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Article

The β-Glucan Receptor Dectin-1 Activates the Integrin Mac-1 in Neutrophils via Vav Protein Signaling to Promote *Candida albicans* Clearance

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SUMMARY

Resistance to fungal infections is attributed to engagement of host pattern-recognition receptors, notably the β-glucan receptor Dectin-1 and the integrin Mac-1, which induce phagocytosis and antifungal immunity. However, the mechanisms by which these receptors coordinate fungal clearance are unknown. We show that upon ligand binding, Dectin-1 activates Mac-1 to also recognize fungal components, and this stepwise process is critical for neutrophil cytotoxic responses. Both Mac-1 activation and Dectin-1- and Mac-1-induced neutrophil effector functions require Vav1 and Vav3, exchange factors for RhoGTPases. Mac-1- or Vav1,3-deficient mice have increased susceptibility to systemic candidiasis that is not due to impaired neutrophil recruitment but defective intracellular killing of *C. albicans* yeast forms, and Mac-1 or Vav1,3 reconstitution in hematopoietic cells restores resistance. Our results demonstrate that antifungal immunity depends on Dectin-1-induced activation of Mac-1 functions that is coordinated by Vav proteins, a pathway that may localize cytotoxic responses of circulating neutrophils to infected tissues.

INTRODUCTION

Infections by opportunistic pathogens are becoming increasingly prevalent due to the growing use of immunosuppressive therapies and acquired immunodeficiency. Seventy five percent of invasive fungal infections are attributed to the otherwise commensal fungi *Candida albicans* (*C. albicans*) and result in 40%–50% mortality (De Rosa et al., 2009). Host resistance relies on neutrophil- and macrophage-mediated fungal recognition and uptake, generation of reactive oxygen species (ROS), and release of proteases that promote fungal killing. These functions, in addition to cytokines and chemokines produced by cells of adaptive and innate immunity keep fungal infections under control (Netea et al., 2008).

C. albicans recognition by neutrophils is attributed to the pattern recognition receptors Dectin-1, Toll-like receptors (TLR), and the CD18 integrin Mac-1 (Netea et al., 2008). Dectin-1 binds to β1,3-glucan, the most abundant polysaccharide in fungal pathogens and is required for fungal resistance in mice (Saijo et al., 2007; Taylor et al., 2007) and humans (Ferwerda et al., 2009). It promotes phagocytosis and ROS generation and produces cytokines in collaborative signaling with TLRs (Netea et al., 2008). Mac-1 (CD11b/CD18, complement receptor 3), a member of the CD18 family of integrins present on neutrophils, macrophages, and other leukocyte subsets, binds and phagocytoses complement-opsonized targets. It also binds β-glucan and mannose structures (Thornton et al., 1996), interacts with *C. albicans*, and internalizes unopsonized zymosan (Ross, 2000), a particulate β-glucan and mannan-rich yeast cell-wall extract (Di Carlo and Fiore, 1958). Unlike Dectin-1 and TLRs, Mac-1 on circulating neutrophils requires activation via "inside-out signaling" to engage its ligands (Hynes, 2002). β-glucan has been reported to bind directly to the membrane-proximal lectin domain of Mac-1 to switch its ligand-binding I domain into an active state (Vetvicka et al., 1996). The tight regulation of the activity of this integrin, known to support neutrophil recruitment and trigger cytotoxic responses, serves to localize the neutrophil's responses and avoid systemic inflammation (Hynes, 2002; Ross, 2000). The relative contribution of Mac-1 to host defense against fungal pathogens is debated. In vitro, there are competing views on the importance of Dectin-1 versus Mac-1 in fungal pathogen recognition and uptake by neutrophils (van Bruggen et al., 2009). Incongruous results on Mac-1's function in fungal clearance in vivo have also been reported (Romani et al., 2004; Soloviev et al., 2011).

Dectin-1 signaling is initiated by phosphorylation of its cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM)-like sequence. Despite recent progress in understanding molecular mechanisms regulating cytokine regulation (Kerrigan and Brown, 2010), signaling pathways responsible for uptake and intracellular killing of *C. albicans* in macrophages are largely undefined. Even less is known about these pathways in neutrophils, the major effector cells early in systemic candidiasis in humans (Ferwerda et al., 2009; Spellberg et al., 2006) and mice (Tuite et al., 2004). In neutrophils, parallels may exist between signaling through Dectin-1 and other ITAM-containing

receptors such as FcγRs, receptors for IgG. Phosphorylation of the ITAMs of FcγRs triggers the assembly of protein complexes containing Syk and Src-family tyrosine kinases. The sequence of events thereafter involves tyrosine phosphorylation of downstream targets of these kinases, including Vav, a guanine exchange factor (GEF) for Rho GTPases; phosphatidylinositol 3-kinase (PI3K); phospholipase C gamma (PLCγ), which triggers a Ca^{2+} flux; and adaptor proteins (e.g., SLP-76). These events culminate in the phosphorylation of phox components of the NADPH oxidase and cytoskeletal changes required for ROS generation and phagocytosis (Berton et al., 2005).

Here, we show that Dectin-1 induces Mac-1 activation and this is required for neutrophil cytotoxic responses, which challenges the view that Mac-1 is directly activated by zymosan (Ross, 2000). Mac-1 activation and downstream responses to zymosan required Vav1,3 proteins and their signaling pathways. The physiological importance of these players was evaluated in a murine model of systemic *C. albicans* blastoconidia infection. Mac-1 and Vav proteins in circulating hematopoietic cells were essential for fungal clearance after neutrophil recruitment, thus placing Mac-1 proximal to actual fungal clearance. We postulate that, as with G protein coupled chemokine receptors (GPCR), which trigger integrin activation through Rap GTPases to localize neutrophil recruitment to sites of inflammation (Sanchez-Madrid and Sessa, 2010), pattern recognition receptors such as Dectin-1 activate Mac-1 through Vav proteins to localize neutrophil cytotoxic responses toward pathogens in host defense.

RESULTS

Dectin-1 Activates Mac-1 in Neutrophils and Both Collaborate in Responses to Fungal Components

A readout of integrin activation is their rapid and inducible binding to ligands (Hynes, 2002). Mac-1 activation can be assayed by the inducible binding of neutrophils to red blood cells opsonized with the Mac-1 ligand complement fragment C3bi (C3-RBC) (Wright and Meyer, 1986). Murine neutrophils bound C3-RBC following treatment with phorbol myristate acetate (PMA) (Figure 1A), which globally activates integrins (Bertram and Ley, 2011). The lack of RBC rosetting in PMA-treated Mac-1-deficient ($Mac\text{-}1^{-/-}$) neutrophils validated this assay as a bonafide readout of Mac-1 activation (Figure 1A). Zymosan treatment resulted in rapid C3-RBC rosetting in murine (Figure 1B) and human neutrophils (Figure S1A). In contrast, an 80% reduction in zymosan-induced RBC rosette formation was observed in Dectin-1-deficient ($Dectin\text{-}1^{-/-}$) murine neutrophils (Figure 1B). Zymosan led to GTP-loading of Rap1GTPase (see Figure 3A), known to be required for inside-out activation of integrins such as Mac-1 (Dupuy and Caron, 2008). Together these data indicate that Dectin-1 engages intracellular signals that trigger Mac-1 activation.

