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EphA2 Is a Neutrophil Receptor for *Candida albicans* that Stimulates Antifungal Activity during Oropharyngeal Infection

Marc Swidergall[1,2,*], **Norma V. Solis**[1,2], **Zeping Wang**[1,6], **Quynh T. Phan**[1,2], **Michaela E. Marshall**[3], **Michail S. Lionakis**[4], **Eric Pearlman**[3], and **Scott G. Filler**[1,2,5,7,*]

[1]Division of Infectious Diseases, Harbor-UCLA Medical Center, Torrance, CA 90502, USA

[2]Institute for Infection and Immunity, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, CA 90502, USA

[3]Institute for Immunology, University of California, Irvine, Irvine, CA 92697, USA

[4]Fungal Pathogenesis Section, Laboratory of Clinical Immunology and Microbiology, National Institute of Allergy and Infectious Diseases (NIAID), NIH, Bethesda, MD 20892, USA

[5]David Geffen School of Medicine at UCLA, Los Angeles, CA 90024, USA

[6]Present address: Center for Infectious Disease Imaging (CIDI), Radiology and Imaging Sciences, NIH Clinical Center, NIH, Bethesda, MD 20892, USA

[7]Lead Contact

SUMMARY

During oropharyngeal candidiasis (OPC), *Candida albicans* proliferates and invades the superficial oral epithelium. Ephrin type-A receptor 2 (EphA2) functions as an oral epithelial cell β-glucan receptor that triggers the production of proinflammatory mediators in response to fungal infection. Because EphA2 is also expressed by neutrophils, we investigated its role in neutrophil candidacidal activity during OPC. We found that EphA2 on stromal cells is required for the accumulation of phagocytes in the oral mucosa of mice with OPC. EphA2 on neutrophils is also central to host defense against OPC. The interaction of neutrophil EphA2 with serum- opsonized *C. albicans* yeast activates the MEK-ERK signaling pathway, leading to NADPH subunit $p47^{phox}$ site-specific phospho-priming. This priming increases intracellular reactive oxygen species production and enhances fungal killing. Thus, in neutrophils, EphA2 serves as a receptor for β-glucans that augments Fcγ receptor-mediated antifungal activity and controls early fungal proliferation during OPC.

*Correspondence: mswidergall@labiomed.org (M.S.), sfiller@ucla.edu (S.G.F.).

AUTHOR CONTRIBUTIONS

M.S. and S.G.F. designed the experiments. Methodology was provided by M.S.L. and E.P. M.S. and N.V.S. performed the experiments. Z.W. and Q.T.P. performed pilot studies. M.E.M. breed the CD18-deficient mice. M.S. and S.G.F. analyzed the data. M.S. and S.G.F. wrote the paper.

DECLARATION OF INTERESTS

S.G.F. is a co-founder of and shareholder in NovaDigm Therapeutics, Inc., a company that is developing a vaccine against mucosal and invasive *Candida* infections.

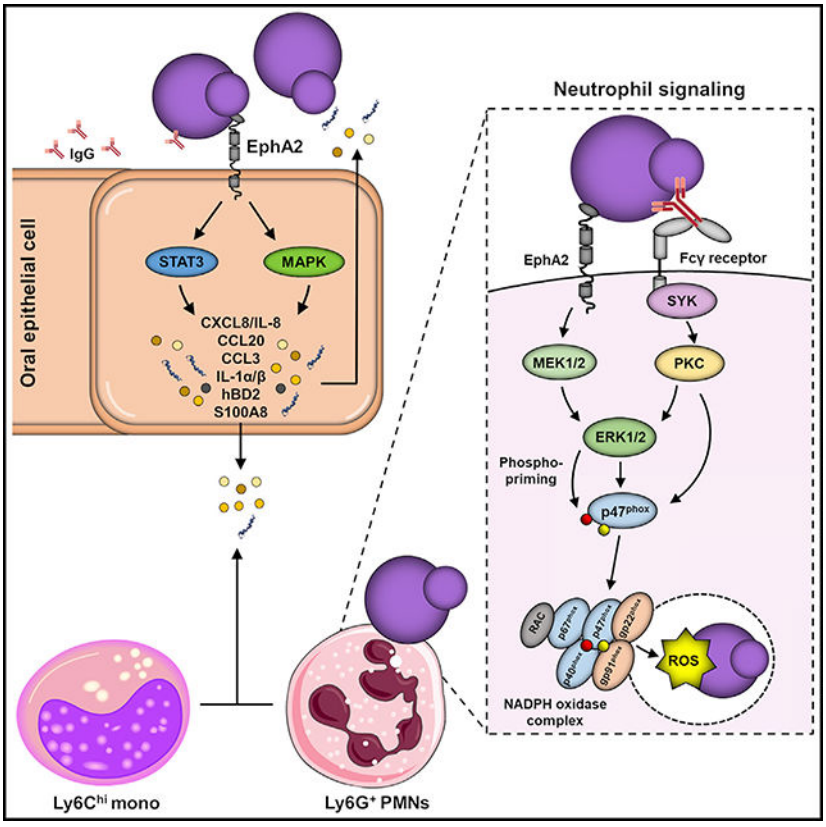
SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.06.020.

In Brief

In oral epithelial cells, EphA2 functions as a β-glucan receptor that triggers the production of proinflammatory mediators in response to oropharyngeal candidiasis. Here, Swidergall et al. show that, in neutrophils, EphA2 recognition of β-glucans augments Fcγ receptor-mediated antifungal activity and prevents fungal proliferation during the initiation of oropharyngeal infection.

Graphical Abstract



INTRODUCTION

The occurrence of fungal infections such as those caused by *Candida albicans* has progressively increased over the last decades (Bongomin et al., 2017; Brown et al., 2012). Invasive candidiasis is associated with mortality rates of 40% to 50%, while mucosal candidal infections such as oropharyngeal candidiasis (OPC) cause significant morbidity in millions of patients who are immunocompromised due to AIDS, neutropenia, diabetes mellitus, or the use of immunosuppressive drugs (Swidergall and Filler, 2017).

In healthy individuals, the collaborative antifungal activity of epithelial cells, phagocytes, and lymphocytes maintains mucosal immunity and prevents fungal proliferation (Verma et al., 2017a). Recognition of components of the fungal cell wall, such as exposed β-glucans, is crucial for inducing host antifungal responses (Dambuza et al., 2017; Levitz, 2010). Masking of β-glucans via mannan is a major mechanism by which fungi avoid recognition

by the host (Ballou et al., 2016). However, immune attack and environmental pressures, such as exposure to hypoxia and echinocandin antifungal drugs, drive cell wall remodeling that unmasks β-glucans and enhances the host inflammatory responses (Hopke et al., 2016). Leukocytes recognize β-glucan via Dectin-1 and complement receptor 3 (CR3; CD11b/CD18), while oral epithelial cells sense exposed β-glucan via ephrin type-A receptor 2 (EphA2) and Dectin-1 (Brown et al., 2002; Swidergall, 2019; Swidergall et al., 2018; van Bruggen et al., 2009). Epithelial β-glucan sensing mediates protective immunity in the oral cavity by distinguishing between fungal colonization and fungal overgrowth (Swidergall et al., 2018). In response to fungal overgrowth, epithelial cells secrete cytokines and chemokines that recruit phagocytes to sites of infection, where they inhibit and eventually kill the proliferating and invading fungi. These phagocytes amplify the proinflammatory response by producing additional cytokines and chemokines (Lionakis and Levitz, 2018; Underhill and Pearlman, 2015). Although neutrophils are vital for host defense against OPC (Huppler et al., 2014), it is incompletely understood how these leukocytes recognize *C. albicans* in the oral cavity. Classical myeloid pattern recognition receptors (PRRs), such as Dectin-1 and TLR2, are dispensable for host defense against OPC (Verma et al., 2017b), suggesting that other neutrophil receptors must sense the presence of *C. albicans* in the oropharynx.

Previously, we reported that EphA2 is an oral epithelial cell receptor for β-glucans. Binding of fungal β-glucans to epithelial cell EphA2 initiates antifungal responses in the oral cavity by inducing cytokine and chemokine production (Swidergall et al., 2018). In the current study, we investigated the role of neutrophil EphA2 in host defense against OPC. We found that during OPC, *EphA2*$^{-/-}$ mice had delayed infiltration of neutrophils and inflammatory monocytes into the oral cavity, resulting in more severe disease compared with wild-type mice. Strikingly, we found that EphA2, in conjunction with Fcγ receptors (FcγRs), is required for neutrophils to limit *C. albicans* proliferation during OPC. The interaction of serum-opsonized yeast with neutrophil EphA2 activates mitogen-activated protein kinase (MAPK) signaling, leading to p47^{phox} priming, increased intracellular reactive oxygen species (ROS) accumulation, and enhanced fungal killing. Thus, in neutrophils, EphA2 serves as a PRR for β-glucans that augments FcγR-mediated antifungal activity and is central to controlling early fungal proliferation during OPC.

RESULTS

EphA2 Signaling Induces Early Leukocyte Infiltration during OPC

The oral epithelium orchestrates the early antifungal response to infection by releasing cytokines and chemokines that recruit leukocytes to the infection site and then prime them for enhanced antifungal activity (Altmeier et al., 2016; Huppler et al., 2014; Swidergall and Ernst, 2014; Swidergall et al., 2018). To investigate the role of EphA2 in the host response to OPC, we determined the oral fungal burden, cytokine-chemokine response, and number of infiltrating neutrophils and inflammatory monocytes after 1 and 2 days of oral infection in wild-type and *EphA2*$^{-/-}$ mice. In agreement with our previous findings (Swidergall et al., 2018), after 1 day of infection, *EphA2*$^{-/-}$ mice had an increased oral fungal burden relative to that of wild-type mice (Figure 1A). This increase was associated with an impaired host

response in the *EphA2*$^{-/-}$ mice, which had lower levels of CCL3, CXCL1/KC, interferon γ (IFN-γ), interleukin (IL)-23 p19, and IL-17A in their oral tissues relative to wild-type mice (Figure 1B). Furthermore, after 1 day of infection, the *EphA2*$^{-/-}$ mice had significantly fewer neutrophils and inflammatory monocytes in their oral tissues compared with the wild-type mice (Figures 1C and 1D; Figure S1). After 2 days of infection, the oral fungal burden of wild-type mice declined, whereas the oral fungal burden of *EphA2*$^{-/-}$ mice remained elevated (Figure 1A). In wild-type mice, the number of neutrophils and inflammatory monocytes declined to near basal levels by day 2, whereas in the *EphA2*$^{-/-}$ mice, the number of these leukocytes increased (Figures 1C and 1D). These results were verified by histopathological analysis, which demonstrated that the tongues of wild-type mice had few visible fungi or leukocytes at day 2 post-infection, whereas the tongues of *EphA2*$^{-/-}$ mice contained numerous fungi and leukocytes (Figure 1E). Thus, during OPC, EphA2 is required for the early recruitment of phagocytes and control of fungal growth.

