CARD9+ microglia promote antifungal immunity via IL-1β- and CXCL1-mediated neutrophil recruitment

Rebecca A. Drummond1,2,3, Muthulekha Swamydas1,3, Vasileios Oikonomou1,4, Ting Zhai1,2, Ivy M. Dambuza5, Brian C. Schaefer6, Andrea C. Bohrer7, Katrin D. Mayer-Barber8, Sergio A. Lira9, Yoichiro Iwakura10, Scott G. Filler11, Gordon D. Brown3, Bernhard Hube10, Julian R. Naglik11, Tobias M. Hohl2 and Michael S. Lionakis1

The C-type lectin receptor–Syk (spleen tyrosine kinase) adaptor CARD9 facilitates protective antifungal immunity within the central nervous system (CNS), as human deficiency in CARD9 causes susceptibility to fungus-specific, CNS-targeted infection. CARD9 promotes the recruitment of neutrophils to the fungus-infected CNS, which mediates fungal clearance. In the present study we investigated host and pathogen factors that promote protective neutrophil recruitment during invasion of the CNS by *Candida albicans*. The cytokine IL-1β served an essential function in CNS antifungal immunity by driving production of the chemokine CXCL1, which recruited neutrophils expressing the chemokine receptor CXCR2. Neutrophil-recruiting production of IL-1β and CXCL1 was induced in microglia by the fungus-secreted toxin Candidalysin, in a manner dependent on the kinase p38 and the transcription factor c-Fos. Notably, microglia relied on CARD9 for production of IL-1β, via both transcriptional regulation of *Il1b* and inlammasome activation, and of CXCL1 in the fungus-infected CNS. Microglia-specific *Card9* deletion impaired the production of IL-1β and CXCL1 and neutrophil recruitment, and increased fungal proliferation in the CNS. Thus, an intricate network of host–pathogen interactions promotes antifungal immunity in the CNS; this is impaired in human deficiency in *CARD9*, which leads to fungal disease of the CNS.

The central nervous system (CNS) is invaded by microorganisms during systemic infections, yet the mechanisms of CNS-specific, antimicrobial immunity remain poorly understood. This is particularly true for CNS fungal infections, which present unmet diagnostic and treatment challenges, leading to unacceptably high mortality rates (>50%). Fungal CNS infection is enhanced by fungus-specific risk factors, including HIV infection, neutropenia, corticosteroid use and Bruton's tyrosine kinase inhibition1. However, the most striking human risk factor for selective CNS fungal infection susceptibility is inherited deficiency of the C-type lectin receptor (CLR)–spleen tyrosine kinase (Syk) adaptor *CARD9*.

*CARD9* relays fungus-sensing signals downstream of the CLR superfamily of pattern recognition receptors, including Dectin-1, Dectin-2, Dectin-3 and Mincle. The kinase Syk is recruited to phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) sequences of CLRs, or their signaling partner FcγR, to form the CARD9–BCL10–MALT1 (BCL is ‘B-cell lymphoma/leukemia’ and MALT is ‘mucosa-associated lymphoid tissue’) signalosome, which activates downstream effectors, including nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), NLRP3 inlammasome and mitogen-activated protein kinase (MAPK) signaling.

*CARD9*-deficient patients manifest fungus-specific infection susceptibility, predominantly in the CNS by *Candida albicans*. We have previously shown that *CARD9* deficiency in humans and mice confers a fungus- and brain-specific defect in neutrophil recruitment, which is detrimental to control of CNS fungal invasion. 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.

In the present study, we systematically investigated host and pathogen factors that promote protective neutrophil influx into the *C. albicans*–infected CNS for a better understanding of the pathogenesis of human CARD9 deficiency. We uncovered an intricate pathway by which the *C. albicans*-secreted toxin Candidalysin engages microglia to produce the interleukin IL-1β and the chemokine ligand CXCL1 for protective recruitment of C-X-C chemokine

---

1Fungal Pathogenesis Section, Laboratory of Clinical Immunology and Microbiology, National Institute of Allergy & Infectious Diseases, National Institutes of Health, Bethesda, MD, USA. 2Infectious Disease Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY, USA. 3Medical Research Council Centre for Medical Mycology at the University of Aberdeen, Aberdeen Fungal Group, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK. 4Department of Microbiology and Immunology, Uniformed Services University, Bethesda, MD, USA. 5Inflammation and Innate Immunity Unit, Laboratory of Clinical Immunology and Microbiology, National Institute of Allergy & Infectious Diseases, National Institutes of Health, Bethesda, MD, USA. 6Infection Control Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA. 7Research Institute for Biomedical Sciences, Tokyo University of Science, Chiba, Japan. 8Division of Infectious Diseases, Department of Medicine, Los Angeles Biomedical Research Institute at Harbor—UCLA, Torrance, CA, USA. 9Department of Microbial Pathogenicity Mechanisms, Leibniz Institute for Natural Product Research and Infection Biology, Hans Knöll Institute Jena, Jena, Germany. 10Friedrich Schiller University, Jena, Germany. 11Centre for Host-Microbiome Interactions, Faculty of Dentistry, Oral and Craniofacial Sciences, King’s College London, London, UK. 12Present address: Institute of Immunology & Immunotherapy, Institute of Microbiology & Infection, University of Birmingham, Birmingham, UK. 13These authors contributed equally: Muthulekha Swamydas, Vasileios Oikonomou, Bing Zhai. *e-mail: r.drummond@bham.ac.uk; lionakism@niaid.nih.gov*
Fig. 1 | Functional redundancy of CARD9-coupled CLRs for protective neutrophil recruitment to the fungus-infected brain. a, Card9−/− mice (n = 4 animals) and their WT controls (n = 4) 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 colony-forming units (c.f.u.)) and fungal growth within the brain at 72 h post-infection (right: dose, 7 × 10^5 c.f.u.). b, Animals of the indicated genotype (WT, n = 9 and Clec7a−/−, n = 6; WT, n = 12 and Clec4n−/−, n = 10; WT, n = 6 and Clec4d−/−, n = 6; WT, n = 6 and Clec4e−/−, n = 8; WT, n = 10 and Clec7a−/−/Fcerlg−/−, n = 9) were intravenously infected with C. albicans SC5314 (2 × 10^5 c.f.u. for Clec4d−/− and Clec7a−/−/Fcerlg−/− and their controls; 1.3 × 10^5 c.f.u. for all others) and analyzed for neutrophil counts by flow cytometry at 24 h post-infection. Fungal burdens in the brain at 24 and 72 h post-infection (WT, n = 9 and Clec7a−/−, n = 6; WT, n = 10 and Clec4n−/−, n = 10; WT, n = 10 and Clec4d−/−, n = 10; WT, n = 6 and Clec4e−/−, n = 4; WT, n = 10 and Clec7a−/−/Fcerlg−/−, n = 9). d, Malt1−/− mice and their littermate controls were infected as above and analyzed for fungal burden in the brain (right: WT, n = 7 and Malt1−/−, n = 5) and neutrophil recruitment to the brain at 24 h post-infection (left: WT, n = 5 and Malt1−/−, n = 5). In all cases, WT refers to appropriate matched control animals for each knockout line for gender, age and genetic background. Individual points represent different mice. Data are pooled from two independent experiments and are shown as mean ± s.e.m., and analyzed using the unpaired, two-tailed, Student’s t-test (a (left), b) or the two-tailed, Mann–Whitney U-test (a (right), c, d). *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001.