The importance of Dectin-1 and Mac-1's relationship was explored in the context of phagocytosis of zymosan. Zymosan phagocytosis was reduced by >90% in both $Dectin\text{-}1^{-/-}$ (Figure 1C) (Taylor et al., 2007) and $Mac\text{-}1^{-/-}$ neutrophils (Figure 1C), while MyD88/TRIF-deficient ($MyD88/TRIF^{-/-}$) neutrophils, which lack signaling through all TLRs, exhibited no defect (Figure 1C). Phagocytosis of serum-opsonized zymosan that leads to C3 deposition, a known Mac-1 ligand, was also Mac-1 dependent (Figure S1B). Zymosan phagocytosis in $Dectin\text{-}1^{-/-}$ neutrophils was evaluated in the presence of high concentrations of Mg^{2+}. This switches the integrin to an active conformation by occupying a metal ion-coordinating site in the ligand-binding I domain and thus bypassing the need for inside-out activating signals to induce ligand binding (Humphries, 1996; Hynes, 2002). Mg^{2+} enhanced phagocytosis in wild-type cells and rescued phagocytosis of unopsonized zymosan in $Dectin\text{-}1^{-/-}$ neutrophils (Figure 1C), indicating that defective phagocytosis in $Dectin\text{-}1^{-/-}$ neutrophils is due to lack of Mac-1 activation. Mg^{2+} did not trigger zymosan phagocytosis in $Mac\text{-}1^{-/-}$ neutrophils (Figure 1C), thus showing the utility of Mg^{2+} treatment in activating Mac-1. A soluble competing β-glucan partially reduced Mg^{2+} induced zymosan phagocytosis in $Dectin\text{-}1^{-/-}$ neutrophils (Figure S1C), thus demonstrating that Mac-1's β-glucan binding activity contributes to zymosan uptake and is responsive to inside-out signaling. Other zymosan polysaccharides described as Mac-1 ligands (Thornton et al., 1996) may contribute to the remaining binding activity. Consistent with Dectin-1 being a major β-glucan receptor, zymosan recognition by neutrophils relied primarily on Dectin-1, as particle binding was markedly reduced only in $Dectin\text{-}1^{-/-}$ and not Mac-1 or $MyD88/TRIF^{-/-}$ neutrophils (Figure 1C). Although a fraction of zymosan remained bound in $Dectin\text{-}1^{-/-}$ neutrophils (Figure 1C), as also previously described (Taylor et al., 2007), this fraction was not internalized (Figure 1C). On the other hand, Mg^{2+}-induced Mac-1 activation in $Dectin\text{-}1^{-/-}$ neutrophils triggered uptake of this surface bound zymosan (Figure 1C), demonstrating that Dectin-1 and Mac-1 are the two primary phagocytic receptors on neutrophils. In contrast to results in neutrophils, zymosan phagocytosis by peritoneal macrophages was unaffected by Mac-1 deficiency (Figure 1D). The altered requirement for Mac-1 in macrophages may be attributed to the finding that besides Dectin-1, macrophages additionally express Dectin-2 (Figure S1D).

Next, the relative contribution of Dectin-1, Mac-1, and TLRs to the neutrophil respiratory burst in response to zymosan was assessed. Zymosan induces the release of extracellular and intracellular ROS (Figure S1E). $Dectin\text{-}1^{-/-}$ neutrophils did not generate ROS in response to zymosan (Figure 1E) as published (Boyle et al., 2011; Taylor et al., 2007), while $Mac\text{-}1^{-/-}$ neutrophils exhibited a partial reduction, as did $MyD88/TRIF^{-/-}$ neutrophils (Figure 1E). On the other hand, Dectin-1 functional blocking monoclonal antibody 2A11 (Figure 1F) or laminarin (competing soluble β-glucan) (Figure 1G) treatment of zymosan-stimulated wild-type neutrophils resulted in reduced but detectable ROS. Under these conditions, ROS was abolished only when Mac-1 was also absent (Figures 1F and 1G). A similar interplay between Dectin-1 and Mac-1 was shown following treatment with curdlan (Figure S1F), a soluble polymer of β1,3-glucan and Dectin-1 agonist (Gringhuis et al., 2009). From this we infer that, although Dectin-1 is the major receptor for ROS production, under conditions of low receptor or ligand density crosstalk between Dectin-1 and Mac-1 is required for optimal ROS generation. That is, Dectin-1 that remains functional following 2A11 or laminarin treatment is likely sufficient to activate Mac-1 and subsequent ROS production.

Vav Proteins Regulate Dectin-1-Induced Mac-1 Activation, Phagocytosis, and the Respiratory Burst

Vav proteins are activated downstream of integrins, immune response receptors, receptor tyrosine kinases, and GPCRs.

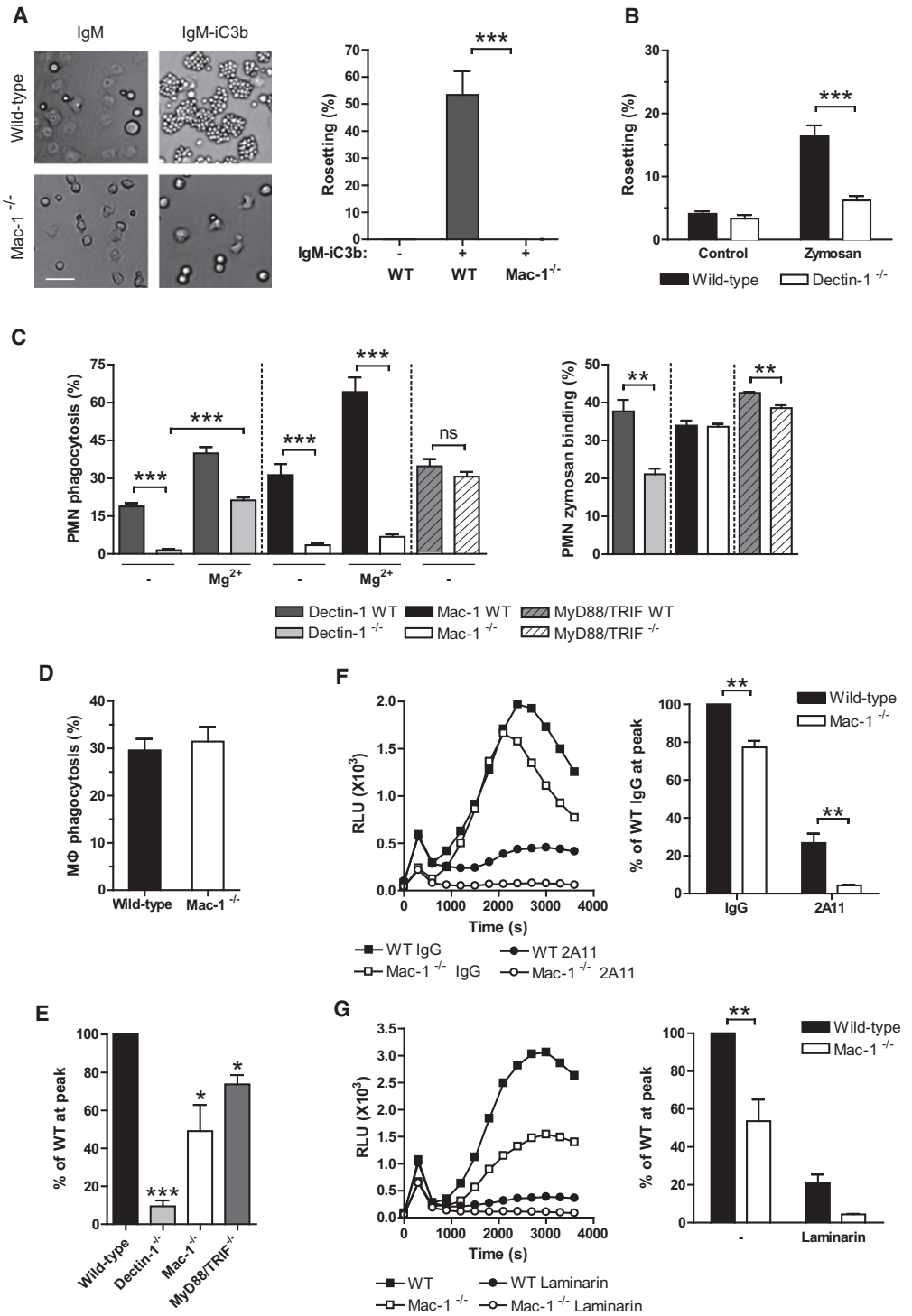

Figure 1. Dectin-1 Activates Mac-1 in Neutrophils and Both Collaborate in Responses to Fungal Components

(A) Wild-type (WT) and Mac-1$^{-/-}$ neutrophils were stimulated with PMA and presented with IgM (negative control) or IgM-iC3b coated RBCs (Bar = 25 μm) and scored for RBC rosetting. (B) Analysis of Mac-1 activation. WT and Dectin-1$^{-/-}$ neutrophils were incubated with IgM-iC3b RBCs in the absence (control) or presence of zymosan. (C) Neutrophils (PMN) from Dectin-1$^{-/-}$, Mac-1$^{-/-}$ or MyD88$^{-/-}$/TRIF$^{-/-}$ mice and their respective wild-type counterparts were incubated with FITC-labeled zymosan ± 10mM $MgCl_2$ (Mg^{2+}). Left panel: Cells at 37°C were evaluated for internalized fluorescent particles. Right panel: Binding of FITC-labeled zymosan was evaluated by FACS analysis in cells at 4°C. (D) Zymosan phagocytosis in Mac-1$^{-/-}$ and WT macrophages (MΦ) was assessed as described for neutrophils. Data represent mean (±SEM) of three to four independent experiments in (A–D). (E) Zymosan-induced ROS was analyzed in Dectin-1$^{-/-}$, Mac-1$^{-/-}$, MyD88$^{-/-}$/TRIF$^{-/-}$, and WT neutrophils. A graph of the mean (±SEM) of the relative light units (RLU) peak value for samples normalized to WT cells is shown. (F) WT and Mac-1$^{-/-}$ neutrophils were treated with zymosan in the presence of dectin-1 antibody 2A11 or IgG control, and real-time ROS generation was