Because EphA2 is expressed on both hematopoietic and nonhematopoietic cells (Finney et al., 2017; Heng and Painter, 2008; Petersen et al., 2012; Suram et al., 2013; Swidergall et al., 2018), we used bone marrow (BM) chimeric mice to dissect the role of EphA2 expressed by these different cell types in host defense against OPC. These mice were generated as diagrammed in Figure 2A and then orally infected with *C. albicans* 8 weeks after successful BM reconstitution (Figure S2). Wild-type mice that were reconstituted with *EphA2*$^{-/-}$ BM and *EphA2*$^{-/-}$ mice that were reconstituted with wild-type BM had significantly more severe OPC, as determined by increased oral fungal burden and greater weight loss, relative to wild-type mice that were reconstituted with wild-type BM (Figures 2B and 2C). In the BM chimeric mice that lacked *EphA2* in either their stromal cells or their myeloid cells, the severity of OPC was similar to that in *EphA2*$^{-/-}$ BM mice that were reconstituted with *EphA2*$^{-/-}$. These results suggest that EphA2 within both hematopoietic and nonhematopoietic compartments is required for protection against OPC.

To determine the biological function of EphA2 in these compartments, we next examined leukocyte accumulation in the oral tissues of the BM chimeric mice after 1 day of OPC. The accumulation of neutrophils in *EphA2*$^{-/-}$ mice reconstituted with wild-type BM was significantly reduced relative to the wild-type mice reconstituted with wild-type BM and similar to *EphA2*$^{-/-}$ mice reconstituted with *EphA2*$^{-/-}$ (Figure 2D). By contrast, the accumulation of neutrophils in wild-type mice reconstituted with *EphA2*$^{-/-}$ BM was similar to that of wild-type mice reconstituted with wild-type BM and significantly greater than *EphA2*$^{-/-}$ mice reconstituted with *EphA2*$^{-/-}$. The accumulation of inflammatory monocytes in the *EphA2*$^{-/-}$ mice reconstituted with wild-type BM and the wild-type mice reconstituted with *EphA2*$^{-/-}$ BM was not significantly different from wild-type mice reconstituted with wild-type BM (Figure 2E). Collectively, these results indicate that EphA2 on stromal cells is required for the normal accumulation of neutrophils in the oral tissues during OPC. They also suggest that although *EphA2*$^{-/-}$ neutrophils are able to accumulate at wild-type levels at sites of OPC, they are unable to control fungal proliferation.

EphA2$^{-/-}$ Neutrophils Have Decreased Antifungal Activity *In Vivo*

The preceding findings suggested that EphA2 may be required for phagocytes to kill *C. albicans.* In addition to being expressed on epithelial cells, EphA2 is known to be expressed on myeloid phagocytes such neutrophils, macrophages, and monocytes (Heng and Painter, 2008; Petersen et al., 2012; Suram et al., 2013; Swidergall et al., 2018). To verify that EphA2 is expressed by murine neutrophils, we stained BM neutrophils with an anti-EphA2 monoclonal antibody. Using flow cytometry and indirect immunofluorescence, we found that EphA2 was expressed on the surface of unstimulated murine neutrophils (Figures 3A and 3B; Figure S3). Furthermore, EphA2 clustering was observed after phagocytosis of serum-opsonized yeast-phase *C. albicans* cells (Figure 3B). EphA2 is a receptor tyrosine kinase that is autophosphorylated on specific tyrosine residues when activated. In resting neutrophils, there was a low level of EphA2 autophosphorylation (Figure 3C; Figure S3). When the neutrophils interacted with *C. albicans,* EphA2 autophosphorylation increased and remained above basal levels for at least 60 min. Thus, neutrophils express EphA2 on their surface, and the receptor is activated by interaction with *C. albicans.*

To investigate the role of EphA2 on phagocytes in host defense against OPC, we treated both wild-type and *EphA2*$^{-/-}$ mice with an anti-Gr1 monoclonal antibody to deplete neutrophils and monocytes. Antibody depletion in wild-type mice (Figure S3) increased their oral fungal burden to the same level as in neutrophil-sufficient *EphA2*$^{-/-}$ mice (Figure 3D; Figure S3). By contrast, phagocyte depletion in *EphA2*$^{-/-}$ mice did not result in a further increase in their already high oral fungal burden, but led to further body weight loss, a marker of disease severity (Figure 3E). These results suggest that innate leukocytes play a central role in the EphA2-mediated host response to OPC.

To determine whether EphA2 enhances the capacity of neutrophils to control the growth of *C. albicans* during OPC, we performed adoptive transfer experiments in which BM neutrophils from wild-type and *EphA2*$^{-/-}$ mice were injected into *CD18*$^{-/-}$ mice after they had been orally infected with *C. albicans* (Figure 3F). The neutrophils of *CD18*$^{-/-}$ mice cannot bind to intercellular adhesion molecule 1 (ICAM-1) on vascular endothelial cells and are therefore unable to migrate out of the bloodstream into infected tissues (Ding et al., 1999; Taylor et al., 2014). As expected, *CD18*$^{-/-}$ mice had more severe OPC than wild-type mice (Figure 3G). When wild-type neutrophils were adoptively transferred into the *CD18*$^{-/-}$ mice, there was a significant reduction in oral fungal burden. By contrast, transfer of *EphA2*$^{-/-}$ neutrophils into these mice did not significantly reduce their oral fungal burden. To determine whether the increased fungal burden in mice that received the *EphA2*$^{-/-}$ neutrophils was due to reduced trafficking of the cells into the infected tissue, we analyzed the accumulation of adoptively transferred neutrophils in the tongues of *CD18*$^{-/-}$ mice after 1 day of infection. We found that similar numbers of wild-type (WT) and *EphA2*$^{-/-}$ neutrophils accumulated in the tongues (Figure 3H; Figure S3), indicating that the inability of *EphA2*$^{-/-}$ neutrophils to control fungal growth during OPC is not due to a defect in migration. Collectively, these data suggest that EphA2 on neutrophils is required to limit fungal proliferation after the neutrophils have arrived at the site of infection.

EphA2 Is Required for Maximal Neutrophil Killing of Serum-Opsonized Yeast

Neutrophils interact with and kill *C. albicans* cells by different mechanisms, depending on the morphology of the organism and whether it is opsonized (Gazendam et al., 2014; Warnatsch et al., 2017). Although mice are immunologically naive to *C. albicans,* the organism can still be opsonized by anti-β-glucan antibodies induced by commensal fungi (Chiani et al., 2009; Ishibashi et al., 2005). We determined the capacity of wild-type and *EphA2*$^{-/-}$ neutrophils to kill *C. albicans* yeast and hyphae in the presence and absence of serum opsonization. Strikingly, *EphA2*$^{-/-}$ neutrophils isolated from either peripheral blood or BM were deficient in killing serum-opsonized *C. albicans* yeast (Figure 4A). However, *EphA2*$^{-/-}$ neutrophils were able to kill both unopsonized yeast and opsonized hyphae, similar to wild-type neutrophils (Figures 4B and 4C). Thus, EphA2 is required for neutrophils to kill opsonized yeast-phase *C. albicans.*

Because EphA2 is also expressed on macrophages (Finney et al., 2017; Suram et al., 2013), we tested the capacity of macrophages from *EphA2*$^{-/-}$ mice to kill opsonized *C. albicans* yeast. *EphA2*$^{-/-}$ BM-derived macrophages killed the organisms, similar to wild-type macrophages (Figure 4D), suggesting that EphA2 is dispensable for macrophage killing of *C. albicans.*

To investigate how EphA2 enhances neutrophil killing of *C. albicans,* we analyzed whether it influences the expression of other neutrophil cell surface receptors, extrinsic neutrophil priming, phagocytosis, and killing of opsonized *Staphylococcus aureus.* We found that EphA2-deficient neutrophils expressed similar levels of Dectin-1, CD11b, CD16/32, CD18, and CD64 compared with wild-type BM neutrophils (Figure S4). To determine whether EphA2 was required for extrinsic priming in which neutrophils release proinflammatory cytokines that prime other neutrophils for enhanced *C. albicans* killing (Summers et al., 2010), we incubated *C. albicans* with a mixture *EphA2*$^{-/-}$ and wild-type neutrophils and determined the extent of fungal killing. We found that adding wild-type neutrophils to *EphA2*$^{-/-}$ neutrophils did not significantly increase fungal killing (Figure 4E), suggesting that *EphA2*$^{-/-}$ neutrophils are not deficient in extrinsic priming. Furthermore, we determined that the phagocytosis of opsonized *C. albicans* yeast by *EphA2*$^{-/-}$ neutrophils was similar to that of wild-type neutrophils (Figure 4F), indicating that EphA2 is required for neutrophils to kill serum-opsonized yeast after they have been phagocytosed. Finally, *EphA2*$^{-/-}$ neutrophils were able to kill serum-opsonized *S. aureus,* similar to wild-type neutrophils (Figure 4G). Thus, the absence of EphA2 does not alter the surface expression of other neutrophil receptors or inhibit extrinsic neutrophil priming or phagocytosis. In addition, deletion of EphA2 does not result in a global defect in the capacity of neutrophils to kill opsonized microorganisms.