**Results**

**CLR functional redundancy during fungal CNS invasion.** As has been previously shown, CARD9 is essential for protective CNS immunity against C. albicans, principally through promoting early neutrophil recruitment to the C. albicans–infected CNS. Collectively, our data unveils complex host–pathogen interactions that recruit protective neutrophils during fungal CNS invasion and reveals the mechanism that underlies CNS fungal susceptibility in CARD9 deficiency.

In Dectin-1 (Clec7a−/−), Dectin-2 (Clec4n−/−), Dectin-3 (Clec4d−/−) and Mincle (Clec4e−/−), and measured brain neutrophil accumulation at 24 h post-infection (see Supplementary Fig. 2). We chose this time point because it is the peak of the neutrophil response in wild-type (WT) animals, and neutrophil depletion at this time point increases susceptibility to fungal invasion of the brain.

Animals individually deficient in CARD9-coupled CLRs recruited neutrophils to the infected brain normally (Fig. 1b). Despite this, we observed increases in fungal burdens of the brain 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 fungal proliferation in the brain. Indeed, brain-infiltrating neutrophils from Dectin-1− and Dectin-2−deficient animals exhibited reduced fungal phagocytosis (see Supplementary Fig. 3), consistent with previous findings.
To activate CARD9-dependent signaling, phosphorylation occurs on the ITAM sequence within the intracellular tail of Dectin-1 or FcRγ, with which Dectin-2, Dectin-3 and Mincl associate. 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−/−Fcer1γ−/−), and found that loss of both Dectin-1 and the FcRγ-coupled CLRs phenocopied Card9 deficiency with greatly decreased neutrophil recruitment and correspondingly increased fungal burdens in the brain (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 fungus-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. Human MALT1 deficiency in addition 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 C. albicans growth in the CNS. Malt1−/− animals recruited neutrophils to the brain similar to the WT; 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, although 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 first infected IL-1 receptor (IL-1R)-deficient mice, because production of IL-1β by human peripheral blood mononuclear cells, after fungal stimulation, is CARD9 dependent, and because IL-1R was previously shown to recruit neutrophils to fungus-infected mucosal tissues and the bacteria-infected brain. After C. albicans challenge, IL-1R-deficient mice phenocopied Card9-deficient mice, with a loss of early brain neutrophil recruitment and accompanying increased fungal burdens in the brain (Fig. 2a,b). Consistent with this, the loss of the IL-1R signaling adaptor MyD88 caused similar defects in neutrophil recruitment and control of fungal proliferation in the infected brain (Fig. 2a,b).

We next assessed which IL-1R ligands were important for driving CNS protection by infecting mice deficient in IL-1α, IL-1β or both. Mice lacking IL-1α had a small reduction in neutrophil numbers and a slight increase in fungal burden of the brain at 24 h post-infection (Fig. 2a,b). However, the lack of IL-1α was compensated for by IL-1β, because Il1a−/− animals recovered and controlled fungal infection of the brain similar to WT by 72 h post-infection (Fig. 2b). In keeping with the critical contribution of IL-1β, mice deficient in IL-1β, or both IL-1α and IL-1β, exhibited significantly reduced neutrophil accumulation and were highly susceptible to fungal invasion of the brain (Fig. 2a,b). Therefore, IL-1β is a critical mediator of neutrophil recruitment to promote control of C. albicans infection in the brain.

Downstream of IL-1R, local chemotactic mediators recruit immune cells to infected tissues. Previously, we have shown that the CNS neutropenia, observed in mouse and human CARD9 deficiency, is not caused by neutrophil-intrinsic chemotaxis defects, but by insufficient local production of soluble chemotactic mediators. However, which of the several chemotaxants 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 and murine subcutaneous phaeohyphomycosis. During systemic C. albicans infection, the chemokine receptor CCR1 drives renal neutrophil accumulation and immune-related kidney destruction, the leukotriene B₄ (LTB₄) receptor LTB₄R1 promotes detrimental pulmonary neutrophil accumulation and CXCR1 mediates neutrophil-dependent fungal killing in the kidney. However, the role of these receptors in CNS anti-Candida immunity is unknown, and CXCR2 and the iMet–Leu–Phe (iMLP) receptor FPR1 have not been examined in relation to anti-Candida defense.

To test the relative dependence on these major neutrophil-targeted chemotaxant receptors to protect the fungus-infected brain, we infected mice deficient in CCR1, CCRX1, CCRX2, LTB₄R1 or FPR1, and measured neutrophil recruitment and fungal burdens in the brain. We found no involvement of the CCL3–CCR1, CXC5–CXC1R1, LTB₄–LBT₄R1 or iMLP–FPR1 axes in controlling fungal brain infection, in line with normal early neutrophil recruitment in infected Ccr1−/−, Cxc1r1−/−, Ltb₄r1−/− and Fpr1−/− animals (Fig. 2c.d and see Supplementary Fig. 4). In contrast, CXCR2-deficient mice had greatly reduced neutrophil accumulation and correspondingly greatly increased fungal growth in the brain (Fig. 2c,d). This data demonstrates the importance of the CXCR2 axis in neutrophil-mediated protection against C. albicans brain infection.

Next, we wondered which CXCR2 ligand could recruit protective neutrophils to the fungus-infected brain. We infected Cxcl1−/− mice that lack expression of the potent neutrophil chemokine CXCL1. Notably, these animals had decreased neutrophil recruitment to the brain post-infection, and exhibited a similar CNS invasion susceptibility phenotype to 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 cerebrospinal fluid is biologically relevant and important.

IL-1β activates CXCL1 in the fungus-infected brain. As 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. 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 cellular sources of CXCL1 in the brain, we infected WT and IL-1β−/− deficient mice and used intracellular flow cytometry. CXCL1 and pro-IL-1β were produced by multiple myeloid phagocytes in the fungus-infected brain, including resident microglia, the most numerous immune cells in the brain, and recruited Ly6C⁺ monocytes that have been implicated in controlling C. albicans CNS invasion, and the neutrophils themselves (Fig. 3b). It is interesting that 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⁺ monocytes isolated from Il1b−/− C. albicans–infected brains produced less CXCL1 when restimulated ex vivo with lipopolysaccharide (LPS), with no differences detected under non-stimulated or zymosan-stimulated conditions. Neutrophil production of CXCL1 did not differ between the two mouse groups (Fig. 3c). Therefore, IL-1β is required for subsequent CXCL1 production from resident microglia and recruited monocytes, which in turn recruits CXCR2-expressing neutrophils to the fungus-infected brain.