Zymosan triggered Vav-Tyr^{174} phosphorylation, a signature of Vav activation (Tybulewicz, 2005) in wild-type neutrophils (Figure 2A). This did not occur in Dectin-1$^{-/-}$ neutrophils (Figure 2A), while it was intact in Mac-1$^{-/-}$ neutrophils, indicating that Vav activation by zymosan is downstream of Dectin-1. Neutrophils deficient in Vav1 and Vav3, the two major Vav proteins in neutrophils (Gakidis et al., 2004), failed to activate Mac-1 in response to zymosan as assessed in the C3-RBC rosetting assay (Figure 2B), even though surface expression of Dectin-1 and Mac-1 was normal (Figure 2B). Moreover, PMA promoted C3-RBC rosetting in both wild-type and Vav1,3-deficient (Vav1,3$^{-/-}$) neutrophils (Figure 2B), demonstrating that Vav is not required for C3-RBC binding per se. Next, pharmacological inhibitors were used to examine three potential pathways engaged by Dectin-1-Vav to activate Mac-1: nonreceptor tyrosine kinases, Ca^{2+}-dependent pathways, and PI3K-mediated signaling. We found that Syk, Src, PLCγ, and intracellular Ca^{2+} were essential for zymosan-induced C3-RBC rosetting, while PI3K and Mitogen-activated protein kinase (MAPK-ERK) were dispensable for this process (Figure 2C), even though inhibitors of these molecules blocked zymosan-induced ROS generation (data not shown). Vav1,3$^{-/-}$ neutrophils failed to uptake zymosan or complement opsonized zymosan (Figure 2D), despite normal zymosan binding (Figure 2D). Like Mac-1, Vav's role was specific for neutrophils, as zymosan phagocytosis was normal in Vav 1,3$^{-/-}$ macrophages (Figure 2E). Finally, Vav1,3$^{-/-}$ neutrophils had no detectable ROS generation in response to zymosan (Figure 2F) or curdlan (Figure S2A).

Fungal Components Activate Vav-Dependent Signals in Neutrophils

We sought to establish signaling events downstream of Dectin-1 and Mac-1 that are Vav dependent. As Vav was required for Dectin-1-mediated integrin activation, we first evaluated whether zymosan induced Rap GTPase activation and whether this required Vav proteins. Zymosan led to GTP-loading of Rap GTPase, which was similar in wild-type and Vav1,3$^{-/-}$ neutrophils (Figure 3A), indicating that Vav is not essential for this step.

Zymosan-induced phosphorylation of Src and Syk kinases, events proximal to Dectin-1 and Mac-1 receptors (Lowell, 2011), was largely intact in Vav1,3$^{-/-}$ neutrophils (Figure 3B). Recruitment and activation of Vav downstream of classical ITAM receptors in neutrophils is associated with activation of phospholipase C (PLCγ) to inositol triphosphate (IP3)-induced Ca^{2+} release, Rac GTPase to p21-activated kinase (PAK) activation, phosphorylation and membrane localization of NADPH oxidase components such as p40(phox), and activation of MAPK (Jakus et al., 2009; Utomo et al., 2006). Engagement of Dectin-1 by zymosan led to the activation of PAK, p40(phox), and the MAPK-ERK as well as pyk-2/RAFTK, a tyrosine kinase related to focal adhesion kinase. Notably, Vav 1,3$^{-/-}$ neutrophils were profoundly defective in each of these responses (Figure 3B), as were Dectin-1$^{-/-}$ and Mac-1$^{-/-}$ neutrophils (Figure 3C), suggesting that Mac-1 and Dectin-1 are upstream of Vav. Further analysis revealed that the Ca^{2+} flux in Vav 1,3$^{-/-}$ cells in response to zymosan was significantly impaired (Figure 3D), consistent with a defect in PLCγ activation. Notably, activation of the Ca^{2+}-sensitive tyrosine kinase pyk-2, placed downstream of Mac-1-dependent adhesive events (Fuortes et al., 1999), was also Vav1,3- (Figure 3B) and PLCγ-Ca^{2+}- (Figure 3E) dependent. In summary, the Vav-PLCγ-Ca^{2+} axis may represent a key hub in integrating signaling pathways through Dectin-1 and Mac-1. Consistent with this, inhibition of PLCγ abolished zymosan-induced ROS (Figure S2B).

Protection against *C. albicans* Requires Mac-1

The physiological role of Mac-1 in clearance of *C. albicans* blastoconidia was assessed, as this process is Dectin-1 dependent (Taylor et al., 2007). Infection with 5×10^4 colony forming units (cfu) (Figure 4A) of *C. albicans* yeast forms resulted in increased mortality in Mac-1$^{-/-}$ mice compared to wild-type animals. The kidney is the chief target organ of *C. albicans* (Brieland et al., 2001), particularly in neutropenic mice (Fulurija et al., 1996). More than a log-fold increase in cfu was recovered from kidneys of Mac-1$^{-/-}$ compared to wild-type animals (Figure 4B), which was associated with an increased number of abscesses and organ enlargement (Figure 4B), coalescing abscesses containing fungal elements, and significant neutrophil infiltration. In contrast, abscesses were virtually absent in wild-type animals (Figure 4C).

The increase in susceptibility to infection could not be attributed to a decrease in renal neutrophil accumulation, as assessed in kidney homogenates (Figure 4D). In tissue sections, neutrophil foci were observed in the interstitium of the cortex, and scattered cells were observed in the medulla of both wild-type and Mac-1$^{-/-}$ renal samples (Figure S3A). To rule out trapping of neutrophils within interstitial vessels in Mac-1$^{-/-}$ mice, a more detailed analysis of renal neutrophil accumulation was pursued in real-time in the kidney cortex, using laser-scanning confocal intravital microscopy. At 4 and 9 hr after infection, no *C. albicans* or extravascular neutrophils were detected (Movie S1). At 16 hr after infection, when neutrophil accumulation was measurable in kidney homogenates (Figure 4D), reproducible *C. albicans* colonization in the cortex was observed. In this area, >80% of neutrophils in wild-type and Mac-1$^{-/-}$ mice were extravascular (Figure 4E) (Movie S1). Notably, at sites of colonization and neutrophil infiltration the vasculature was damaged, as rhodamine-dextran perfusion (a readout of capillary patency) was diminished in these areas.

Mac-1$^{-/-}$ neutrophils failed to kill unopsonized *C. albicans* ex vivo (Figure 4F). This was specific for neutrophils as macrophages lacking this integrin exhibited normal intracellular killing (Figure 4F). Thus, Mac-1 is dispensable for renal neutrophil accumulation following fungal infection, but is essential for antifungal cytotoxic functions of tissue-recruited neutrophils. Next, we compared CD18-deficient mice with the same expressing

monitored. Representative ROS profiles and a graph, plotted as described in (E) is shown. The reduction in ROS in Mac-1$^{-/-}$ neutrophils following IgG isotype control treatment was less significant than without (E), and may result from the engagement of FcγRs by the antibody. (G) ROS generation was monitored in WT and Mac-1$^{-/-}$ neutrophils treated with zymosan in the presence or absence of laminarin. Data are shown as in (F). *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$ (one-way or two-way ANOVA with Bonferroni post test [B, C, E, F, G]; unpaired t test [A, D]). See also Figure S1.