Hyphal cells are the predominant *C. albicans* morphotype during later time points of OPC (Solis and Filler, 2012; Solis et al., 2017). If lack of EphA2 only influences the capacity of neutrophils to kill opsonized yeast, then EphA2 deficiency should have less of an impact at later time points, when hyphae predominate and few yeast-phase organisms are present. To test this hypothesis, we orally infected wild-type and *EphA2*$^{-/-}$ mice and monitored their weight over time. We also determined their oral fungal burden after 4 days of infection. Relative to wild-type mice, *EphA2*$^{-/-}$ mice lost significantly more weight during the first 3

days of infection, but their weight returned to wild-type levels by day 4, at which time both strains of mice had essentially cleared the infection (Figures 4H and 4I). Thus, EphA2 is important for host defense against *C. albicans* during the initial phases of OPC but is dispensable after prolonged infection.

Serum-opsonized yeast are recognized by FcγRs expressed by neutrophils, monocytes, and macrophages, which trigger intracellular signaling pathways that result in the killing of phagocytosed *C. albicans* (Gazendam et al., 2016). To verify the importance of FcγRs in neutrophil killing of *C. albicans,* we blocked the FcγRs CD16, CD32, and CD64 using specific antibodies. In the absence of FcγR signaling, neither wild-type nor *EphA2*$^{-/-}$ neutrophils were able to kill serum-opsonized *C. albicans,* partly because of decreased phagocytosis (Figures 4J and 4K), thus highlighting the central role of FcγRs in mediating neutrophil killing of this organism. To determine whether early control of *C. albicans* in the oral cavity depends on FcγR signaling in phagocytes, we orally infected mice deficient in Fc-gamma receptor 1 (FcγRI; CD64). After 2 days of infection, *FcγRI*$^{-/-}$ mice had a significantly greater oral fungal burden relative to wild-type mice (Figure 4L). These results support the model that FcγR signaling is critical for controlling early fungal proliferation in the oral cavity.

The EphA2-MAPK Axis Primes p47^{phox} to Enhance Intracellular ROS Production

Killing of serum-opsonized *C. albicans* occurs when FcγRs recognize antibodies on the fungal surface, thereby activating protein kinase C (PKC) and inducing the production of ROS (Gazendam et al., 2014). To investigate the post-phagocytic mechanisms of EphA2-dependent *C. albicans* killing, we measured intracellular ROS production by neutrophils as they interacted with opsonized *C. albicans* yeast *in vitro.* Wild-type neutrophils generated a large amount of intracellular ROS in response to *C. albicans,* whereas *EphA2*$^{-/-}$ neutrophils generated significantly less ROS (Figure 5A). ROS is produced by the NADPH oxidase enzyme complex, which is composed of two transmembrane proteins (p22^{phox} and gp91^{phox}) and four cytosolic proteins (p47^{phox}, p67^{phox}, p40^{phox}, and the Rac guanosine triphosphatases [GTPases]) that assemble at membrane sites upon cell activation (El-Benna et al., 2009). By indirect immunofluorescence, we analyzed the localization of p47^{phox} in wild-type and *EphA2*$^{-/-}$ neutrophils. We found that there was reduced accumulation of p47^{phox} around the phagosomes of *EphA2*$^{-/-}$ neutrophils that had ingested serum-opsonized *C. albicans* yeast (Figures 5B and 5C). By contrast, the localization of membrane-bound gp91^{phox} in the *EphA2*$^{-/-}$ neutrophils was similar to that of wild-type cells (Figure S5).

p47^{phox} is activated on selective sites by several protein kinases, such as PKC, protein kinase A (PKA), and the MAPK ERK, which in turn leads to localization of the protein to the phagocyte membrane (El Benna et al., 1996, 2009; Fontayne et al., 2002). In oral epithelial cells, fungal recognition by EphA2 activates MEK½, an upstream element of ERK signaling (Roberts and Der, 2007; Swidergall et al., 2018). We therefore investigated the extent of activation of MEK½, and ERK½ in neutrophils in response to serum-opsonized *C. albicans* yeast and found that the fungus strongly induced MEK and ERK phosphorylation in wild-type neutrophils, but not *EphA2*$^{-/-}$ neutrophils (Figure 5D; Figure S5). The spleen tyrosine kinase (SYK) is essential for FcγR signaling in macrophages and neutrophils (Kiefer et al.,

1998). PKC-δ, a known downstream target of FcγR signaling, activates both ERK and p47^{phox} (Bey et al., 2004; Gazendam et al., 2014; Limnander et al., 2011; Ueda et al., 1996). Therefore, we tested whether EphA2 is required for activation of SYK and PKC-δ in response to serum-opsonized *C. albicans*. Exposure to *C. albicans* induced the phosphorylation of SYK and PKC-δ in both wild-type and *EphA2*$^{-/-}$ neutrophils (Figure 5E; Figure S5), suggesting that EphA2 influences the activity of p47^{phox} independent of the SYK-PKC axis. PKC can be activated via a receptor-independent mechanism using phorbol myristate acetate (PMA) to induce ERK activation and subsequent ROS production (Karlsson et al., 2000). To verify that ERK can be activated and ROS can be produced in the absence of EphA2, we stimulated wild-type and *EphA2*$^{-/-}$ neutrophils with PMA and found that the magnitude of ERK phosphorylation in *EphA2*$^{-/-}$ neutrophils was similar to that in wild-type cells (Figure 5F; Figure S5). Moreover, addition of PMA to both wild-type and *EphA2*$^{-/-}$ neutrophils induced similar amounts of intracellular ROS accumulation (Figure 5G). Collectively, these results indicate that EphA2 signals in parallel to PKC through MEK½ to enhance ERK activation. Activation of ERK is required for neutrophils to respond to serum-opsonized *C. albicans*, because inhibition of ERK abolished neutrophil killing of these organisms (Figure 5H).

Neutrophils can be primed by host factors, as well as microbial products such as lipopolysaccharide (LPS), for enhanced antimicrobial responses (Dang et al., 1999; Forehand et al., 1989). ERK½ primes the neutrophil oxidative burst by phosphorylating p47^{phox} at Ser345 (Dang et al., 2006). Immunoblot analysis showed that although exposure to opsonized *C. albicans* yeast induced phosphorylation of the p47^{phox} Ser345 priming site in wild-type neutrophils, this response was markedly reduced in *EphA2*$^{-/-}$ neutrophils (Figure 5I; Figure S5). By contrast, phosphorylation of Ser304, which is required for NADPH oxidase activation (El-Benna et al., 2009), was not diminished in the absence of EphA2 (Figure S5). These data indicate that the impaired capacity of *EphA2*$^{-/-}$ neutrophils to kill serum-opsonized *C. albicans* is due to failure of p47^{phox} priming, which results in reduced production of intracellular ROS.

On oral epithelial cells, EphA2 specifically senses surface-exposed β-glucan on fungi (Swidergall et al., 2018). Next, we determined whether β-glucan, the fungal ligand of EphA2, is able to induce priming of p47^{phox} in murine neutrophils. Immunoglobulin G (IgG)-coated Sepharose beads were not able to induce phosphorylation Ser345, while zymosan, the particulate form of β-glucan, strongly activated phosphorylation of this residue, both by itself and especially in combination with IgG beads (Figure 5J; Figure S5). Collectively, these results support the model that β-glucan recognition via EphA2 is required for maximal activation of the MEK-ERK signaling pathway, which in turn primes p47^{phox}, leading to enhanced intracellular ROS production and oxidant-mediated killing of opsonized *C. albicans* yeast.

DISCUSSION

Neutrophils play a key role in host defense against OPC (Altmeier et al., 2016; Huppler et al., 2014). *In vitro,* neutrophils kill *C. albicans* by two distinct mechanisms, depending on whether the organism has been opsonized (Gazendam et al., 2014). Phagolysosomal killing

of unopsonized *C. albicans* depends on CR3 recognition and signaling via SYK, phosphatidylinositol 3-kinase (PI3K), and caspase recruitment domain-containing protein 9 (CARD9) but is independent of NADPH oxidase activity. By contrast, phagolysosomal killing of serum-opsonized *C. albicans* depends on the FcγRs, PKC, and ROS generated by the NADPH oxidase system. Our studies reveal that EphA2 not only is required for recruitment of phagocytes to foci of oral infection but also is a critical enhancer of the neutrophil defense against OPC by priming $p47^{phox}$ to boost FcγR-mediated ROS production and subsequent killing of phagocytosed serum-opsonized yeast.

When *C. albicans* proliferates on and invades oral epithelial cells, it activates distinct host signaling pathways that lead to an antifungal response consisting of production of antimicrobial peptides and secretion of chemokines and proinflammatory cytokines (Conti et al., 2016; Moyes et al., 2010; Swidergall et al., 2018). We have reported that exposed β-glucan on the *C. albicans* surface binds to EphA2 on oral epithelial cells, stimulating these cells to mount an antifungal response (Swidergall et al., 2018). Our current finding that $EphA2^{-/-}$ mice, and $EphA2^{-/-}$ mice reconstituted with wild-type BM, had delayed accumulation of neutrophils and inflammatory monocytes in the oropharynx during OPC indicates that EphA2 signaling is required for the early recruitment of these phagocytes. This defect in phagocyte recruitment was likely due to the absence of chemotactic signals produced by the stromal cells. It was not due to defective chemotaxis of $EphA2^{-/-}$ phagocytes, because neutrophils from $EphA2^{-/-}$ mice accumulated in the oral cavity, similar to wild-type neutrophils during OPC when they were adoptively transferred into $CD18^{-/-}$ mice and when wild-type mice were reconstituted with $EphA2^{-/-}$ BM.

Two lines of data indicate that EphA2 is central to the capacity of neutrophils to prevent *C. albicans* overgrowth during OPC. First, the oral fungal burden of $EphA2^{-/-}$ mice was similar to that of mice depleted of phagocytes. Second, the adoptive transfer of $EphA2^{-/-}$ neutrophils into $CD18^{-/-}$ mice did not reduce their oral fungal burden. Our *in vitro* studies demonstrated that although $EphA2^{-/-}$ neutrophils had reduced capacity to kill opsonized yeast-phase *C. albicans,* they retained the ability to kill both opsonized hyphae and unopsonized yeast. This finding suggests that during OPC, opsonized yeast-phase organisms are present, at least during the initial stages of infection. These organisms are likely opsonized by either secretory immunoglobulin A (sIgA) or IgG, the two principal antibody classes present in the saliva in the oral cavity (Brandtzaeg, 2013).