Candidalysin is a fungal virulence factor in the brain. Use of genetically deficient mice allowed us to map the host pathway promoting protection against C. albicans brain infection, in which
IL-1β–IL-1R–MyD88 signaling activates CXCL1 production by resident microglia and recruited monocytes to mobilize neutrophils into the CNS. To identify the pathogen-associated factors that induce this protective host pathway, we infected animals with C. albicans strains lacking known virulence factors and assessed neutrophil recruitment and IL-1β and CXCL1 production in the infected brain.

C. albicans hyphae are the predominant CNS-invasive morphological forms of C. albicans and hyphal formation is associated with important virulence traits such as toxin and protease production, adhesion, invasion and immune system activation. Thus, we first asked whether neutrophil recruitment was impaired during infection with the hgc1Δ/Δ C. albicans strain which cannot form filaments in vivo. Indeed, infection with hypha-deficient hgc1Δ/Δ C. albicans significantly impaired neutrophil recruitment and enhanced fungal invasion of the CNS tissue relative to the isogenic WT C. albicans strain (Fig. 4a). Thus, it is striking that filamentation is not required for C. albicans invasion of brain tissue, in contrast to other organs such as the kidney.

Candidalysin is a recently described peptide toxin encoded by ECE1 and expressed exclusively by C. albicans hyphae. Candidalysin has been shown to mediate epithelial cell damage via pore formation in the plasma cell membrane, resulting in IL-1α release and proinflammatory cytokine production. Hence, Candidalysin-null mutants were highly attenuated in murine oropharyngeal and vulvovaginal candidiasis models. Instead, we found that lack of Candidalysin-specific to the candidalysin peptide because mutant strains deficient in the entire gene (ecec1Δ/Δ), or specifically in the Candidalysin-encoding portion of the gene (ecec1Δ/Δ + ECE1Δ/Δ), were both hypervirulent for C. albicans brain invasion (Fig. 4b).

The increased ability of the Candidalysin-null mutants to proliferate within the brain correlated directly with the degree of neutrophil recruitment. We found an almost complete absence of neutrophils in the brains of WT animals infected with Candidalysin-null strains, and observed hyphal forms growing in the brain parenchyma without neutrophilic reaction (Fig. 4c). In contrast, the Candidalysin-producing parental strain and the re-integrate 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 pathogen17.

**Fig. 3 | Production of CXCL1 is dependent on IL-1β in the fungus-infected brain.** a, WT (n = 6–7 animals), Cxcl1−/− (n = 5) and Il1b−/− (n = 6) animals were infected as in Fig. 1; the brains were isolated at 24 h post-infection and analyzed for CXCL1 or IL-1β production by ELISA. Data are pooled from two independent experiments and were analyzed using the unpaired, two-tailed, Student’s t-test. b, The relative proportions of myeloid cell populations (gated within live CD45+ singlets) in the uninfected (n = 6) and 24 h infected WT (n = 6) brain (left), and the relative proportion of myeloid cell populations producing CXCL1 (n = 3) or pro-IL-1β (n = 9) in the 24 h infected brain (right). For the latter, total CD45+CXCL1 (or IL-1β)+ cells were first gated and then the cell types were defined within this initial gate with lineage markers (see below), using samples from the unstimulated condition. Data are shown as the mean ± s.e.m. c, WT (n = 3) and Il1b−/− mice (n = 4) were infected with 2 × 10^5 c.f.u. of C. albicans and the brain cells analyzed for CXCL1 production by intracellular flow cytometry 24 h later. Brain cells were restimulated ex vivo with 62.5 µg ml−1 depleted zymosan or 1 µg ml−1 LPS for 4 h in the presence of 5 µg ml−1 brefeldin A. Representative plots from the LPS-stimulated condition are gated on microglia (top: CD45intLy6G−CD11b+) and neutrophils (bottom: CD45+Ly6C−Ly6G−CD11b+), showing corresponding Cxcl1−/− cells to gating controls. In all panels, ‘wild type’ refers to appropriate matched control animals for each knockout line for gender, age and genetic background. Each symbol represents an individual mouse. Data are shown as mean ± s.e.m., and were analyzed using the unpaired, two-tailed, Student’s t-test. *P < 0.05, **P < 0.01.
We wondered next whether other secreted proteins associated with *C. albicans* hyphae also activate protective neutrophil responses in the brain. Secreted aspartyl proteases (Saps) are enzymes with extracellular proteolytic activity linked to virulence\(^2\). *C. albicans* Saps promote neutrophil recruitment during vulvovaginal candidiasis in mice\(^3,4\). Expression of the SAP4–6 subfamily is coordinately regulated with hyphal formation\(^5\), so we tested whether these hyphae-associated Saps contributed toward virulence during brain invasion. WT animals infected with the triple-deficient strain sap4/5/6\(\Delta\Delta\) had comparable fungal burdens in the brain to animals infected with the complemented control strain (Fig. 4c). 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\(^3,4\) (Fig. 4e). Therefore, protective CNS neutrophil recruitment is activated by candidalysin, and not by other enzymes secreted by *C. albicans* hyphae.

**Candidalysin drives microglial IL-1β and CXCL1 in vivo.** Next we sought to define the Candidalysin-responsive, CNS-immune cells post-infection. We infected WT mice with the parental strain of *C. albicans* (BWP17) or the Candidalysin-null strain (ece1\(\Delta\Delta\)), and analyzed IL-1β and CXCL1 production at 24 h post-infection. Although all brain phagocytes produced both IL-1β and CXCL1, microglia were the only population to exhibit dependence on Candidalysin, because microglia isolated from ece1\(\Delta\Delta\)-infected brains produced significantly less IL-1β and CXCL1 ex vivo (Fig. 5a,b). Instead, Ly6Chi monocytes and neutrophils did not exhibit a significant increase in IL-1β synthesis upon Candidalysin treatment (Fig. 5a,c). We hypothesized that other, as-yet unidentified, fungal factors activate this pathway in these phagocytes. Together, our data shows that Candidalysin acts on microglia to stimulate IL-1β release, which then drives the CXCL1 production that is required for protective neutrophil CNS recruitment.