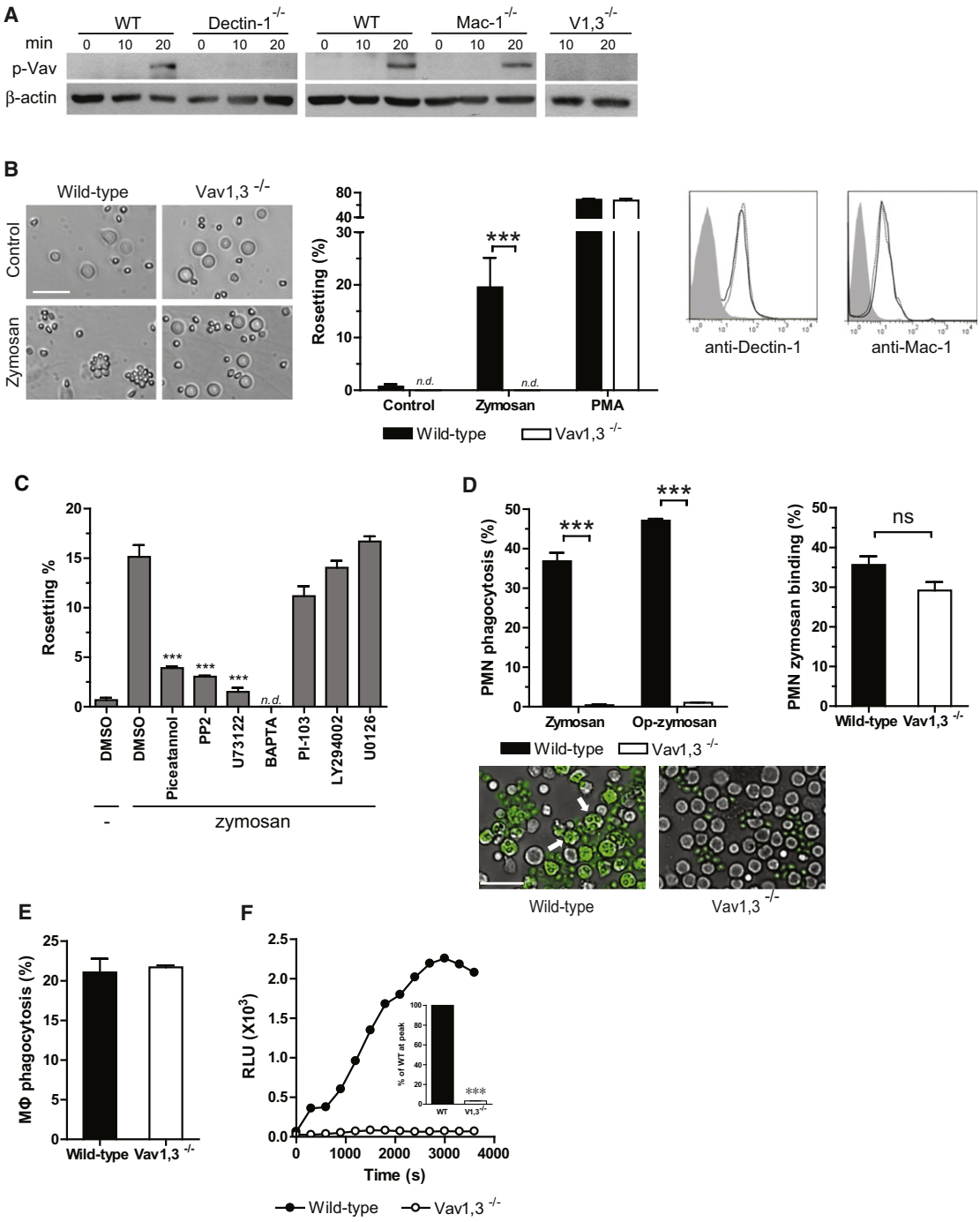

Figure 2. Vav Activated by Dectin-1 Is Required for Mac-1 Activation and Function

(A–F) (A) Lysates of WT, Dectin-1$^{-/-}$, and Mac-1$^{-/-}$ neutrophils stimulated with zymosan for the times in min were analyzed for Vav-Tyr^{174} phosphorylation. (B) Analysis of iC3b-RBC rosetting in wild-type and Vav1,3$^{-/-}$ neutrophils following treatment with vehicle control, zymosan, or PMA. Representative pictures and a graph of the mean (±SEM) of three experiments are shown. FACS analysis of Dectin-1 and Mac-1 expression in wild-type (solid line) and Vav1,3$^{-/-}$ (dotted line) neutrophils compared to IgG isotype control is shown. (C) C3-RBC rosetting was analyzed in unstimulated (-) and zymosan-stimulated wild-type cells treated with vehicle control (DMSO) or inhibitors of Syk (Piceatannol), Src (PP2), PLCγ (U73122), PI3K (PI-103, LY294002), or ERK (U0126) or a chelator of intracellular Ca^{2+} (BAPTA). Mean (±SEM) of results is given. n.d. = non-detectable. (D) Upper left and bottom panel: Phagocytosis of FITC-labeled unopsonized or iC3b opsonized zymosan by WT and Vav1,3$^{-/-}$ neutrophils (PMN). Representative pictures are shown for unopsonized zymosan (Bar = 25 μm). Upper right panel: Binding of FITC-labeled zymosan was evaluated by FACS analysis. Mean (±SEM) of results is given. (E) Zymosan phagocytosis in WT and Vav1,3$^{-/-}$ macrophages (MΦ). Mean (±SEM) of results is given. (F) Respiratory burst of WT and Vav1,3$^{-/-}$ neutrophils in response to zymosan. Representative ROS profiles and graph of the relative light units (RLU) peak value for samples (inset) is shown. n = 5 independent experiments. ***p < 0.001 (one-way or two-way ANOVA with Bonferroni post-test [B, C, D, upper left panel]; one sample t test [F]; unpaired t test [D, upper right panel]). See also Figure S2.

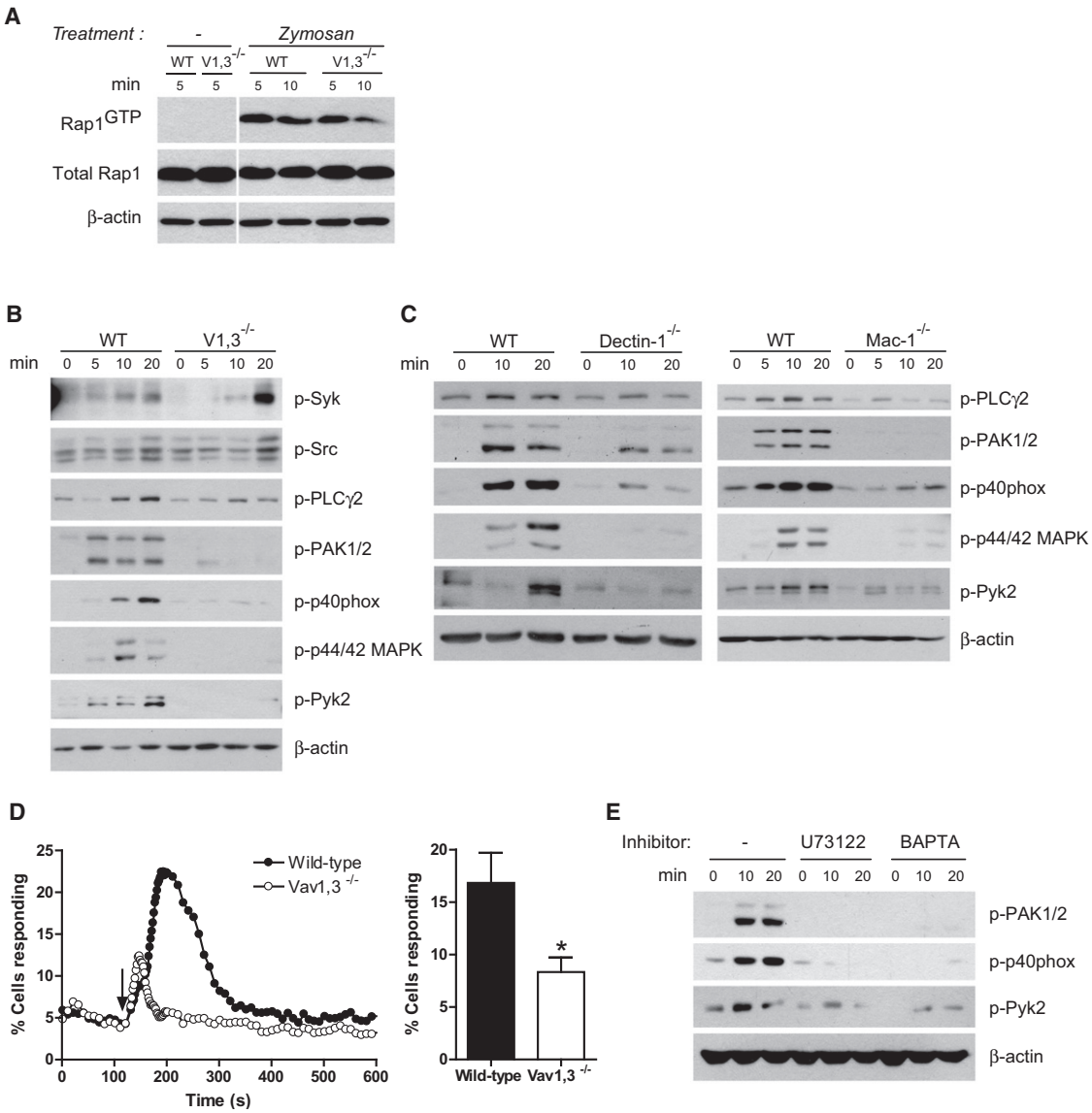

Figure 3. Vav Is Dispensable for Zymosan-Induced Rap GTPase Activation but Is Required for ITAM-Based Signals