Our data show that in neutrophils, EphA2 signaling plays a key role in augmenting FcγR-mediated antifungal activity. We found that FcγR is required for neutrophils to kill *C. albicans in vitro* and that mice lacking the FcγRI were also highly susceptible to OPC. These results are consistent with those of Gazendam and colleagues, who found that blocking FcγR greatly impaired the capacity of neutrophils to inhibit the growth of *C. albicans in vitro* (Gazendam et al., 2014). Here we show that the early neutrophilic antifungal responses in the oral cavity depend on FcγR signaling. When serum-opsonized *C. albicans* yeast interacted with FcγR in $EphA2^{-/-}$ neutrophils, there was virtually no phosphorylation of MEK½, reduced phosphorylation of ERK½, and absent phosphorylation of $p47^{phox}$ on Ser345. As a result, $p47^{phox}$ was not primed, leading to reduced intracellular ROS accumulation and decreased *C. albicans* killing. Thus, EphA2 functions in parallel to

FcγR to enhance ERK½ phosphorylation, which augments $p47^{phox}$ priming, leading to increased intracellular ROS production and neutrophil killing of opsonized *C. albicans* yeast. In epithelial cells, EphA2 binds to and is activated by fungal β-glucans, leading to enhanced secretion of proinflammatory cytokines (Swidergall et al., 2018). Our current data show that in neutrophils, EphA2 recognition of fungal β-glucans is necessary for $p47^{phox}$ priming and production of fungicidal levels of ROS in response to FcγR ligation.

The finding that *EphA2*$^{-/-}$ neutrophils could still kill opsonized *S. aureus* suggests that $p47^{phox}$ can also be primed by an EphA2-independent pathway. Extracellular host factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor alpha (TNF-α), can also prime $p47^{phox}$ and enhance intracellular ROS production (Dang et al., 2006). How EphA2 interacts with these host factors to prime neutrophils for enhanced microbicidal activity is unknown and a topic of future investigation.

Although we found that the EphA2-FcγR-MAPK pathway is required for neutrophils to protect against OPC, studies of CARD9 knockout mice and humans with CARD9 deficiency suggest that the CR3-SYK-CARD9 pathway is dispensable for host defense against this mucosal infection. Both mice and humans with CARD9 deficiency are resistant to OPC (Bishu et al., 2014; Drewniak et al., 2013; Drummond et al., 2018). However, CARD9 is required for host defense against disseminated candidal infections, particularly in the brain (Drummond et al., 2015). These results indicate that distinct sets of neutrophil receptors recognize *C. albicans* at different anatomic sites.

Although EphA2 was required for maximal neutrophil killing of opsonized *C. albicans* yeast, it was dispensable for the killing of opsonized hyphae. When neutrophils ingest yeast, the organisms are contained within the phagolysosome and trigger intracellular ROS production. However, when neutrophils encounter larger organisms, such as hyphae, that are too large to be contained within the phagolysosome, ROS is produced extracellularly, amplifying IL-1β expression and leading to neutrophil swarming (Warnatsch et al., 2017). In *C. albicans* yeast, β-glucans are exposed at the sites of bud scars, whereas in hyphae, β-glucans are largely masked by mannans (Gantner et al., 2005). The lack of β-glucan exposure by hyphae provides a potential explanation for why *EphA2*$^{-/-}$ neutrophils retained wild-type capacity to kill this *C. albicans* morphotype. Collectively, these results reinforce the concept that neutrophils use different receptors to sense and respond to different growth forms of *C. albicans* in different niches within the host.

Because EphA2 recognizes β-glucans on pathogenic fungi other than *C. albicans*, such as *Aspergillus fumigatus* and *Rhizopus oryzae* (Swidergall et al., 2018), it is probable that it enhances the neutrophil response to these organisms too. Moreover, EphA2 has been found to recognize *Plasmodium, Chlamydia trachomatis,* and several viruses (Chen et al., 2018; Kaushansky et al., 2015; Lupberger et al., 2011; Subbarayal et al., 2015; TerBush et al., 2018; Zhang et al., 2018). However, the studies with these organisms were performed using normally nonphagocytic host cells, such as hepatocytes or epithelial cells. Our discovery that EphA2 enhances FcγR-mediated activity during *C. albicans* infection suggests that this protein may also mediate the response of myeloid cells to other microbial pathogens.

STAR★METHODS

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Scott G. Filler (sfiller@ucla.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

For *in vivo* animal studies, age-and sex matched mice were used. Animals were bred/housed under pathogen-free conditions at the Los Angeles Biomedical Research Institute, and UC Irvine. For strain specific information please see section "Mouse Model of Oropharyngeal Candidiasis" and "Generation of BM chimeric mice." In all of the mouse studies, the animals were randomly assigned to the different treatment groups. The researchers were not blinded to the experimental groups because the primary endpoint (oral fungal burden) was an objective measure of disease severity.

Ethics Statement—All animal work was approved by the Institutional Animal Care and Use Committee (IACUC) of the Los Angeles Biomedical Research Institute and UC Irvine.

Strains—*C. albicans* SC5314 (Fonzi and Irwin, 1993), a GFP expressing strain (Levitin et al., 2007), and *S. aureus* RN6390 (Kahl et al., 2000) were used. For use in the experiments, the *C. albicans* cells were grown for 18 h in yeast extract-peptone dextrose (YPD) at 30°C and the *S. aureus* cells were grown in brain heart infusion broth at 37°C. The next day, the cells were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS), and counted using a hemacytometer (C. *albicans*) or a spectrophotometer (*S. aureus)*.

METHOD DETAILS

Mouse Model of Oropharyngeal Candidiasis—*EphA2*$^{-/-}$ (B6.129S6-Epha2^{tm1Jrui}/J) mice were provided by A. Wayne Orr, LSU Health Sciences Center, Shreveport, LA (Finney et al., 2017), and *CD18*$^{-/-}$ (B6.129S7-Itgb2^{tm1Bay}/J) mice were purchased from The Jackson Laboratory. Deficient mice were bred under pathogen-free conditions at the Los Angeles Biomedical Research Institute and UC Irvine. C57BL/6 control mice were obtained from The Jackson Laboratory. The *EphA2*$^{-/-}$ and C57BL/6 control mice were cohoused for at least 1 week before the experiments. Male 7 week old Fcer1g (*Fc*γ*RI*$^{-/-}$) mice and age-and sex matched C57BL/6 mice were purchased from Taconics. OPC was induced in all strains of mice as described previously (Solis and Filler, 2012; Solis et al., 2017). For inoculation, the animals were sedated, and a swab saturated with 2×10^7 *C. albicans* cells was placed sublingually for 75 min. Mice were sacrificed after 1, 2 and 4 days of infection, respectively. The tongues were harvested, weighed, homogenized and quantitatively cultured. For histopathologic analysis, thin sections of paraffin-embedded tongues were stained with periodic acid-Schiff stain (PAS). The researchers were not blinded to the experimental groups because the primary endpoint (oral fungal burden) was an objective measure of disease severity.

Generation of BM chimeric mice—Bone marrow chimeric mice were generated as previously described (Lionakis et al., 2013). Briefly, for BM cell transfers, femurs and tibias

from 6- to 8-week-old donor wild-type (*EphA2*$^{+/+}$; CD45.1; or CD45.2) and *EphA2*$^{-/-}$ (CD45.2) mice were removed aseptically and BM was flushed using cold PBS supplemented with 2 mM EDTA. Six week-old recipient wild-type (CD45.1) and *EphA2*$^{-/-}$ (CD45.2) mice were irradiated with 9 Gy and were reconstituted 6 hours after irradiation with 2.5×10^6 *EphA2*$^{+/+}$ CD45.2 (WT→WT), *EphA2*$^{+/+}$ CD45.1 (wild-type→*EphA2*$^{-/-}$), or *EphA2*$^{-/-}$ CD45.2 (*EphA2*$^{-/-}$→wild-type CD45.1 or *EphA2*$^{-/-}$) BM cells by lateral tail-vein injection. Mice were given enrofloxacin (Victor Medical) in the drinking water for the first 4 weeks of reconstitution before being switched to antibiotic-free water. Chimeras were infected with *C. albicans* 8 weeks after transplantation. Prior to infection, we confirmed that mice reconstituted with congenic BM stem cells had achieved a satisfactory level of chimerism by assessing the number of CD45.1 and CD45.2 leukocytes in the blood, using flow cytometry (Figure S2).

Cytokine and Chemokine Measurements *In Vivo*—To determine the whole tongue cytokine and chemokine protein concentrations, the mice were orally infected with *C. albicans.* The mice were sacrificed after one day post-infection, and their tongues were harvested, weighed and homogenized. The homogenates were cleared by centrifugation as previously described (Break et al., 2015; Swidergall et al., 2018) and the concentration of inflammatory mediators was measured using a multiplex bead array assay (R&D Systems).

Flow Cytometry of Infiltrating Leukocytes—Immune cells in the mouse tongues were characterized as described elsewhere (Sparber and LeibundGut-Landmann, 2017). Briefly, mice were orally infected with *C. albicans* strain SC5314. After 1 and 2 days of infection, the animals were administered a sub lethal anesthetic mix intraperitoneally. The thorax was opened, and a part of the rib cage removed to gain access to the heart. The vena cava was cut open and the blood was washed out by slowly injecting 10 mL PBS into the right ventricle. The tongue was harvested and cut into small pieces in 100 μL of ice-cold PBS. 1 mL digestion mix (4.8 mg/ml Collagenase IV; Worthington Biochem, and 200 μg/ml DNase I; Roche Diagnostics, in 1x PBS) was added after which the tissue was incubated at 37°C for 45 min. The resulting tissue suspension was then passed through a 100 μm cell strainer. The single-cell suspensions were then incubated with rat anti-mouse CD16/32 (2.4G2; BD Biosciences) for 10 minutes (1:100) in FACS buffer at 4°C to block Fc receptors. For staining of surface antigens, cells were incubated with fluorochrome-conjugated (FITC, PE, PE-Cy7, allophycocyanin [APC], APC-eFluor 780,) antibodies against mouse CD45 (30-F11; BD Biosciences), Ly6C (AL-21; BD Biosciences), Ly6G (1A8, BioLegend), CD11b (M1/70; eBioscience), and CD90.2 (30-H12; BioLegend). After washing with FACS buffer, the cell suspension was stained with a LIVE/DEAD fluorescent dye (7-AAD; BD Biosciences) for 10 minutes (1:500). The stained cells were analyzed on a 2-laser LSRII flow cytometer (BD Biosciences), and the data were analyzed using FACS Diva (BD Biosciences) and FlowJo software (Treestar). Only single cells were analyzed, and cell numbers were quantified using PE-conjugated fluorescent counting beads (Spherotech).