**Candidalysin drives differing glial IL-1β–CXCL1 ex vivo.** To gain mechanistic insights into how microglia respond to Candidalysin, we cultured the microglia cell line BV-2 (ref. \(^2\)) in the presence of synthetic Candidalysin, and measured IL-1β and CXCL1 in the supernatants. In line with our in vivo work, we found time- and dose-dependent IL-1β production by BV-2 cells in response to Candidalysin (Fig. 6a). However, we did not detect CXCL1 from BV-2 cells stimulated under these conditions. We first considered that this could be due to Candidalysin-induced damage which may
prevent BV-2 cells from producing CXCL1 after IL-1β secretion. Indeed, as shown for epithelial cells, Candidalysin mediated dose-dependent cell damage to BV-2 microglia (Fig. 6b). Alternatively, additional signals beyond IL-1β, derived from non-microglial CNS cells, might be required for microglial CXCL1 induction, acting in trans. To test this hypothesis, we co-cultured BV-2 cells with immortalized C8-D1A astrocytes in the presence of Candidalysin and measured IL-1β and CXCL1 in the supernatants. We chose astrocytes because they are known to respond to IL-1β to produce inflammatory mediators, including CXCL1, in other models of CNS inflammation. We found that astrocytes responded to Candidalysin to produce CXCL1 (Fig. 6c), but not IL-1β (data not shown), and that CXCL1 production greatly increased when astrocytes and microglia were co-cultured (Fig. 6c). To confirm that microglia are a relevant cellular source of CXCL1 detected during microglia–astrocyte co-culture, we performed intracellular staining for CXCL1 and found BV-2 microglia to be 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.

Next we 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 MKP1 (ref. 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 MKP1 and c-Fos (Fig. 6c), and chemical inhibition of p38 or c-Fos greatly reduced IL-1β release from Candidalysin-stimulated BV-2 cells (Fig. 6f). Therefore, microglia produce IL-1β in response to Candidalysin via activation of p38 and c-Fos.

The microglial 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 intervention25–27. We first examined whether CARD9 deficiency causes developmental defects in resident microglia, but found no defects in abundance or activation markers at a steady state in Card9−/− microglia, which accumulated in similar numbers to WT microglia after fungal infection (see Supplementary Fig. 5). As 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, because microglia highly express CARD9 and we previously found reduced transcription of CXC chemokines by Card9−/− microglia harvested from the C. albicans−infected brain5. We infected Card9−/− and Card9−/+ animals with WT Candidalysin-expressing C. albicans, isolated phagocytes from the brain, and measured pro-IL-1β and CXCL1 production after ex vivo restimulation. We found greatly decreased frequencies of CXCL1+ and pro-IL-1β+ cells in the fungus-infected Card9−/− brain, and these decreases mapped to microglia (Fig. 7a,b).

As production and secretion of mature IL-1β depend 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 sorted
Fig. 7 | CARD9 is required for microglial pro-IL-1β transcription, inflammasome activation and CXCL1 production in the fungus-infected brain.

**a,b.** Card9+/+ (n = 13) and Card9−/− (n = 13) animals were infected with 2 × 10^5 c.f.u. of WT C. albicans (BWP17), and brain cells were 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) show pooled data from four independent experiments, analyzed using the two-tailed, unpaired, Student’s t-test. The data are shown as mean ± s.e.m. (a) or with minimum/maximum values (whiskers), the 25th/75th percentiles and the median (b). c, Microglia were sorted by FACS from pooled Card9+/+ (n = 4) and Card9−/− (n = 4) animals at 24 h post-infection and were analyzed using the unpaired, two-tailed, Student’s t-test for Il1b expression by quantitative PCR with reverse transcription (qRT-PCR). d,e, The indicated proteins from FACS-sorted microglia were analyzed by immunoblot (caspase and IL-1β blots: WT, n = 6 and Card9−/−, n = 7; c-Fos blot: WT, n = 10 and Card9−/−, n = 10; NLRP3 blot: WT, n = 8 and Card9−/−, n = 8). Graphs in d and e represent the band pixel density normalized to the WT control and are shown with mean ± s.e.m., analyzed using the unpaired, two-tailed, Student’s t-test. Example blots are representative of three independent FACs sorts/experiments; pooled data are shown in the graphs above. f, Nlrp3−/− animals and their WT controls were infected with 1.3 × 10^6 c.f.u. C. albicans and analyzed using the unpaired, two-tailed, Student’s t-test for neutrophil recruitment to the brain 24 h later (left: WT, n = 9 and Nlrp3−/−, n = 8) and the two-tailed, Mann–Whitney U-test for fungal brain burdens at 72 h post-infection (right: WT, n = 14 and Nlrp3−/−, n = 14), as described in Fig. 1. *P < 0.05, **P < 0.01, ***P < 0.005.

Microglia from WT- and Card9−/−-infected brains using FACs, and examined Il1b transcription using qRT–PCR, and levels of pro-IL-1β and cleaved and pro-caspase-1 using immunoblotting. We found greatly 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 after a viral infection. We also found greatly reduced cleaved caspase-1 in Card9−/−.
Microglial CARD9 deletion causes CNS fungal invasion. Next we directly examined the impact of genetic Card9 deletion, specifically within microglia, by utilizing mice that expressed tamoxifen-inducible Cre recombinase under the Cx3cr1 promoter (Cx3cr1CreER)\(^{32}\). These mice have been used to genetically manipulate long-lived CX3CR1 microglia, while leaving short-lived CX3CR1 monocytes and monocyte-derived macrophages unaffected. We bred Cx3cr1CreER/+/– animals to Card9-flxed mice\(^{3}\), tamoxifen pulsed the progeny to activate Cre expression, and waited 4–6 weeks to allow replenishment of short-lived, non-microglial CX3CR1+ cells from the bone marrow, whereas long-lived microglia remained Card9-deficient (see Supplementary Fig. 6). C. albicans infection of Card9\(^{+/–}\)Cx3cr1CreER/+/– animals revealed an important dependence on Card9 expression by the long-lived CX3CR1+ cellular compartment for control of fungal growth in the brain (Fig. 8a), although renal fungal control was unaffected in microglia-specific, conditional Card9\(^{−/−}\)mice (Fig. 8a).

To analyze whether the susceptibility to brain infection in Card9\(^{+/–}\)Cx3cr1CreER/+/– mice was related to a neutrophil recruitment defect, we quantified neutrophils within the infected brains of Card9\(^{+/–}\)Cx3cr1CreER/+/– mice and their Cre-negative littermates. We found that microglial deletion of Card9 greatly reduced the protective early influx of neutrophils into the fungus-infected brain (Fig. 8b), which correlated with greatly decreased expression of microglial pro-IL-1β and CXCL1 in the conditional Card9\(^{−/−}\)mice (Fig. 8c). Together, our data show that CARD9-expressing microglia orchestrate control of fungal invasion of the brain by responding to fungus-secreted Candidalysin to produce IL-1β-induced CXCL1, which recruits CXCR2-expressing neutrophils that mediate CNS fungal clearance (see Supplementary Fig. 7).