(A–C) Wild-type (WT) and Vav1,3-deficient (V1,3$^{-/-}$) neutrophils were stimulated with zymosan for times given in minutes (min) and cell lysates were prepared. (A) Western blots of GTP-loaded Rap1 (Rap1^{GTP}; activated Rap1) as compared with total Rap1 and β-actin. (B) Western blot analysis of phosphorylation (p-) of Syk and Src, PLCγ2, PAK1/2, p40(phox), MAPK, and Pyk2. β-actin served as a loading control. (C) Dectin-1$^{-/-}$ and Mac-1$^{-/-}$ neutrophils were analyzed similarly to that described in (B). For all western blots representative data from 1 of 3 experiments is shown. (D) Indo-1 preloaded wild-type and Vav1,3$^{-/-}$ neutrophils were stimulated with zymosan (arrow). Ca^{2+} flux was evaluated by measuring a ratio of fluorescence signal at 405 nm to 485 nm over time. Data shown is a representative profile of the percentage of responding cells as a function of time. A graph of the mean (±SEM) of the maximum percentage of responding cells from four independent experiments is given. *$p < 0.05$ (unpaired t test). (E) Western blot analysis as described in (B) of wild-type cells stimulated with zymosan and pretreated without (-) or with an inhibitor to PLCγ (U73122) or a chelator of intracellular Ca^{2+} (BAPTA). See also Figure S2.

human CD18 selectively in neutrophils. The latter reinstates expression of CD18 integrins including Mac-1 on neutrophils to 50% of wild-type levels and markedly restores zymosan phagocytosis (Figures S3B–S3E). As observed with Mac-1$^{-/-}$ mice, the kidney fungal burden was much higher in CD18 null mice compared to wild-type counterparts (Figure 4G), which was not a consequence of defective renal neutrophil recruitment (Figure 4H). Importantly, restoration of CD18 in neutrophils resulted in a significant reduction in fungal burden compared to CD18$^{-/-}$ mice (Figure 4G). These data reveal the importance of neutrophil CD18 in fungal resistance at a step downstream of neutrophil recruitment.

Protection against *C. albicans* Requires Vav Proteins

The importance of Vav proteins in *C. albicans* clearance was evaluated. Inoculation with 5×10^4 cfu resulted in significant mortality in Vav1,3$^{-/-}$ mice compared to wild-type counterparts (Figure 5A), as did 1×10^5 cfu (Figure S4). Results in

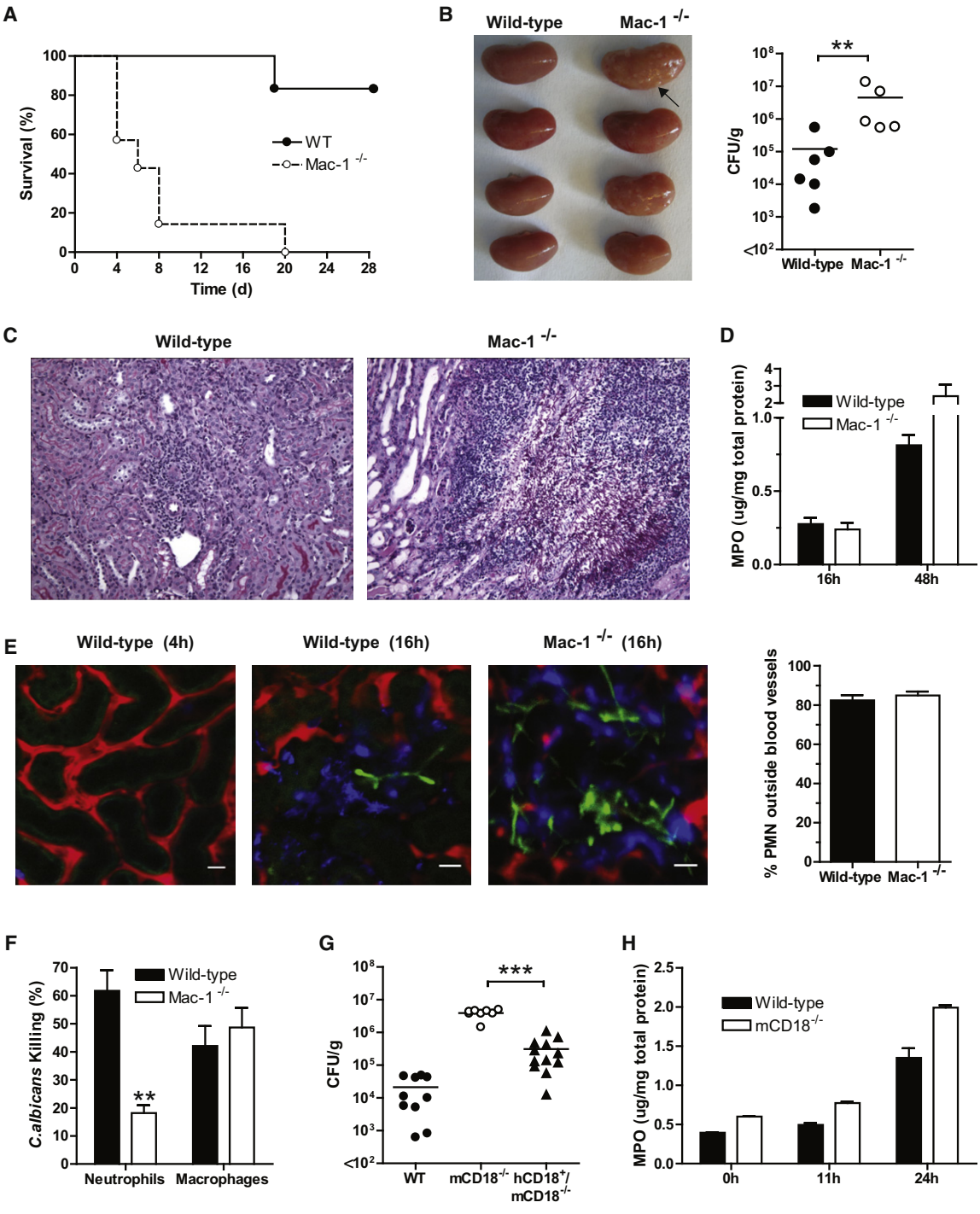

Figure 4. Mac-1 Is Required for Resistance to Systemic *C. albicans* Infection

(A) Mac-1$^{-/-}$ mice and their wild-type cohorts (n = 8 in each) were infected intravenously with 5 × 10^4 *C. albicans* and monitored daily for survival. p = 0.0011 (log-rank test). (B) Kidneys were harvested at day 4 after infection for gross morphology and visual inspection of abscesses (arrow) (left panel), and for quantitation of fungal burden in kidney homogenates (right panel). Each symbol represents data from an individual animal. (C) A representative photomicrograph of periodic acid Schiff (PAS)-stained kidney sections from wild-type and Mac-1$^{-/-}$ mice is shown. Within kidney tissues from Mac-1$^{-/-}$ mice, large collections of neutrophils (abscess formation) surrounded by mixed inflammatory cells are present with abundant fungal forms, which are virtually absent in kidneys from wild-type mice. (D) Kidneys harvested from mice at 16 and 48 hrs after infection were homogenized and myeloperoxidase enzyme (MPO) activity was determined and normalized to total protein content. Mean (±SEM) of results is given. (E) Confocal intravital microscopy of wild-type and Mac-1$^{-/-}$ mice at the indicated times after injection with live GFP-labeled *C. albicans*. Mice were also given rhodamine-dextran to delineate blood vessels and anti-Gr-1 to identify neutrophils. Left panels: Representative pictures show neutrophils (blue), blood vessels (red), and *C. albicans* fungal elements (green). *Right panel:* Quantitation of percent of neutrophils outside of blood vessels. Data represent mean (±SEM) of three independent experiments. (F) Analysis of intracellular killing of live unopsonized *C. albicans* by neutrophils

Vav1,3$^{-/-}$ mice phenocopied Mac-1$^{-/-}$ mice, including an increase in kidney fungal burden (Figure 5B), the presence of renal abscesses containing fungal elements (Figure 5C), and robust neutrophil accumulation in the extravascular space at all time points tested (Figures 5D and 5E). Ex vivo, Vav1,3$^{-/-}$ neutrophils failed to kill *C. albicans* (Figure 5F), while macrophages deficient in Vav1,3 retained this function (Figure 5F). The importance of Vav specifically in hematopoietic cells in antifungal resistance in vivo was tested by generating mice chimeric for Vav1,3 expression by bone marrow transplantation. Lack of Vav in circulating hematopoietic cells led to increased kidney fungal burden, which was comparable to that in mice lacking Vav in all cells, while Hrc presence of Vav in circulating cells provided resistance to infection (Figure 5G).

