3 × 10^5 C8-D1A astrocytes, and CXCL1 production analyzed in the supernatant by ELISA (n = 10 individual culture wells) or by intracellular flow cytometry (n = 3-5 individual culture wells; data shown with the mean). In **d**, microglia and astrocytes were distinguished by CD45 staining, and CXCL1 production assessed within $CD45^+$ (microglia) and $CD45^-$ (astrocyte) gates. Histogram is gated on $CD45^+$ microglia. **e,** To measure cFos and pMKP1/2 activation, BV-2 cells were stimulated with the indicated Candidalysin concentrations for 30 or 120 min and BV-2 cells then lysed and analyzed for cFos and pMKP1/2 by immunoblot, normalizing to β-actin. Immunoblots shown are representative of 2 independent experiments. **f,** BV-2 cells were cultured in the presence of the indicated cFos and p38 inhibitors for 2 h prior to stimulating with 20 μM Candidalysin, and IL-1β measured in the supernatant by ELISA after 24 h (data shown as mean +/- SEM; n = 6 individual culture wells). All data is pooled from 2 independent experiments and analyzed by one-way ANOVA. *P<0.05, **P<0.01, ***P<0.005, ****P<0.0001.

Fig. 7: CARD9 is required for the production of IL-1β, via regulation of pro-IL-1β transcription and inflammasome activation, and of CXCL1 in the fungal-infected brain. **a,b,** *Card9*$^{+/+}$ (n = 13 animals) and *Card9*$^{-/-}$ (n = 13 animals) animals were infected with 2 × 10^5 CFU wild-type *C. albicans* (BWP17), and brain cells isolated 24 h later. Brain leukocytes were restimulated as in Fig. 4, and intracellular staining for pro-IL-1β and CXCL1 analyzed by flow cytometry in total CD45$^+$ cells (LPS-stimulated condition shown) (**a**) or microglia alone, normalized to *Card9*$^{+/+}$ results (**b**). Panels **a,b** show pooled data from 4 independent experiments, analyzed with two-tailed unpaired t-test. Data shown as mean +/- SEM (**a**) or with minimum/maximum values (whiskers), the 25th/75th percentiles and the median (**b**). **c,** Microglia were FACS-sorted from pooled *Card9*$^{+/+}$ (n = 4 animals) and *Card9*$^{-/-}$ animals (n = 4 animals) at 24 h post-infection and analyzed by unpaired two-tailed t-test for *Il1b* expression by qRT-PCR, or **d,e,** the indicated proteins by immunoblot (Caspase and IL-1β blots; WT n = 6 animals, *Card9*$^{-/-}$ n = 7 animals; cFos blot; WT n = 10 animals, *Card9*$^{-/-}$ n = 10 animals; NLRP3 blot; WT n = 8 animals, *Card9*$^{-/-}$ n = 8 animals;). Graphs in (**d,e**) represent the band pixel density normalized to the wild type control, and are shown with mean +/- SEM and analyzed by unpaired two-tailed student t-tests. Example blots are representative of 3 independent FACS sorts/experiments; pooled data is shown in the graphs above. **f,** *Nlrp3*$^{-/-}$ animals and their wild-type controls were infected with 1.3 × 10^5 CFU *C. albicans* and analyzed by unpaired two-tailed t-tests for neutrophil recruitment to the brain 24 h later (left; WT n = 9 animals, *Nlrp3*$^{-/-}$ n = 8 animals) and by two-tailed Mann-Whitney U-test for fungal brain burdens at 72 h post-infection (right; WT n = 14 animals, *Nlrp3*$^{-/-}$ n = 14 animals), as described in Fig. 1. **P*<0.05, ***P*<0.01, ****P*<0.005.

Fig. 8: CARD9 is required specifically in microglia for neutrophil recruitment and control of fungal invasion in the CNS. a, *Card9*$^{fl/fl}$*Cx3cr1*$^{CreER-/-}$ and *Card9*$^{fl/fl}$*Cx3cr1*$^{CreER+/-}$ littermates (n=8-13) were tamoxifen-pulsed at 4-5 weeks of age, left to rest for 4-6 weeks and then infected with 1.3 × 10^5 CFU *C. albicans* (SC5314) intravenously and analyzed for brain and kidney fungal burdens (*Card9*$^{fl/fl}$*Cx3cr1*$^{CreER-/-}$ n = 8-10 animals; *Card9*$^{fl/fl}$*Cx3cr1*$^{CreER+/-}$ n = 8 animals), **b,** neutrophil recruitment to the brain at 24 h post-infection (*Card9*$^{fl/fl}$*Cx3cr1*$^{CreER-/-}$ n = 13 animals; *Card9*$^{fl/fl}$*Cx3cr1*$^{CreER+/-}$ n = 9 animals), and **c,** intracellular staining for pro-IL-1β and CXCL1, as described in Fig. 3 (*Card9*$^{fl/fl}$*Cx3cr1*$^{CreER-/-}$ n = 8 animals; *Card9*$^{fl/fl}$*Cx3cr1*$^{CreER+/-}$ n = 8 animals). Data is pooled from 2-4 independent experiments and is shown as mean +/- SEM, analyzed by two-tailed Mann-Whitney U-tests (panel **a**) or two-tailed unpaired t-tests (panel **b, c**). **P*<0.05, ***P*<0.01.