Discussion

In the present study, we demonstrated the critical contribution of CARD9-mediated IL-1β and CXCL1 in recruiting protective neutrophils to the fungus-infected CNS. We identified microglia as major producers of CARD9-dependent IL-1β and CXCL1 during C. albicans CNS invasion, and the fungus-secreted toxin Candidalysin as a critical pathogen-derived factor activating this pathway. Our study offers 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 a mortality rate greater than 50% despite therapy\(^{31}\). Neutropenia is the major predisposing factor for systemic candidiasis and Candida CNS invasion in particular\(^{31,34}\). Moreover, CNS invasion is prevalent during systemic candidiasis in low-birthweight neonates and also occurs as an iatrogenic complication after neurosurgical procedures\(^{35,36}\). Strikingly, CARD9 deficiency is a primary immunodeficiency disorder characterized by heightened susceptibility to fungal infections, of which CNS candidiasis is a hallmark\(^{3,34}\). CARD9 deficiency is the only known primary immunodeficiency disorder that causes fungus-specific infection susceptibility without other infectious or non-infectious manifestations, and the only one that causes fungal disease in which CNS is a primary target tissue\(^{17}\). We have previously demonstrated that Candida CNS disease in CARD9 deficiency is caused by a fungus- and brain-specific defect in neutrophil recruitment\(^{7}\). CNS neutropenia is now confirmed in several CARD9-deficient patients with CNS candidiasis\(^{37,38}\). None the less, how CARD9 mediates protective neutrophil trafficking into the fungus-infected CNS remained unclear.

Our analysis of mice deficient in several CLRs, cytokine and chemokine circuits uncovered: (1) the functional redundancy
among CLR s, which may suggest the presence of yet-undiscovered CARD9-coupled receptors for driving tissue-specific antifungal defense; (2) 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 (3) 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 meningitis39 and fungal pneumonia40, and in the present study we reveal its importance for recruiting neutrophils during CNS fungal infection, principally through binding CXCL1. In contrast, CCR1, CXCR1 and LTB4R1 are dispensable despite their regulation of neutrophil recruitment and function in other C. albicans−infected tissues35,41. These studies further underscore the organ- and context-specific dependence on chemotactic molecules for protective host immunity.

We have shown that IL-1β is required for CXCL1 induction, in line with earlier work showing IL-1β-induced CXCL1 production controlling neutrophil accumulation during bacterial peritonitis and autoimmune, traumatic or bacterial neuroinflammation39,41,42. Importantly, microglia are the primary myeloid cellular source of IL-1β-dependent CXCL1 production in vivo. During oral candidiasis, IL-1β is also required for neutrophil accumulation to the oral mucosa, as we have shown for systemic infection in the brain. However, further attesting to the presence of tissue-specific, anti-Candida immune response cues, the IL-1β-dependent response in the oral epithelium is largely controlled by IL-1α release via c-Fos activation, whereas we found that IL-1α plays a modest role in controlling fungal invasion of the brain. In fact, IL-1α release by both oral and vaginal epithelial cells is driven by exposure to the fungus-secreted toxin Candidalysin43,44.

Candidalysin enables the establishment of C. albicans mucosal infections, because Candidalysin-deficient strains are avirulent in these models44–47. In contrast, we found that Candidalysin-deficient strains are hypervirulent for the brain, associated with decreased IL-1β and CXCL1 production and impaired neutrophil recruitment. These results indicate that Candidalysin is not only a classic virulence factor, but also an immune modulator, which exerts context-specific effects on the immune system. We propose that this dual function of Candidalysin is the result of a co-evolutionary event: the fungus developed an efficient toxin to damage host membranes and, in response, the host evolved a sensitive Candidalysin detection system to defend against this common mucosal pathogen. Whether Candidalysin is recognized by a specific microglicial innate receptor to mediate the protective IL-1β–CXCL1 axis is unclear, because the toxin mediates cellular damage that could also activate glial cells. Therefore, identification of how host epithelial and immune cells recognize Candidalysin merits investigation.

We found that Candidalysin selectively activates microglia for IL-1β and CXCL1 production, a self-renewing macrophage population that contributes toward neuroinflammation in neurodegenerative disorders, and promotes pathogen and dead cell clearance within the CNS45. It is interesting that, downstream of Candidalysin-induced microglial IL-1β secretion, which occurs via c-Fos activation, we show that additional signals derived from astrocytes acting in trans are required for microglia to secrete CXCL1. Whether direct microglia–astrocyte contact is required or astrocyte-derived soluble factors acting on microglia are needed remains unknown. Indeed, microglia are known to interact with astrocytes to drive or suppress inflammation46,47. Therefore, the molecular factors that drive microglia–astrocyte cross-talk within the fungus-infected brain warrant further investigation.

Last, we examined the dependence on CARD9 for the microglia-mediated, IL-1β–CXCL1-dependent pathway that recruits protective neutrophils, using fully CARD9-deficient and conditional microglia-specific Card9−/− mice. We showed that CARD9 is critical for both c-Fos activation and the production of both IL-1β and CXCL1 by microglia in the infected CNS, operating at the levels of both transcriptional pro-IL-1β regulation and inflammasome activation for IL-1β generation, with NLRP3 being at least partly involved, as shown with microsor mus infection40. Together, this data sheds light on the pathogenesis of inherited CARD9 deficiency by outlining a pathway of CARD9-dependent microglial production of sequential IL-1β and CXCL1, which recruits protective neutrophils into the fungus-infected CNS.

Future studies should examine whether Card9 promotes microglial innate functions beyond orchestrating neutrophil recruitment such as fungus uptake and killing. Of note, the phenotype of microglia-specific, conditional, knockout mice is less severe than that of Card9−/− mice, which may reflect the important role of astrocytes expressing Card9 post-infection4, in priming microglial CXCL1 production. Future work should examine whether non-CNS, tissue-resident macrophages, such as Kupffer cells, differentially depend on Card9 for IL-1β–CXCL1-mediated neutrophil recruitment, as previously shown for differential macrophage and dendritic cell-induced, Card9-dependent, tumor necrosis factor-α; this will help further understanding of the CNS specificity of fungal infections 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 autoimmune and malignant diseases48,49. Surveillance of Syk inhibitor-treated patients and research into 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 mechanistic light on the pathogenesis of inherited CARD9 deficiency.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41590-019-0377-2.