DISCUSSION

An absence of Dectin-1 or its effector Card9 increases incidences of mucocutaneous candidiasis in patients (Ferwerda et al., 2009; Glocker et al., 2009). Moreover, leukocyte adhesion deficiency I (LADI) patients that lack expression of Mac-1 and other members of the CD18 family have recurrent localized and disseminated Candidal and Aspergillus infection (Lekstrom-Himes and Gallin, 2000) that correlates with a failure of their neutrophils to respond to zymosan (Ross et al., 1987). Our results demonstrate that Dectin-1 activation of Mac-1 on recruited neutrophils is essential for neutrophil phagocytic functions associated with fungal clearance and likely serves as an early amplification loop to localize and contain fungal pathogens (Figure 6). The lack of invasive candidiasis in patients lacking Dectin-1 and Card9 may be because, in the absence of Dectin-1, Mac-1 activation is superseded by other pattern recognition receptors in an evolving infection. In support of this, Mac-1 activation by Mg^{2+} restored phagocytosis in Dectin-1$^{-/-}$ neutrophils. Moreover, Dectin-1-deficient human neutrophils were reported to exhibit normal yeast phagocytosis in the presence of serum and under adherent conditions (Ferwerda et al., 2009), which have the ability to activate Mac-1 independently of Dectin-1. In addition to serving as a pattern recognition receptor, Mac-1 binds complement iC3b-opsonized targets. Under opsonizing conditions as well, Mac-1 and Vav proteins were required for zymosan uptake. The Dectin-1 to Mac-1 pathway described herein may be specific for the yeast forms of *C. albicans* prevalent in human invasive candidiasis (Larone, 2002), as the molecular mechanisms involved in clearance of hyphae and conidia fundamentally differ. For example, although yeast forms engage Dectin-1, hyphae forms shield β-glucans by mannans, thus preventing activation of Dectin-1 signaling pathways (Netea et al., 2008). The molecular mechanisms of clearance may also diverge as hyphae trigger neutrophil spreading and extracellular release of ROS, while conidia fungal forms promote phagocytosis-induced ROS (Boyle et al., 2011). In showing the specificity of the Dectin-1-Vav-Mac-1 axis for neutrophil- but not macrophage-mediated antifungal activities, our results suggest that this mechanism evolved to localize cytotoxic responses of circulating neutrophils to infected tissues. There are potentially broad implications of this paradigm in neutrophil-mediated host defense.

Before the identification of Dectin-1, Mac-1 was considered the major fungal recognition receptor on phagocytes in vitro (Ross, 2000). This was reiterated in a recent study that reported the dominance of Mac-1 in fungal responses in human neutrophils; however, Dectin-1 function was only partially blocked (60%–70%) using anti-hDectin-1 antibody (van Bruggen et al., 2009). In light of our results, it is plausible that zymosan engagement of the remaining Dectin-1 is likely sufficient, and required, to activate Mac-1. Integrin activation is associated with alterations in integrin conformation that is coupled to changes in monomeric affinity, a process mediated by Rap GTPases. It also correlates with integrin clustering, which leads to an increase in the number of bonds that the integrin can form, a process regulated by the cytoskeleton (Hynes, 2002). Although Dectin-1-induced Mac-1 activation was Vav1,3-dependent, Rap1 GTPase activation was not sufficient for integrin activation in the absence of Vav. Thus, we postulate that Vav is not required for changes in the affinity of Mac-1 following zymosan stimulation, but may be responsible for PLCγ-induced Ca^{2+} flux and cytoskeletal changes required for integrin clustering. Indeed, inhibition of PLCγ or Ca^{2+} abolished zymosan-induced Mac-1 activation.

Both Dectin-1- and Mac-1-deficient mice exhibit decreases in survival following *C. albicans* infection. In Dectin-1$^{-/-}$ animals, this has been attributed to a decrease in neutrophil recruitment, albeit the analysis was done following acute infection in the peritoneum (Taylor et al., 2007) and not in the infected kidney, the primary target organ of infection and predictor of mortality in invasive candidiasis (Brieland et al., 2001; Odds, 1988). A deficiency in Mac-1, all CD18 integrins, or Vav 1,3 didn't impact renal neutrophil influx. Abundant extravasated neutrophils were observed in proximity to *C. albicans* in the renal interstitium of both Mac-1$^{-/-}$ and Vav1,3$^{-/-}$ mice. Thus Mac-1 and indeed CD18 integrins are not required for renal neutrophil recruitment, suggesting that recruitment to the specialized vasculature of this organ has distinct requirements for leukocyte adhesion receptors (Mayadas et al., 1999). The lack of effect of Mac-1 deficiency on neutrophil recruitment contrasts with the conclusion in the *C. albicans* hyphae model. However, in this case as well, recruitment was examined in the peritoneum after local introduction of hyphae (Soloviev et al., 2011). Despite normal neutrophil recruitment in Mac-1$^{-/-}$ and Vav1,3$^{-/-}$ mice, a significant increase in fungal colonization in the kidney that correlated with defects in *C. albicans* intracellular killing was observed. Together our results suggest a model wherein Dectin-1-induced Mac-1 activation via Vav proteins in renal-accumulated neutrophils promotes the phagocytic clearance of *C. albicans* (Figure 6). Contrary to our study, a previous study showed a small reduction in fungal burden in the kidney in Mac-1$^{-/-}$ mice following *C. albicans* blastoconidia infection, although survival was not specifically measured (Romani et al., 2004). The analysis on a mixed background mouse strain and use of a different fungal

and macrophages. Data represent mean (±SEM) of four experiments. (G) Kidney fungal burden at day 4 in wild-type, CD18-deficient (mCD18$^{-/-}$) and CD18$^{-/-}$ mice with human CD18 restored in neutrophils (hCD18^{+}/mCD18$^{-/-}$). (H) Renal neutrophil accumulation was evaluated in wild-type and CD18$^{-/-}$ mice at indicated time points after *C. albicans* infection. Data represent mean (±SEM) of three independent experiments $^{**}p < 0.01$; $^{***}p < 0.001$ (unpaired t test [F]; Mann-Whitney test [B, G]). See also Figure S3 and Movie S1.