Antibody Depletion—Mice were injected intraperitoneally with 80 μg anti-Gr-1 (RB6–8C5, eBiosciences) or isotype control (BioXcell) monoclonal antibodies 24 h prior to inoculation with *C. albicans* (Day 0). The degree of depletion was assessed in peripheral

blood that was obtained by cardiac puncture. The percentage of neutrophils was determined by enumeration of a blood smear stained with modified Giemsa stain solution (Sigma-Aldrich), counting 100–200 cells per sample. The absolute neutrophil count (ANC) was determined by multiplying the percentage of neutrophils (mature and band forms) by total leukocyte counts, which were determined by counting tryptophan blue stained cells using a hemacytometer.

Neutrophil Isolation from Mouse Bone Marrow—Mouse neutrophils were purified from bone marrow cells using negative magnetic bead selection (MojoSort, BioLegend). In brief, bone marrow cells were flushed from femurs and tibias using sterile RPMI 1640 medium supplemented with 10% FBS and 2 mM EDTA onto a 50 mL screw top Falcon tube fitted with a 100 μm filter (Swamydas et al., 2015). Cells were washed with 1X MojoSort buffer (1X PBS, 0.5% BSA, 2mM EDTA). Neutrophils were isolated according to the manufacturer's instructions. Bone marrow-enriched neutrophils had > 90% purity and > 90% viability as determined by flow cytometry (*in vitro* experiments). In the adoptive transfer experiments, the purity exceeded 94%.The remaining ~10% of cells were identified as $CD45^+$ $CD19^+$ (B cells) < 2.72%, $CD45^+$ $CD3^+$ (T cells) < 0.24%, $CD45^+$ $Ly6C^{hi}$ (inflammatory monocytes) < 0.31%, $CD45^+$ MHC II^+ (dendritic cells) < 2.37%, and $CD45^-$ (stromal cells) < 4.00%.

Neutrophil Isolation from Mouse Peripheral Blood—Peripheral blood was collected via cardiac puncture, anticoagulated with EDTA, and then layered over Ficoll-Histopaque 1077 (Sigma Aldrich). After centrifugation (4°C, 1500 rpm) the supernatant was discarded, and red blood cells were lysed using ACK lysis buffer (GIBCO). After 5 min cold PBS was added and cells were washed twice. The isolated neutrophils were resuspended in RPMI 1640 medium + 2% inactivated mouse serum (Gemini Bio-Products).

Adoptive Transfer of Neutrophils—Adoptive transfer of neutrophils was performed as described elsewhere (Leal et al., 2012). Briefly, male and female *CD18*$^{-/-}$ mice aged 6–12 weeks were orally infected as described above. After 6 hours post infection 5×10^6 naive donor bone marrow neutrophils from *EphA2*$^{-/-}$ and C57BL/6 (wild-type) mice were injected into the tail vein of $CD18^{-/-}$ recipient mice. After 1 and 2 days of infection, mice were euthanized, and the oral fungal burden was determined.

Surface Staining of Neutrophil Receptors—Mouse neutrophils were isolated as described above. Purified neutrophils were incubated with anti-mouse CD16/32 (2.4G2; BD Biosciences) blocking antibody, followed by anti-mEphA2 (PE conjugated; FAB639P; R&D Systems), anti-Dectin-1 (PE conjugated; Clone RH1; BioLegend), anti-CD11b (PE conjugated; Clone M1/70; BioLegend), anti-CD18 (PE conjugated; Clone M18/2; BioLegend), anti-CD64 (PE conjugated; Clone X54–5/7.1; BioLegend), and surface expression was measured using flow cytometry. For CD16/32 staining neutrophils were stained with anti-CD16/32 (PE conjugated; Clone 93; BioLegend) without Fc-block.

Neutrophil Killing Assays—Neutrophils from mice were isolated as described above. Neutrophil killing of *C. albicans* yeast was determined by CFU enumeration. Briefly, unopsonized or serum-opsonized (2% heat-inactivated mouse serum; Gemini Bio-Products)

C. albicans SC5314 yeast cells were incubated with isolated neutrophils in a 1:20 ratio for 3 hours. Neutrophils were lysed with 0.02% Triton X-100 in ice-cold water for 5 minutes, diluted and remaining *Candida* cells were quantitatively cultured. In some experiments, Fcγ receptors were blocked using antibodies against CD16/32 (2.4G2; BD Biosciences), and CD64 (clone X54–5/7.1; BioLegend). To determine the effect of ERK inhibition during killing BM-neutrophils were incubated with 1 μM SCH772984 (Cayman Chemical Company) for 45 min prior to infection. The capacity of neutrophils to kill serum-opsonized *S. aureus* RN6390 was also determined by quantitative culture using a *S. aureus* to neutrophil ratio of 10:1 and an incubation time of 30 minutes. The capacity of neutrophils to kill *C. albicans* hyphae was determined using the alamarBlue (Invitrogen) reduction as a measure of fungal inactivation. Neutrophils were incubated in duplicate wells of flat bottom 96-well plates containing hyphae that had been grown for 3 hours with serum opsonization, at a neutrophil to *C. albicans* ratio of 1:4 at 37°C. After 2.5 hours, the neutrophils were lysed with 0.02% Triton X-100 in water for 5 minutes, after which the *C. albicans* hyphae were washed twice with PBS and incubated with 1 × alamarBlue (Invitrogen) for 18 hours at 37°C. Optical density at a wavelength of 570nm and 600nm was determined. Neutrophil killing capacity was calculated as the amount of alamarBlue reduced by wells containing *C. albicans* hyphae incubated with and without neutrophils.

BM-derived macrophages—BM-derived macrophages were generated by culturing BM cells obtained as described above from wild-type and *EphA2*$^{-/-}$ mice in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine (Mediatech), 20 mM HEPES, 1% penicillin/streptomycin and 40 ng/ml M-CSF (Peprotech Inc.) for 5 to 7 days.

Neutrophil Phagocytosis—For phagocytosis assays, 5×10^5 neutrophils from wild-type or *EphA2*$^{-/-}$ mice were incubated with 5×10^5 serum-opsonized *C. albicans* yeast cells for 30 minutes at 37°C on fibronectin-coated circular glass coverslips in 24-well tissue culture plates. Phagocytosed yeast cells were quantified using a differential fluorescence assay as outlined elsewhere (Liu et al., 2016; Phan et al., 2007). Briefly, 5×10^5 BM neutrophils were incubated with 5×10^5 *C. albicans* expressing GFP. After 30 min cells were fixed using 3% paraformaldehyde for 10 min. Coverslips were washed 3x in PBS and incubated for 30 min with anti-Candida Alexa 568 Ab. The coverslips were viewed with an epifluorescence microscope, and the number of phagocytosed organisms per high-power field was determined, counting at least 100 organisms per coverslip. Phagocytosed *C. albicans* appeared green (GFP), while non-phagocytosed fungal cells were both green (GFP) and red (anti-Candida Alexa 568).

Indirect Immunofluorescence—To determine protein localization within the neutrophils, the cells were incubated in RPMI 1640 medium containing 2% heat-inactivated mouse serum for 30 min on fibronectin coated coverslips. The cells were then incubated with opsonized *C. albicans* CAI4-GFP for 30 min. Next, they were fixed with 3% paraformaldehyde, blocked with 10% BSA, followed by anti-mouse CD16/32 (2.4G2; BD Biosciences), and incubated with antibodies against EphA2 (D4A2, #6997; Cell Signaling), and p47^{phox} (A-7; Santa Cruz), followed by an Alexa 488-conjugated mouse anti-rabbit

antibody. To visualize F-actin, the cells were also stained with Phalloidin-IFluor 405 (Abcam). The cells were then imaged by confocal microscopy.

Immunoblotting—Neutrophils were incubated with opsonized *C. albicans* at a multiplicity of infection of 5 or with 10 μg/mL IgG Sepharose beads (#3420; Cell Signaling) and/or 10 μg/mL zymosan (depleted, tlrl-zyd; InvivoGen). At various time points, the cells were washed with cold PBS, collected by centrifugation, and boiled in sample buffer. The lysates were separated by SDS-PAGE, and phosphorylation was detected by immunoblotting with specific antibodies against pEphA2 (#6347, Cell Signaling), pSer345 $p47^{phox}$ (orb126026; biorbyt), pSer304 $p47^{phox}$ (PA5–36773; Thermo Fisher), pp44–42 (#4370; Cell Signaling), pPKC (#2055; Cell Signaling). Next, the blot was stripped, and the total amount of each protein was detected by immunoblotting with antibodies against EphA2 (D4A2, Cell Signaling, or clone 233720; R&D Systems), $p47^{phox}$ (A-7; Santa Cruz), p44/42 (#3085R; BioVision), and PKC (#9616; Cell Signaling). Each experiment was performed at least 3 times.

QUANTIFICATION AND STATISTICAL ANALYSIS

At least three biological replicates were performed for all *in vitro* experiments unless otherwise indicated. Data were compared by Mann-Whitney corrected for multiple comparisons using GraphPad Prism (v. 8) software. P values < 0.05 were considered statistically significant.