Received: 25 May 2018; Accepted: 12 March 2019; Published online: 17 April 2019

References
1. Lionakis, M. S. & Levitz, S. M. Host control of fungal infections: lessons from basic studies and human cohorts. Annu. Rev. Immunol. 36, 157–191 (2018).
2. Drummond, R. A. & Lionakis, M. S. Mechanistic insights into the role of C-type lectin receptor/CARD9 signaling in human antifungal immunity. Front. Cell Infect. Microbiol. 6, 39 (2016).
3. Glöcker, E. O. et al. A homozygous CARD9 mutation in a family with susceptibility to fungal infections. N. Engl. J. Med. 361, 1727–1735 (2009).
4. Lanternier, F. et al. Deep dermatophytosis and inherited CARD9 deficiency. N. Engl. J. Med. 369, 1704–1714 (2013).
5. Drummond, R. A. et al. CARD9-dependent neutrophil recruitment protects against fungal invasion of the central nervous system. PLoS Pathog. 11, e1005283 (2015).
6. Li, X. et al. The β-glucan receptor Dectin-1 activates the integrin Mac-1 in neutrophils via Vav protein signaling to promote Candida albicans clearance. Cell Host Microbe 10, 603–615 (2011).
7. Drewniak, A. et al. Invasive fungal infection and impaired neutrophil killing in human CARD9 deficiency. Blood 121, 2385–2392 (2013).
8. Altmeyer, S. et al. IL-1 coordinates the neutrophil response to C. albicans in the oral mucosa. PLOS Pathog. 12, e1005882 (2016).
9. Karki, R. et al. Concerted activation of the AIM2 and NLRP3 inflammasomes orchestrates host protection against Aspergillus infection. Cell Host Microbe 17, 357–368 (2015).
10. Biondo, C. et al. The interleukin-1β/CXCL1/2/neutrophil axis mediates host protection against group B streptococcal infection. Infect. Immun. 82, 4508–4517 (2014).
35. McCarthy, M. W., Kalasauskas, D., Petraitis, V., Petraitiene, R. & Walsh, T. J. Microglia promote learning-dependent synapse pulmonary capillaritis during lethal fungal sepsis. Cell Host Microbe 23, 121–133.e124 (2018).

36. Swamydas, M. et al. CXCR1-mediated neutrophil degranulation and fungal killing promote Candida clearance and host survival. Sci. Trans. Med. 8, 322ra310–322ra310 (2016).

37. Ngo, L. Y. et al. Inflammatory monocytes mediate early and organ-specific innate defense during systemic candidiasis. J. Infect. Dis. 209, 109–119 (2014).

38. Erwig, L. P. & Gow, N. A. R. Interactions of fungal pathogens with phagocytes. Nat. Rev. Microbiol. 14, 163–176 (2016).

39. Zheng, X., Wang, Y. & Wang, Y. Hgc1, a novel hypha-specific G1 cyclin-related protein regulates Candida albicans hyphal morphogenesis. EMBO J. 23, 1845–1856 (2004).

40. Moyes, D. L. et al. Candidalysin is a fungal peptide toxin critical for mucosal infection. Nature 532, 64–68 (2016).

41. Verma, A. H. et al. Oral epithelial cells orchestrate innate type 17 responses to Candida albicans through the virulence factor candidalysin. Sci. Immunol. 2, eaam8834 (2017).

42. Richardson, J. P. et al. Candidalysin drives epithelial signaling, neutrophil recruitment, and immunopathology at the vaginal mucosa. Infect. Immun. 86, e00645–17 (2017).

43. Naglik, J. R., Challacombe, S. J. & Hube, B. Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. Microbiol. Mol. Biol. Rev. 67, 400–423 (2003).

44. Gabrielli, E. et al. In vivo induction of neutrophil chemotaxis by secretory aspartyl proteinases of Candida albicans. Virulence 7, 819–825 (2016).

45. Pericollini, E. et al. Secretory aspartyl proteinases cause vaginitis and can mediate vaginitis caused by Candida albicans in mice. mBio 6, e00724–15 (2015).

46. Hannamagaru, R., Aldrich, A. & Kilian, T. Critical role for the AIM2 inflammasome during acute CNS bacterial infection. J. Neurochem. 129, 704–711 (2014).

47. Prinz, M., Erny, D. & Hagemeyer, N. Ontogeny and homeostasis of CNS myeloid cells. Nat. Immunol. 18, 385–392 (2017).

48. Shinozaki, Y. et al. Transformation of astrocytes to a neuroprotective phenotype by microglia via P2Y1 receptor downregulation. Cell Rep. 19, 1151–1164 (2016).

49. Rothhammer, V. et al. Microglial control of astrocytes in response to microbial metabolites. Nature 557, 724–728 (2018).

50. Mao, L. et al. Pathogenic fungus Microsporum canis activates the NLRP3 inflammasome. Infect. Immun. 82, 882–892 (2014).

51. Goodridge, H. S. et al. Differential use of CARD9 by Dectin-1 in macrophages and dendritic cells. J. Immunol. 182, 1146–1154 (2009).

52. Weiblatt, M. E. et al. An oral spleen tyrosine kinase (Syk) inhibitor for rheumatoid arthritis. N. Engl. J. Med. 363, 1303–1312 (2010).

53. Flynn, R. et al. Targeting Syk-activated B cells in murine and human chronic graft-versus-host disease. Blood 125, 4085–4094 (2015).

Acknowledgments
This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Disease, National Institutes of Health, as well as NIH grants awarded to T.M.H. (no. R01 AI093808), S.G.F. (no. R01AI124566) and S.A.L. (no. R01CA161373). Additional funding was provided by the Burroughs Wellcome Fund (awarded to T.M.H.), the Wellcome Trust (nos. 102705 and 097377; awarded to G.D.B.), the MRC Centre for Medical Mycology and the University of Aberdeen (no. MR/N006364/1; awarded to G.D.B.). The authors additionally thank C. Huaman for their care and screening of the Malt1−/− mice, which were a kind gift to B.C.S. from T. Mak and the University Health Network (Canada), and D. McGavern and F. Crews for providing the murine glial cell lines.

Author contributions
R.A.D. and M.S.L. designed the study. R.A.D., M.S., V.O., B.Z. and I.M.D. performed the experiments. R.A.D., M.S., V.O., B.Z., I.M.D., T.M.H. and M.S.L. analyzed the data. 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. wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41590-019-0377-2.

Reprints and permissions information is available at www.nature.com/reprints.

Correspondence and requests for materials should be addressed to R.A.D. or M.S.L.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature America, Inc. 2019.
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 I4BS facility of the National Institutes of Health (NIH, Bethesda, MD, USA), the Memorial Sloan-Kettering Cancer Center Comparative Medicine Shared Center, or Resources (New York, USA) or the Medical Research Facility at the University of Amsterdam (UK). The following strains (and their respective WT controls/littermates) were obtained from the NIAID Taconic contract: Clec7a+/+, IL1R+/−, IL1b−/−, Fpr1−/−. All other strains and their respective controls/littermates were bred in-house at the NIH (Clec7a−/−, Clec7a+/+, Myd88−/−, Ccr1−/−, Cx3cr1−/−, Il1a−/−, Il1b−/−, Il6r−/−, Nramp3−/−, Card9+/CreERT2/+;−/−, Mec1−/−;−/−); Memorial Sloan-Kettering Cancer Center (Clec7a+/−;Ecerlg−/−, University of Aberdeen (Clec4d−/−)) or Uniformed Services University of the Health Sciences (Malt1−/−). Mice homozygous for the Card9−/− allele were purchased from the Wellcome Trust Sanger Institute (EUCCOM Project No. 44813), and these animals were bred with the FLPer deleter strain (Jackson Laboratories) to remove the FRT-flanked, knockout, first cassette, generating Card9−/− homozygous mice (referred to as Card9+/−). Homozygous Card9−/− animals were bred with heterozygous Clec7a+/− transgenic animals (Jackson Laboratories) to generate Card9+/CreERT2+/−;Clec7a+/−;−/− mice and littermate controls. Soon after weaning (~5–6 weeks old), Card9+/CreERT2+/−;Clec7a+/−;−/− mice and their controls were treated with two 10 mg 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 (NIAID).