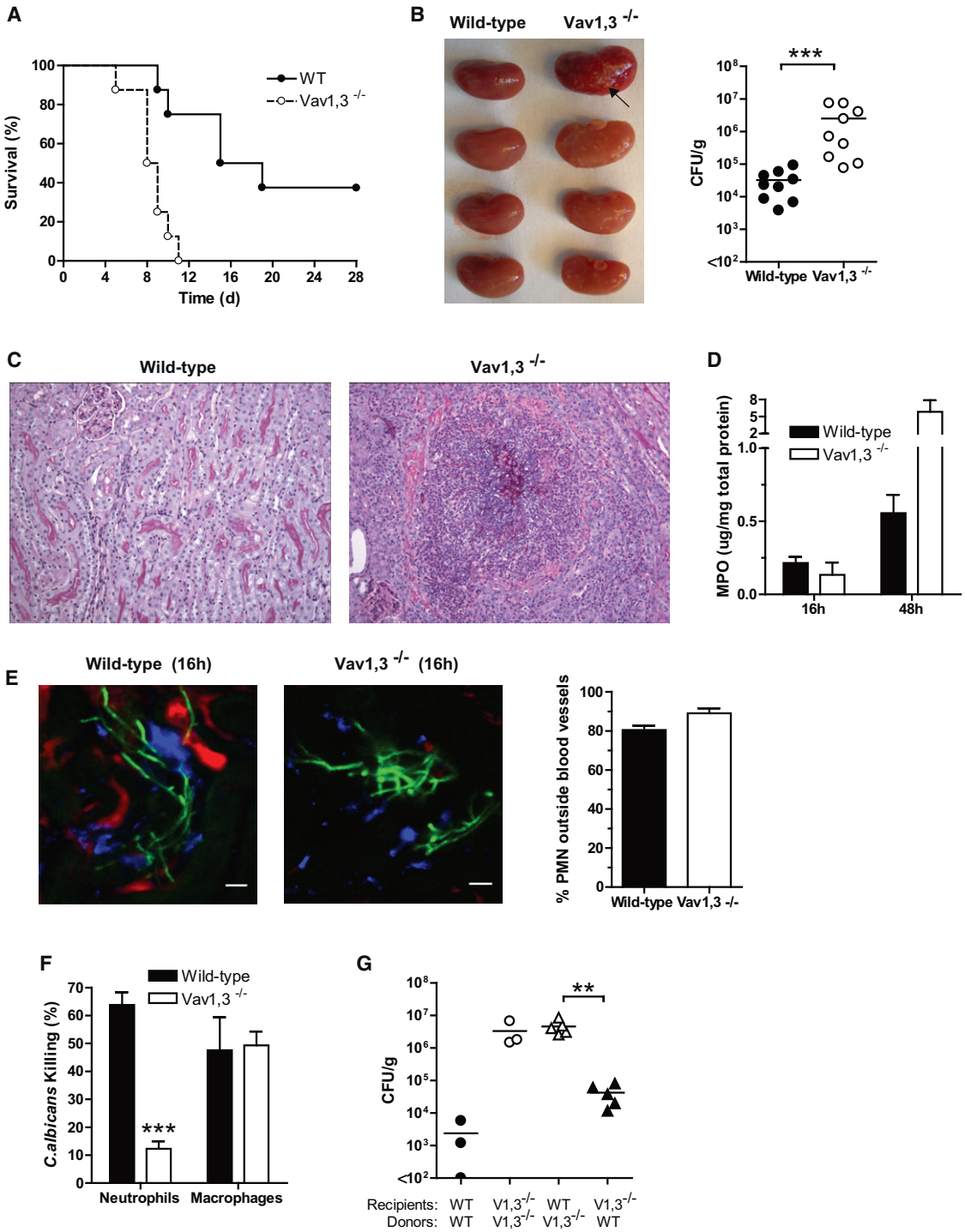

Figure 5. Vav1,3 Proteins Are Required for Resistance to *C. albicans* Infection

(A) Survival curves of wild-type and Vav1,3-deficient mice infected intravenously with 5×10^4 cfu of *C. albicans* (n = 8 in each). p = 0.0009 (log-rank test). (B) Images of kidneys harvested from wild-type and Vav1,3$^{-/-}$ mice 4 days after infection (left panel) and quantification of fungal burden in kidney homogenates (right panel). (C) A representative photomicrograph of PAS-stained renal sections from wild-type and Vav1,3$^{-/-}$ mice is shown. (D) Evaluation of tissue neutrophil accumulation in kidney at the indicated days after infection. Mean (±SEM) of results is given. (E) Laser-scanning confocal intravital microscopy of wild-type and Vav-1,3$^{-/-}$ mice as described in Figure 4E. Data represent mean (±SEM) of three independent experiments. (F) Analysis of intracellular killing of live unopsonized *C. albicans* by neutrophils and macrophages. Data represent mean (±SEM) of three independent experiments. (G) Wild-type (WT) and Vav 1,3 deficient (V1,3$^{-/-}$) recipient mice were reconstituted with bone marrow from wild-type or Vav1,3$^{-/-}$ donors. At day 4 after *C. albicans* innoculation, the cfu in kidney homogenates was determined. **p < 0.01; ***p < 0.001 (unpaired t test [F]; Mann-Whitney test [B, G]). See also Figure S4.

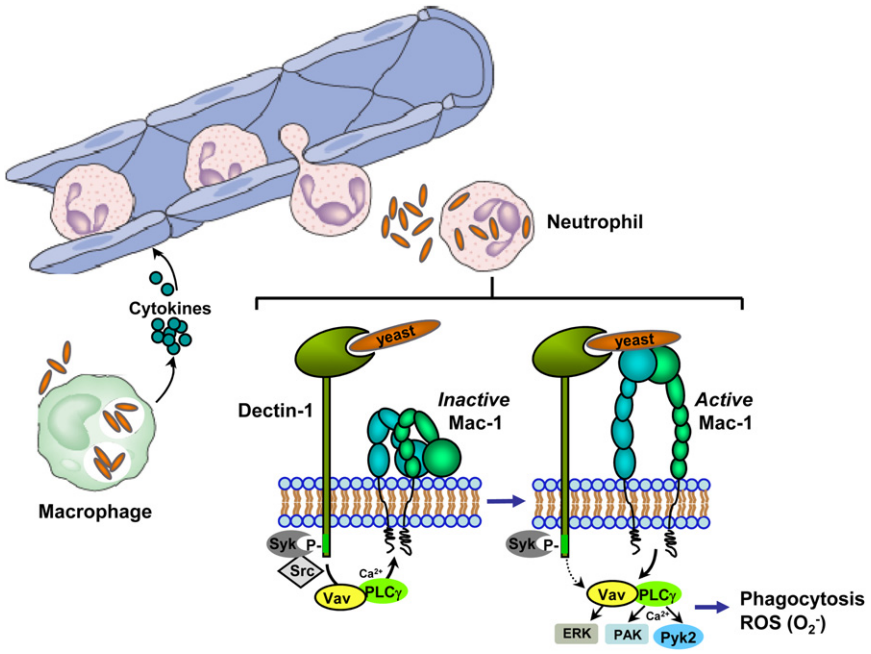

Figure 6. Model of Neutrophil-Mediated Antifungal Cytotoxicity

C. albicans blastoconidia (yeast) engage macrophages, which generate cytokines that activate the endothelium to recruit circulating neutrophils. Engagement of Dectin-1 on transmigrated neutrophils leads to phosphorylation (-P) of its ITAM by Src kinases, and recruitment and phosphorylation of Syk kinase, leading to Vav/PLCγ activation and Ca^{2+} release. These steps result in Mac-1 activation and its binding to *C. albicans*. Ligand-bound Mac-1 recruits p-Vav and contributes to the Dectin-1-initiated signaling complexes, leading to activation of downstream targets including Pyk-2, PAK, and ERK. These events together promote cytoskeletal rearrangement required for phagocytosis and activation of the NADPH oxidase to produce reactive oxygen species (ROS).

strain from that used in the current study may account for such a disparity (Dostert and Tschopp, 2007).

Vav activated by Dectin-1 is pivotal in relaying signals from fungal components. Syk and Src kinases are activated by zymosan and serve as docking sites for Vav proteins, which in other cell types are key hubs in signaling to downstream effectors that link to cytoskeletal rearrangements. Vav-Tyr^{174} phosphorylation may facilitate stepwise relief of its autoinhibition, leading not only to GEF activity but also allowing it to interact with other molecules to form a functional signaling complex (Bustelo, 2000). Vav phosphorylation was observed in Mac-$1^{-/-}$ neutrophils, yet the phosphorylation of molecules anticipated to be downstream of Vav, such as PLCγ or PAK, was suppressed. One explanation is that signaling downstream of Mac-1 involves a signaling pathway other than the Vav-PLCγ axis. However, it is also likely that, in addition to phosphorylation, Vav's localization into microsignalosomes (Weber et al., 2008) that may be supported by integrin-containing lipid-rich domains is required for the recruitment of signaling molecules such as PLCγ (Nakayama et al., 2008; Rodriguez et al., 2003) and PAK (Krautkrämer et al., 2004). Our work suggests that Vav controls Ca^{2+} release. Reconstitution of Vav null T cells with Vav1 lacking its GEF domain rescues several T cell receptor responses, including Ca^{2+} mobilization (Miletic et al., 2009). By extension, both Vav's GEF and adaptor functions may play essential roles in Dectin-1-dependent signaling. The overlap of signaling pathways impaired in Mac-1 and Vav1,$3^{-/-}$ neutrophils and the absolute requirement for Vav proteins in zymosan-induced ROS, a Dectin-1 dependent process, indicate that in addition to integrin activation, Vav1,3 are recruited into a complex that is required for signaling downstream of Dectin-1 and activated Mac-1 (Figure 6).