DATA AND CODE AVAILABILITY

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- EphA2 is a receptor for β-glucans on oral epithelial cells and neutrophils
- Epithelial cell EphA2 recruits phagocytes to foci of oral *C. albicans* infection
- Neutrophil EphA2 primes $p47^{phox}$ to enhance production of reactive oxygen species
- Neutrophil EphA2 augments killing of opsonized *C. albicans* yeast

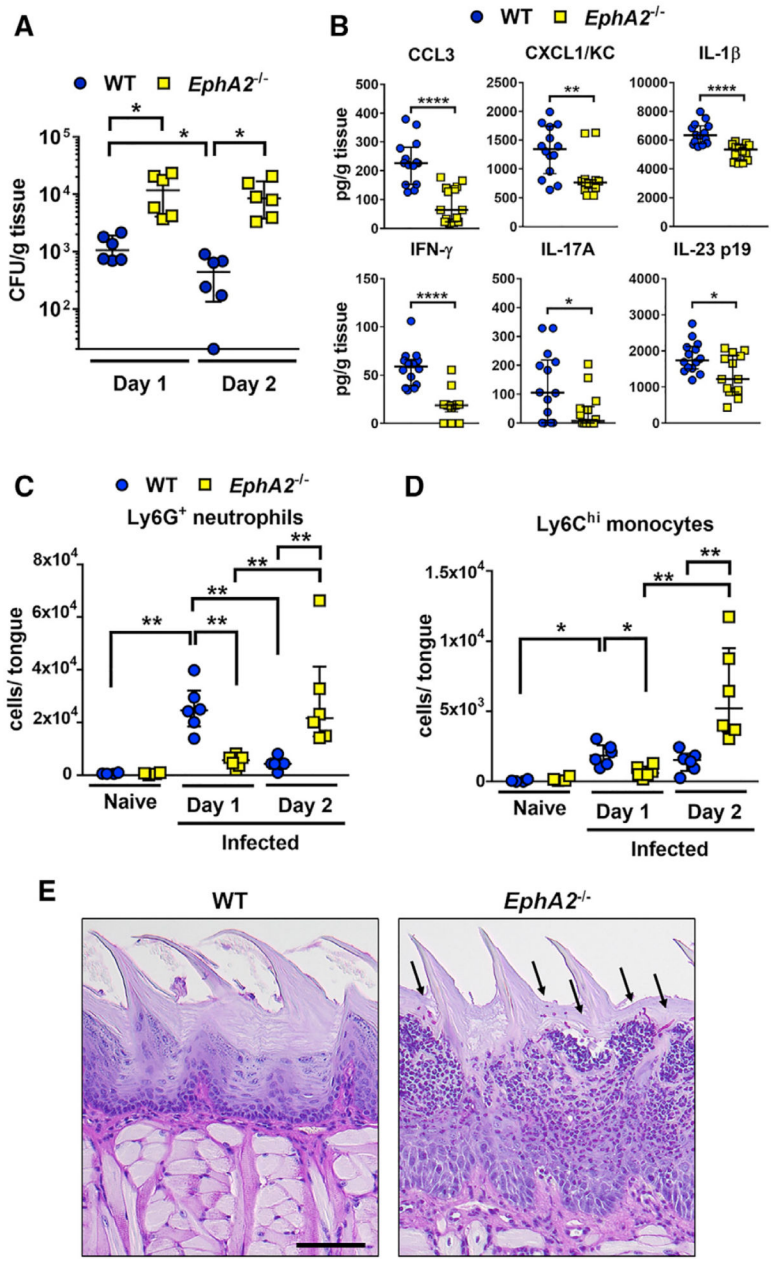

Figure 1. EphA2 Signaling Orchestrates Early Phagocyte Recruitment during OPC

(A) Oral fungal burden of immunocompetent wild-type and *EphA2*$^{-/-}$ mice after 1 and 2 days of OPC. Results are median ± interquartile range of six mice per group from two independent experiments. *$p < 0.05$ (Mann-Whitney test corrected for multiple comparisons). The y axis is set at the limit of detection (20 colony-forming units [CFUs] per gram of tissue).

(B) Level of chemokines and cytokines in the tongue homogenates of immunocompetent wild-type and *EphA2*$^{-/-}$ mice with OPC after 1 day of infection. Scatterplots show median and interquartile range of seven mice in each group, tested in duplicate in two independent experiments. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ (Mann-Whitney test corrected for multiple comparisons).

(C and D) Myeloid phagocyte infiltration in tongues of immunocompetent wild-type and *EphA2*$^{-/-}$ mice after 1 and 2 days of infection (n = 6 for infected mice, n = 4 for naive mice). Results are median ± interquartile range from combined results of two independent experiments. *p < 0.05, **p < 0.01 (Mann-Whitney test corrected for multiple comparisons). See Figure S1 for the gating strategy during flow cytometric analysis.

(E) Histopathology of the tongue of wild-type and *EphA2*$^{-/-}$ mice with OPC after 2 days of infection. Scale bar, 100 μm. Results are representative of 2 mice from the same experiment. Arrows indicate the *C. albicans* cells.

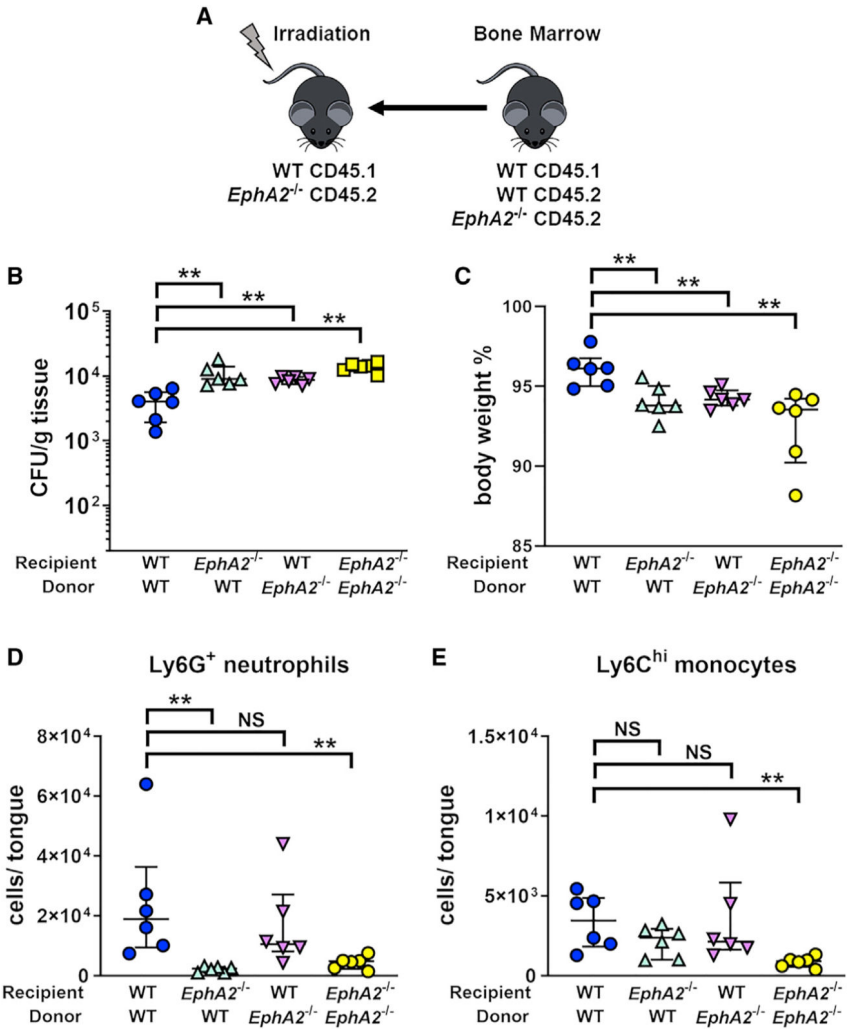

Figure 2. EphA2 in Hematopoietic and Nonhematopoietic Cells Contributes to Resistance against Acute OPC

(A) Scheme of bone marrow chimeric mice.

(B-E) Oral fungal burden (B), body weight (C), and innate phagocyte recruitment (D and E) after 1 day of oral *C. albicans* infection in the BM chimeras. Results are median ± interquartile range of six mice per group from a single experiment. **p < 0.01, NS, not significant (Mann-Whitney test corrected for multiple comparisons).

See also Figure S2.

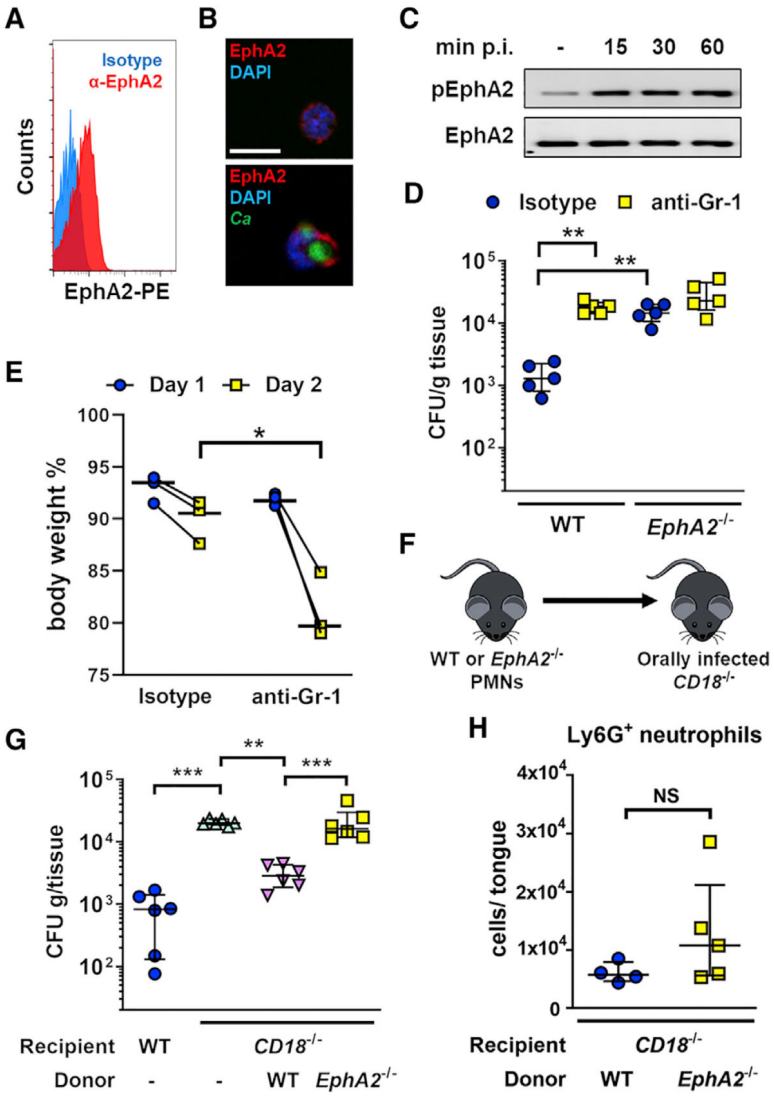

Figure 3. *EphA2*$^{-/-}$ Neutrophils Have Decreased Capacity to Limit Fungal Proliferation *In Vivo*

(A) Flow cytometry of EphA2 surface expression on wild-type neutrophils. Histograms of 5 × 10^5 events are shown.