Candidiasis model and fungal burden determination. Candida albicans strains used in this study were SC5314, BWP17, cEcl1AΔα, cEcl1AΔα + ECE1, ecelΔA + Aece1Δgαα (ref. 7), CAI4 + Cplp10 and spafA5Δ6Δα, and hgc1αAΔ and hgc1αAΔ + HGC1 (ref. 8). 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 c.f.u for analysis at 24 h post-infection, or 7 × 10^5 c.f.u. for analysis at 72 h post-infection, unless otherwise stated in the corresponding figure legends. For analysis of fungal burdens in the brain, animals were euthanized and brains weighed, homogenized in PBS and serially diluted before plating on 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 previously described methods15, resuspended in PBS and stained with live/dead fluorescent dye (Invitrogen) for 10 min on ice. Cells were then stained with fluorescein isothiocyanate-conjugated antibodies in the presence of anti-CD11b/32 and 0.5% bovine serum albumin (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 Bioscience). 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 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 a protease inhibitor cocktail (Roche). Homogenized brains were centrifuged twice to remove debris and the resulting supernatants snap-frozen in liquid nitrogen and stored at −80 °C before analysis. IL-1β and CXCL1 concentrations in the homogenates were determined by ELISA (R&D Systems), following the manufacturers’ instructions.

Ex vivo restimulations and intracellular FACS analysis. Animals were infected with 2 × 10^5 c.f.u. of the indicated C. albicans strain intravenously, and brain leukocytes isolated 24 h later. For these experiments, brains were first digested in RPMI medium supplemented with 0.8 mg/ml Dispase (Gibco), 0.2 mg/ml Collagenase Type 4 (Worthington) and 0.1 mg/ml DNase (Roche) at 37 °C for 30 min, then pipetted vigorously to create a homogeneous suspension. These suspensions were centrifuged (1,500 r.p.m. × 5 min, 4 °C), the pellets re-suspended in 5 ml 40% Percoll (GE Healthcare) and centrifuged again at 1,700 r.p.m. for 20 min at 4 °C to remove myelin. Cell pellets were washed in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin (Invitrogen), and added to FACS tubes for stimulations. Cells were incubated for 4 h at 37 °C in the presence of 5 μg/ml brefeldin A (Sigma), 5 μg/ml monensin (Sigma), and 0.25 μg/ml mAbs (for flow cytometry and intracellular cytokine staining for intracellular cytokines using the eBioscience Foxp3 staining kit, and staining for CXCL1 (IC5432R, from R&D Systems) or pro-IL-1β (NTEN3, from eBioscience) performed overnight at 4 °C.

Samples were washed once in PBS/0.5% BSA/0.01% sodium azide before acquisition using the BD Fortessa instrument equipped with BD FACs Diva software (BD Biosciences). FlowJo (TreeStar) was used for the final analysis. CXCL1+ and pro-IL-1β+ cells were determined by employing similar staining and gating in animals deficient in these mediators (CXCL1−/−, I1β−/−) as negative controls.

Cell culture and Candida lysin stimulations. BV-2 cells were kindly provided by F. Crews (University of North Carolina School of Medicine). BV-2, D1A, and Flt3−/− macrophages were kindly provided by D. McGavern (NINDS, NIH). Details about the validation and use of these cell lines are provided in the Life Sciences Reporting Summary.

24-well plate and incubated overnight at 37 °C. BV-2 cells and Candidalysin were added as described above. In both experiments, supernatants (50 ml) were collected and assayed for IL-1β, TNFα, IL-6, IL-18, IL-23, IL-2, IL-22, IL-8, IFNγ, IL-12p70, IL-10, CXCL1, and CXCL2 by ELISA (R&D Systems), and CXCL1 staining by intracellular flow cytometry or immunoassay.

Immunoassay analysis. Whole-cell lysates were suspended in RIPA buffer containing protease and phosphatase inhibitors (Thermo Scientific). Lysates were separated in SDS–PAGE and transferred to a 0.2-μm nitrocellulose membrane (Bio-Rad Laboratories). The membrane was incubated with the following primary antibodies: phospho-MKP1/MKP2 polyclonal (Ser84, Ser91) (Thermo Scientific) and c-Fos (Cell Signaling), IL-1β (3A6) (Cell Signaling), caspase-1 p20 (Casper-1; Adipogen Life Sciences, Thermo Scientific) and NLRP3 (D48T; Cell Signaling). Normalization was performed by probing the membrane with antibody to β-actin (Cell Signaling). Chemiluminescence detection was performed using Clarity Western ECL Substrate (Bio-Rad Laboratories) and the ChemiDoc MP Imaging System (Bio-Rad).

FACS/MACS sorting of microglia. WT animals were infected with 1.3 × 10^5 c.f.u. SC5314 and were euthanized at 24 h post-infection. Brains were digested as above and leukocytes stained with sterile antibodies14. Ly6C+ monocytes (CD45+CD11b+Ly6C+Ly6G−) and microglia (CD45+CD11b+Ly6G+Ly6C−) were FACS sorted into sterile sorting buffer (Hanks’ balanced salt solution supplemented with 2 mM EDTA, 10% FCS, 100 μM l-penicillin, 100 μg/ml streptomycin) using an FACS Aria instrument for downstream qRT–PCR and immunoblot analyses. Purity of cells was greater than 95%, on average. In some experiments (qRT–PCR of CLRs in brain-resident microglia; see Supplementary Fig. 1), microglia were instead sorted by magnetic separation using anti-CD11b microbeads (Miltenyi). Cells were then centrifuged (1,500 r.p.m., 5 min, 4 °C) and resuspended in TRIZol for mRNA purification or radioimmunoprecipitation assay buffer for downstream immunoblot analysis. Depending on the experiment, up to five 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) following the manufacturer’s protocol. Purified RNA was used as a template for cDNA generation using the SCRT cDNA SuperMix kit (Quanta Biosciences) with oligo(dT) and random primers. Quantitative PCR was performed using 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 glyceraldehyde 3-phosphate dehydrogenase transcript levels using the ΔΔCT method. TaqMan primers/probes (Clec7a, Clec4n, Clec4d, Clec4e, I1b, 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 using the Kolmogorov–Smirnov test and analyzed accordingly using the two-tailed, unpaired Student’s t-test or the two-tailed, Mann–Whitney U-test. In cases where multiple data-sets were analyzed, two-way ANOVA was used with Bonferroni’s correction. In all cases, P < 0.05 was considered significant. Further details relating to power calculations, randomization and blinding are described in the Life Sciences Reporting Summary.
Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

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

References
50. Ruland, J., Duncan, G. S., Wakeham, A. & Mak, T. W. Differential requirement for Malt1 in T and B cell antigen receptor signaling. *Immunity* 19, 749–758 (2003).