Mac-1 and Vav proteins were not required for phagocytosis or *C. albicans* killing in macrophages, even though Dectin-1 contributes to phagocytosis in this cell type (Taylor et al., 2007). The differential requirement for Mac-1 may reflect a need for tighter regulation of cytotoxic functions of circulating cells, such as neutrophils versus macrophages (Hynes, 2002). Dectin-1 alone may serve as a phagocytic receptor in macrophages, as its stable expression in nonprofessional phagocytes can result in zymosan uptake (Herre et al., 2004). Alternatively, Dectin-2 (Saijo and Iwakura, 2011) present on macrophages but not on neutrophils may replace Mac-1's role. The dispensable role of Vav proteins in macrophages may be attributed to differential signaling requirements in these cells versus neutrophils, which is consistent with Dectin-1 signaling being cell context-dependent (Goodridge et al., 2009; Herre et al., 2004).

In view of the findings that both Dectin-1 and Mac-1 have been implicated in fungal responses, our data provide a mechanistic explanation for how these two receptors coordinate fungal-induced neutrophil cytotoxic responses. Importantly, we demonstrate that Dectin-1 activation of Mac-1 via Vav proteins and PLCγ/Ca^{2+} represents a pathway for integrin activation relevant to host defense. The in vivo findings allow us to clearly establish a role for Mac-1 and Vav 1,3 in cytotoxic responses of recruited neutrophils, thus offering Vav as an attractive therapeutic target for enhancing tissue-localized immunity to fungal and potentially other microbial infections.

EXPERIMENTAL PROCEDURES

Mice

Vav1,3-deficient (Vav1,$3^{-/-}$) mice (Fujikawa et al., 2003) were backcrossed to C57BL/6J for six generations and maintained with similarly derived wild-type (WT) counterparts. Mac-1-deficient (Mac-$1^{-/-}$)/B6F9 mice (Hirahashi et al., 2006), MyD88/TRIF (MyD$88^{-/-}$TRIF$^{-/-}$)-deficient mice (Yamamoto et al., 2003), and Dectin-1 -deficient (Dectin-$1^{-/-}$) mice backcrossed to C57BL/6 (Saijo et al., 2007) were as described, and C57BL/6J wild-type mice served as their controls. All mice were bred and maintained in virus- and antibody-free facilities. Dectin-$1^{-/-}$ mice (Taylor et al., 2007) backcrossed to 129sv/ev were maintained as described (Parti et al., 2010), and control 129sv/ev mice for these animals were purchased from Taconic. All presented data were derived from neutrophils from Dectin-$1^{-/-}$ mice, described in Taylor et al. (2007). The data were confirmed in neutrophils harvested from Dectin-$1^{-/-}$ mice, described in Saijo et al., (2007), and neutrophils only from these animals

were used in the zymosan-binding assay. All the experiments were approved by the Harvard Medical School Animal Care and Use Committee.

Reagents and Antibodies

Curdlan, zymosan, laminarin, PMA, luminol, and cytochalasin D were obtained from Sigma-Aldrich. The following Abs were used for western blot analysis: rabbit anti-p-Syk, rabbit anti-p-Src, rabbit anti-p-Akt, rabbit anti-p-PAK1/2, rabbit anti-p-Pyk2, rabbit anti-p-p40(phox), and mouse anti-p-p44/42 MAPK (Cell Signaling Technology); rabbit anti-Rap1, rabbit anti-p-Vav (Santa Cruz Biotechnology); and β-actin (Sigma). Rat anti-Dectin-1 (2A11; AbD serotec) and rat IgG control (BD Biosciences) were used in functional blocking assays.

Respiratory Burst, Phagocytosis and iC3b-SRBC Rosetting

Mature bone marrow derived neutrophils were isolated as described (Hirahashi et al., 2006). Peritoneal macrophages were collected by lavage from mice injected intraperitoneally 4 days before with 3% thioglycollate. Peripheral blood human neutrophils from healthy donors were isolated using density gradients. Real-time ROS generation was monitored by luminol-enhanced chemiluminescence. Neutrophils (3×10^6 cells) were transferred to PBS plus Ca^{2+}/Mg^{2+} containing 50 μM luminol. Curdlan or zymosan was added at 100 μg/ml. Dectin-1 antibody 2A11 or IgG control was added at 10 μg/ml. Chemiluminescence was measured using an AutoLumat LB-953 tube luminometer (Berthold). For phagocytosis, FITC-zymosan was incubated with cells at a 10:1 ratio at 37°C for 30 min. A fluorescence microscope was used to assess the percentage of cells with ≥1 internalized FITC-zymosan.

Zymosan-binding assay was adopted from Taylor et al. (2007). After 20 min on ice, neutrophils and FITC-zymosan at 10:1 with 0.5% BSA were centrifuged at 350 g and left for 1hr on ice before resuspension and FACs analysis.

C3-RBC rosetting: Sheep red blood cells (RBC) (Lampire) were incubated with anti-sheep RBC IgM (Cedarlane) followed by incubation without or with C5-deficient serum (Sigma). Neutrophils were treated with PMA or zymosan for 15 min at 37°C. Cells were washed, C3-RBCs were added, the samples were spun down at 800 rpm and incubated at 37°C for 15 min. After washing, the percentage of neutrophils with ≥2 rosetted RBCs was determined.

Rap1 Activation and Calcium Flux Assay

GTP-loaded Rap1 in neutrophils treated with zymosan was determined in pull-down assays as previously described (Ghandour et al., 2007) with additional 5 mM Diisopropylfluorophosphate in the lysis buffer.

For Ca^{2+} flux, 2×10^7 neutrophils in PBS plus Ca^{2+}/Mg^{2+} were incubated with 2 μM Indo-1 AM and 0.04% F-127 (Invitrogen) at 37°C for 45 min. 2×10^6 cells were washed and stimulated with zymosan. The Ca^{2+} flux was monitored on a LSRII flow cytometer in real time for 8 min. Data were analyzed with Flowjo software.

In Vitro *C. albicans* Killing Assay

This assay was based on a published protocol (Johnnidis et al., 2008). 5×10^5 *C. albicans* (CA) were incubated with or without 5×10^5 phagocytes in 96-well plates (Falcon) in RPMI media. Surviving CA were incubated with Alamar blue (Invitrogen) at 1:10 dilution in PBS and fluorescence was measured using a SpectraMax M2 plate reader (Molecular Devices). Killing was calculated as [1- (Number of CA incubated with phagocytes)/(Number of CA incubated without phagocytes)].

In Vivo Model of Systemic Candidiasis

C. albicans (SC5314; American Type Culture Collection) were cultured and mice were infected with blastoconidia (yeast forms) essentially as described (Hirai et al., 2006; Taylor et al., 2007). After infection, mice were weighed and monitored daily. Mice were euthanized if they lost >20% of their body weight. In a separate group, the kidneys were harvested 4 days after infection. The left kidneys were photographed and homogenized for enumeration of fungal burden. The right kidneys were fixed for histological analysis. For intravital microscopy, mice were given 1×10^5 live GFP-labeled *C. albicans* (Cormack et al., 1997).

For tissue neutrophil accumulation, an MPO assay was performed as previously described (Huang et al., 2004). MPO activity in supernatants of homogenized kidney tissue was measured with TMB substrate kit (Pierce). MPO protein quantity was calculated based on a recombinant mouse Myeloperoxidase standard (R&D Systems). Total protein content was measured with BCA Protein Assay Kit (Pierce) and MPO quantity was normalized to protein content.

In Vivo Confocal Microscopic Imaging of Neutrophils in the Kidney Cortex

Mice were anesthetized by intraperitoneal injection of ketamine-xylazine, and intravital microscopy of the kidney was performed essentially as described (Fan et al., 2010). Briefly, mice were placed on a heated plate of a motorized x-y-z translational stage. The anti-Gr-1 antibody conjugated with Alexa 647 was injected intravenously for neutrophil staining. After surgical exteriorization of the left kidney, 70 kDa TRITC-dextran (50 μl of 5% w/v) was injected intravenously to stain blood vessels. A coverslip was applied to the top of the exposed kidney and the tissue was imaged with a laser-scanning confocal microscope with a 40× 0.6 N.A. objective. Images were acquired within 1 hr after the kidney exteriorization.

Statistical analysis

ANOVA with Bonferroni post tests was used when making multiple statistical comparisons on a single data set. Two-tailed unpaired t test was used for analysis of two groups. For the analysis of nonparametrically distributed data, the two-tailed Mann-Whitney test was used. Survival data were analyzed with the log-rank test. Results were considered statistically significant with p values of less than 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one movie, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chom.2011.10.009.

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