(B) Wild-type neutrophils were either uninfected (top panel) or infected for 30 min with GFP-expressing *C. albicans* (bottom panel) and then stained with anti-mouse EphA2 (mEphA2) antibody (Ab) (red) and DAPI (blue). Scale bar, 10 μm.

(C) Immunoblot analysis showing the time course of EphA2 phosphorylation in wild-type neutrophils that had been infected with yeast-phase *C. albicans* SC5314 for the indicated time points.

(D) Oral fungal burden of wild-type and *EphA2*$^{-/-}$ mice that had been treated with either isotype control or anti-Gr-1 antibody and then orally infected with *C. albicans* for 2 days. Results are median ± interquartile range of five mice per group from two independent experiments. **p < 0.01 (Mann-Whitney test corrected for multiple comparisons). The y axis is set at the limit of detection.

(E) Body weight of *EphA2*$^{-/-}$ mice that had been treated with either isotype control or anti-Gr-1 antibody and then orally infected with *C. albicans* for 2 days. Results show median of three mice per group from two independent experiments. *$p < 0.05$ (Mann-Whitney test).
(F) Scheme of neutrophil adoptive transfer of orally infected *CD18*$^{-/-}$ mice. PMNs, polymorphonuclear neutrophils.
(G) Oral fungal burden 2 days post-infection of immunocompetent wild-type and *CD18*$^{-/-}$ mice after adoptive transfer of wild-type or *EphA2*$^{-/-}$ neutrophils. Results are median ± interquartile range of six mice per group from two independent experiments. **$p < 0.01$, ***$p < 0.001$ (Mann-Whitney test corrected for multiple comparisons). The y axis is set at the limit of detection (20 CFUs per gram of tissue).
(H) Neutrophil counts after 1 day of infection in the tongues of *CD18*$^{-/-}$ mice receiving wild-type or *EphA2*$^{-/-}$ neutrophils. Results are median ± interquartile in a single experiment ($n = 4–5$). NS, not significant (Mann-Whitney test).
See also Figure S3.

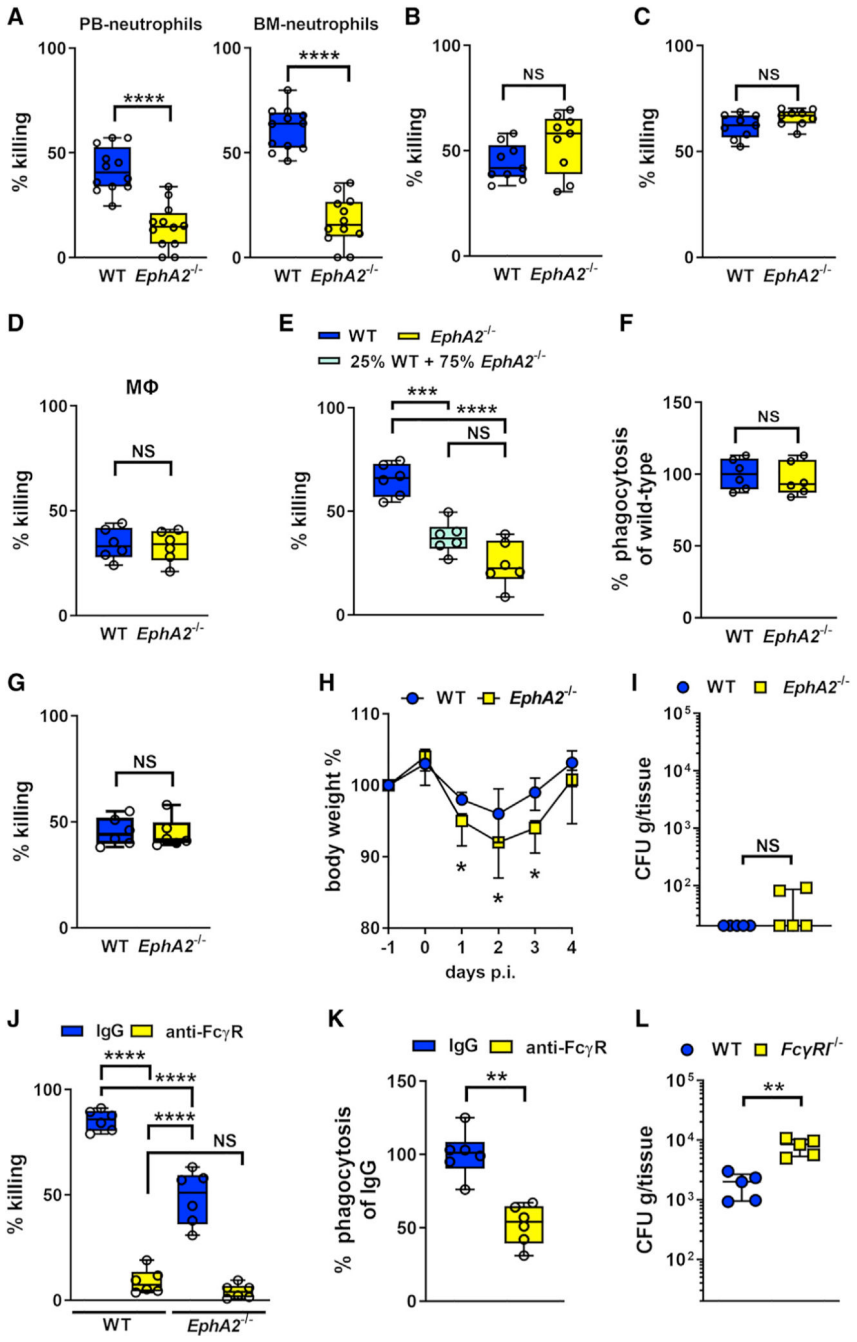

Figure 4. EphA2 Is Required for Maximal Fungal Killing of Serum-Opsonized Yeast-Phase *C. albicans*

(A) Killing of serum-opsonized yeast-phase *C. albicans* by peripheral blood (PB) and bone marrow (BM) neutrophils isolated from wild-type and *EphA2*$^{-/-}$ mice. The percentage of organisms killed was determined by colony counting. Results are means ± SD from 4 experiments, each performed in triplicate. ****$p < 0.0001$ (Mann-Whitney test).

(B) Killing of unopsonized *C. albicans* yeast by neutrophils from the indicate mouse strains. NS, not significant.

(C) Neutrophil killing of opsonized *C. albicans* hyphae.

(D) Killing of serum-opsonized yeast-phase *C. albicans* by bone marrow-derived macrophages.
(E) Killing of opsonized yeast *C. albicans* by a mixed wild-type and *EphA2*$^{-/-}$ population. Results are means ± SD from 3 independent experiments, each performed in duplicate. ***p < 0.001; ****p < 0.0001 (Mann-Whitney test corrected for multiple comparisons).
(F) Phagocytosis of opsonized *C. albicans* yeast by wild-type and *EphA2*$^{-/-}$ neutrophils. NS, not significant. Data were normalized to the phagocytosis of wild-type neutrophils.
(G) Neutrophil killing of serum-opsonized *S. aureus.*
(H) Body weight of wild-type and *EphA2*$^{-/-}$ mice after oral infection with *C. albicans.* Results are median ± interquartile range of 5 mice per strain in a single experiment. *p < 0.05 (Mann-Whitney test for each day).
(I) Oral fungal burden of wild-type and *EphA2*$^{-/-}$ mice after 4 days of OPC. Results are median ± interquartile range of five mice per group in a single experiment. **p < 0.01 (Mann-Whitney test). The y axis is set at the limit of detection (20 CFUs per gram of tissue).
(J) FcγR signaling is required for killing of serum-opsonized *C. albicans* yeast. The neutrophil FcγRs, CD16, CD32, and CD64 were blocked using specific Abs, after which the killing of serum-opsonized yeast-phase *C. albicans* was determined by colony counting. Results are means ± SD from 4 experiments in triplicate. ****p < 0.0001, NS, not significant (Mann-Whitney test).
(K) FcγR signaling is required for yeast phagocytosis. The neutrophil FcγRs, CD16, CD32, and CD64 were blocked using specific Abs, after which phagocytosis of opsonized *C. albicans* yeast by wild-type was determined. Results are means ± SD from 3 independent experiments, each performed in duplicate. **p < 0.01 (Mann- Whitney test).
(L) Oral fungal burden of wild-type and *FcyRI*$^{-/-}$ mice after 2 days of OPC. Results are median ± interquartile range of five mice per group in a single experiment. **p < 0.01 (Mann-Whitney test). The y axis is set at the limit of detection.
See also Figure S4.