51. Tay, T. L. et al. A new fate mapping system reveals context-dependent random or clonal expansion of microglia. *Nat. Neurosci.* 20, 793–803 (2017).

52. Goldmann, T. et al. A new type of microglia gene targeting shows TAK1 to be pivotal in CNS autoimmune inflammation. *Nat. Neurosci.* 16, 1618–1626 (2013).

53. Lionakis, M. S., Lim, J. K., Lee, C. C. R. & Murphy, P. M. Organ-specific innate immune responses in a mouse model of invasive candidiasis. *J. Innate Immun.* 3, 180–199 (2011).

54. Cougnoux, A. et al. Microglia activation in Niemann–Pick disease, type C1 is amendable to therapeutic intervention. *Hum. Mol. Genet.* 27, 2076–2089 (2018).
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

| Data collection     | BD FACS Diva |
|---------------------|--------------|
| Data analysis       | FlowJo 9.9.6, GraphPad Prism 7.0 |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All raw data associated with the figures is available on request, without restrictions.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf
Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
In each of the animal experiments, we aimed to have \( n = 6 \) per experiment group, since our power calculations indicated that this sample size would allow for a detection of a 30% difference in the means with a probability of greater than 95%, assuming a standard deviation of around 19% and minimum power 0.8. These calculations are based on years of experience with these models and measuring these parameters using our chosen methods. When the desired sample size was not available (due to mouse breeding and availability), we used what was available and repeated the experiment several times independently to increase sample size number closer to the desired number. The same power calculations were applied to determine sample size for the cell line-based experiments.

**Data exclusions**
No data exclusions.

**Replication**
All experiments were repeated at least twice and up to 4 times, to ensure reproducibility of the results. Data from each experiment was pooled and raw data is presented in the figures. In cases where the experiment-to-experiment variability was higher, data was normalized to the appropriate control and pooled data presented in this way, after ensuring that individual experiments had a similar pattern of results and gave similar statistical results as the pooled normalized data. There were no experiments that we could not replicate independently in this study.

**Randomization**
In all experiments, the assignment of littermate controls to cages, the placement of the experimental cage within the animal facility and the order in which infections/analysis procedures were performed were done at random.

**Blinding**
In some experiments, mouse blinding was not possible due to the nature of mouse breeding (i.e. when WT lines were used for infection with different Candida strains - see methods for details). In some of the experiments that used littermate controls (e.g. Card9fl/fl-Cx3cr1-CreER animals, Card9 KO - see methods for details), animals were assigned a 4-digit number and analyzed in a blinded using the ID number. Genotype was assigned at the final analysis stage (i.e. when graphing the data). All other data were acquired and analyzed in a non-blinded fashion because it did not involve subjective measurements.

### Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

#### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | Antibodies            |
| ☒   | Eukaryotic cell lines  |
| ☒   | Palaeontology         |
| ☒   | Animals and other organisms |
| ☒   | Human research participants |
| ☒   | Clinical data         |

#### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | ChIP-seq              |
| ☒   | Flow cytometry        |
| ☒   | MRI-based neuroimaging |

#### Antibodies

**Antibodies used**
Anti-mouse antibodies (all used at a 1:100 dilution) used in this study were:
- CD45 (30-F11), CD11b (M1/70), pro-IL-1β (NTJEN3) from eBiosciences
- Ly6G (1A8), Ly6C (AL-21) from BD Biosciences
- CXCL1 (IC4S32R) from RnD Systems
- pMPK1/2 (polyclonal) from Thermo Scientific
- cFos, IL-1β (3A6), Nlrp3 (D4D8T), b-actin from Cell Signaling
- Caspase 1 p20 (Casper-1) from Adipogen Life Sciences
- CD16/32 blocking antibody (24G2) from BD Biosciences

**Validation**
All antibodies against surface-expressed markers used in this study have been previously validated by the manufacturer, as stated on their associated product webpages, and by our own lab in previous experiments. We validated the use of intracellular anti-cytokine antibodies by staining animals deficient in these cytokines, as described in the Methods and shown in the figures.

#### Eukaryotic cell lines

**Policy information about cell lines**

**Cell line source(s)**
BV-2 microglia were obtained from Dr Fulton Crews (University North Carolina at Chapel Hill). BV-2 were originally made by Dr Elizabeth Blasi and are deposited in the Biological Bank of the IRCCS San Martino University Hospital (Italy). C8-1DA were originally from ATCC and validated/obtained from the McGavern lab at NINDS (NIH).
Authentication

Expression of common microglia markers CD45, CD11b and Cx3CR1 by BV-2 microglia were tested by flow cytometry. We did not personally validate the C8-1DA cell line, except for showing their lack of expression of the hematopoietic cell marker CD45.

Mycoplasma contamination

Cell lines were not tested for mycoplasma; all cells grew well and health/viability was checked prior to each experiment.

Commonly misidentified lines

No misidentified/contaminated cell lines used in this study. (See ICLAC register)

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals

Sex-matched mice were used at 8-12 weeks of age, using either male or female mice depending on availability. The mouse lines that were on the C57Bl/6 background were: Card9fl/flCx3cr1-CreER, Card9-/-, Clec7a-/-, Clec4n-/-, Clec4e-/-, MyD88-/-, Ccr1-/-, Cxcr1-/-, Il1a-/-, Il1b-/-, Il1r-/-, Clec4d-/-, Malt1-/-, Fpr1-/-, Ltb4r1-/-, Clec7a/-Fcerg-/-, Nlrp3-/-.
The only mouse line on the BALB/c background were the Cxcr2-/- animals.

Wild animals

No wild animals used.

Field-collected samples

No field-collected samples used.

Ethics oversight

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

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☑ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☑ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
☑ All plots are contour plots with outliers or pseudocolor plots.
☑ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

All sample preparations are described in detail in the Methods section, or (in the case of isolating brain leukocytes) described in more detail in Lionakis et al (2011) Journal Innate Immunity.

Instrument

All samples were acquired on a 5-lazer BD LSR Fortessa.

Software

The flow cytometer used to collect the data was BD FACSDiva, and the final analysis was completed using FlowJo (TreeStar).

Cell population abundance

The abundance of microglia and monocytes (the only populations sorted in this study) are shown in Fig 3b and the gating strategy in Fig S2. Post-sort analyses confirmed the purity of our sorted populations as >95%.

Gating strategy

Our gating strategy is defined in Fig S2 and defined in the methods.

☑ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.