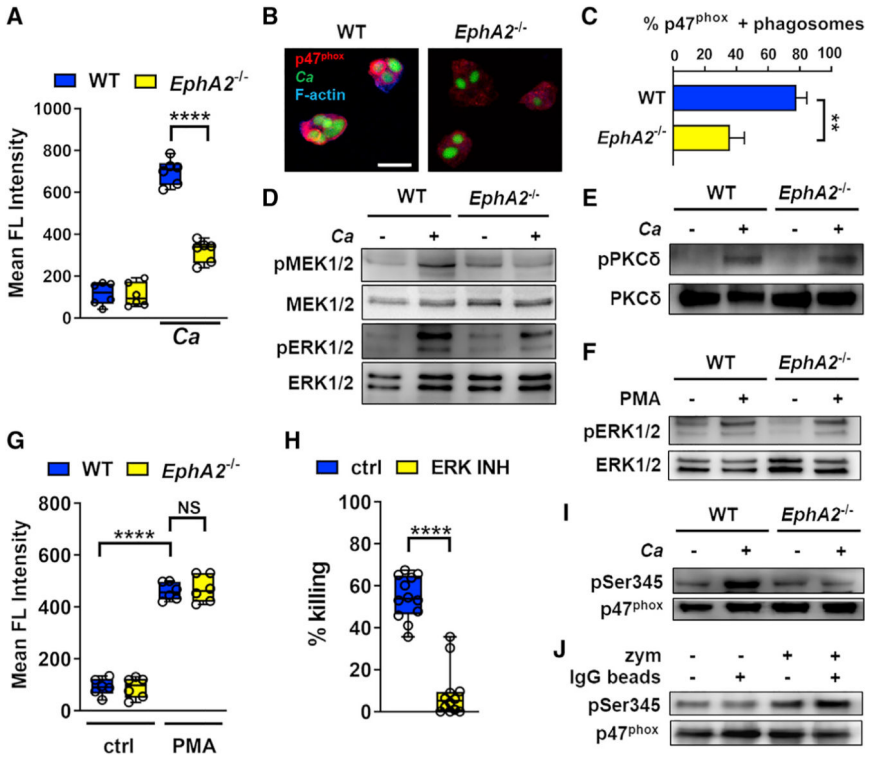

Figure 5. EphA2 Activates the MEK-ERK MAPK Module to Prime p47^{phox}

(A) Intracellular ROS accumulation measured by mean fluorescence (FL) intensity in wild-type and *EphA2*$^{-/-}$ neutrophils after45 min of infection with opsonized *C. albicans* yeast. Results are median ± interquartile range of neutrophils from 3 mice per strain, each tested in duplicate. ****p < 0.0001 (Mann-Whitney test corrected for multiple comparisons).

(B) Effects of EphA2 on localization of p47^{phox} in neutrophils infected with *C. albicans*. Wild-type and *EphA2*$^{-/-}$ neutrophils were incubated with opsonized GFP-expressing *C. albicans* yeast for 30 min, fixed, and stained for p47^{phox} (red) and F-actin (blue). Scale bar, 10 μm.

(C) Percentage of phagosomes containing *C. albicans* and surrounded by p47^{phox}. Results are median ± interquartile of 60 neutrophils per mouse strain in three independent experiments. ****p < 0.01 (Mann-Whitney test).

(D) Representative immunoblot of MEK½ and ERK½ phosphorylation in wild-type and *EphA2*$^{-/-}$ neutrophils that had been infected with yeast-phase *C. albicans* for 30 min.

(E) Representative immunoblot of PKC-δ phosphorylation in wild-type and *EphA2*$^{-/-}$ neutrophils that had been infected with yeast-phase *C. albicans* SC5314 for 30 min with a 5:1 ratio.

(F) Representative immunoblot of ERK½ phosphorylation in wild-type and *EphA2*$^{-/-}$ neutrophils stimulated with 50 nM PMA for 30 min.

(G) Intracellular ROS accumulation (mean fluorescence [FL] intensity) of wild-type and *EphA2*$^{-/-}$ neutrophil stimulated with PMA for 45 min. Results are median ± interquartile range of neutrophils from 3 mice per strain, each tested in duplicate. ****p < 0.0001 (Mann-Whitney test corrected for multiple comparisons).

(H) Effects of inhibiting ERK with the specific inhibitor SCH7729884 on the killing of *C. albicans* by bone marrow neutrophils isolated from wild-type mice. The percentage of organisms killed was determined by colony counting. Results are median ± interquartile from 4 experiments in triplicate. **p < 0.01, ****p < 0.0001 (Mann-Whitney test).
(I) Representative immunoblot analysis of Ser345 $p47^{phox}$ phosphorylation in wild-type and *EphA2*$^{-/-}$ neutrophils that had been infected with yeast-phase *C. albicans* for 30 min.
(J) β-glucan induces $p47^{phox}$ priming. Wild-type BM neutrophils were incubated with 10 μg/mL of Sepharose IgG beads and/or 10 μg/mL of zymosan (depleted) for 30 min. Representative immunoblot analysis of Ser345 $p47^{phox}$ phosphorylation.
See also Figure S5.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Mouse CD16/CD32 (Mouse BD Fc Block)	BD Biosciences	Cat# 553142 RRID:AB_394657
PE anti-mouse CD16/32 antibody	BioLegend	Cat# 101308, RRID:AB_312807
Rat Anti-CD45 Monoclonal Antibody, Allophycocyanin Conjugated, Clone 30-F11	BD Biosciences	Cat# 559864, RRID:AB_398672
Rat Anti-Ly-6C Monoclonal Antibody, FITC Conjugated, Clone AL-21	BD Biosciences	Cat# 553104 RRID: AB_394628
PE/Cy7 anti-mouse Ly-6G antibody	BioLegend	Cat# 127618, RRID:AB_1877261
CD11b Monoclonal Antibody (M1/70), APC-Cyanine7	eBioscience	Cat# A15390 RRID:AB_2534404
PE anti-mouse/human CD11b antibody	BioLegend	Cat# 101207, RRID:AB_312790
PE anti-mouse CD18 antibody	BioLegend	Cat# 101407, RRID:AB_312816
PE anti-mouse CD90.2 antibody	BioLegend	Cat# 105308, RRID:AB_313179
PE anti-mouse CD369 (Dectin-1, CLEC7A) antibody	BioLegend	Cat# 144304, RRID:AB_2561501
APC/Cyanine7 anti-mouse CD45.1 antibody	BioLegend	Cat# 110715, RRID:AB_313504
FITC anti-mouse CD45.2 antibody	BioLegend	Cat# 109805, RRID:AB_313442
Ly-6G (Gr-1) Monoclonal Antibody (RB6–8C5)	eBiosciences	Cat# 14–5931–82, RRID:AB_467730
InVivoMab rat IgG2b isotype control antibody	Bio X Cell	Cat# BE0090, RRID:AB_1107780
PE anti-mouse CD64 (FcRI) antibody	BioLegend	Cat# 139303, RRID:AB_10613467
Purified anti-mouse CD64 (FcRI) antibody	BioLegend	Cat# 139301, RRID:AB_10612757
MHC Class II (I-A/I-E) Monoclonal Antibody (M5/114.15.2), FITC	eBiosciences	Cat# 11–5321–82, RRID:AB_465232
APC anti-mouse CD19 antibody	BioLegend	Cat# 115511, RRID:AB_313646
APC anti-mouse CD3 antibody	BioLegend	Cat# 100235, RRID:AB_2561455
Mouse EphA2 Phycoerythrin mAb (Clone 233720) antibody	R&D	Cat# FAB639P, RRID:AB_2099100
EphA2 (D4A2) XP Rabbit mAb antibody	Cell Signaling Technology	Cat# 6997, RRID:AB_10827743
Phospho-EphA2 (Ser897) (D9A1) Rabbit mAb antibody	Cell Signaling Technology	Cat# 6347, RRID:AB_11220420
MEK1/2 Antibody	Cell Signaling Technology	Cat# 9122, RRID:AB_823567
Anti-MEK1 / 2, phospho (Ser217 / Ser221) Antibody, Unconjugated	Cell Signaling Technology	Cat# 9121, RRID:AB_331648
p44/42 MAPK (Erk1/2) Antibody	BioVision	Cat# 3085R-100, RRID:AB_10989057
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody	Cell Signaling Technology	Cat# 4370, RRID:AB_2315112
PKCdelta (D10E2) Rabbit mAb antibody	Cell Signaling Technology	Cat# 9616, RRID:AB_10949973
Phospho-Zap-70 (Tyr319)/Syk (Tyr352) (65E4) Rabbit mAb antibody	Cell Signaling Technology	Cat# 2717F RRID: AB_2218658
Syk (D3Z1E) XP Rabbit Antibody	Cell Signaling Technology	Cat# 13198 RRID: AB_2687924
Phospho-PKCdelta (Tyr311) Antibody	Cell Signaling Technology	Cat# 2055, RRID:AB_330876
p47 phox (phospho-Ser345) antibody	Biorbyt	orb126026
Phospho-p47phox (Ser304) Polyclonal Antibody	Thermo Fisher Scientific	Cat# PA5–36773, RRID:AB_2553720
p47-phox (A-7) antibody	Santa Cruz Biotechnology	Cat# sc-17844, RRID:AB_627987
Mouse IgG (Sepharose Bead Conjugate) antibody	Cell Signaling Technology	Cat# 3420, RRID:AB_1549744
Chemicals, Peptides, and Recombinant Proteins		

REAGENT or RESOURCE	SOURCE	IDENTIFIER
7-AAD	BD Biosciences	Cat# 559925
2% heat-inactivated mouse serum	Gemini Bio-Products	Cat#100–113
SCH772984	Cayman Chemical Company	Ca# 19166 CAS# 942183–80–4
alamarBlue	Invitrogen	Cat# DAL1025
Zymosan (depleted)	InvivoGen	Cat# tlrl-zyd Lot# DZN-40–01
Recombinant Murine M-CSF	Peprotech Inc	Cat# 315–02
H2DCFDA	Invitrogen	Cat# D399
Critical Commercial Assays		
MojoSort Mouse Neutrophil Isolation Kit	BioLegend	Cat# 480058
Luminex Mouse Magnetic Assay	R&D	Cat# LXSAMSM
Experimental Models: Organisms/Strains		
C. albicans SC5314	Fonzi and Irwin, 1993	N/A
C. albicans-GFP	Levitin et al., 2007	N/A
S. aureus RN6390	Kahl et al., 2000	N/A
Epha2^{tm1Jrui}/J	Finney et al., 2017	N/A
C57BL/6J	The Jackson Laboratory	RRID: IMSR_JAX:000664
B6.129S7-*Itgb2*^{tm1Bay}/J	The Jackson Laboratory	RRID:IMSR_JAX:002128
B6.129P2-*Fcer1g*^{tm1Rav} N12	Taconics	RRID:IMSR_TAC:583
Software and Algorithms		
FlowJo V10	Treestar	https://www.flowjo.com/
GraphPad Prism V8	GraphPad	https://www.graphpad.com/
Other		
Luminex multiplex analyzer	Luminex	https://www.luminexcorp.com
BD LSR II	BD Biosciences	https://www.bd.com
Leica TCS SP8 Confocal system	Leica	https://www.leica-microsystems.com