Calcineurin inhibitors, such as cyclosporine A (CsA) and FK506, are potent immunosuppressants used clinically for the treatment of a range of immune-mediated diseases, such as the rejection of solid organ allografts. CsA is best characterized for its ability to inhibit effector T cell functions, predominantly by preventing the activation of the NFAT transcription factors (Hogan et al., 2003). Blocking the activation of NFATs prevents the transcription of many characteristic T cell effector cytokines, such as IL-2 (Northrop et al., 1994).

There are four calcium-responsive members of the NFAT family, designated NFATc1 through NFATc4 (Mactan, 2005). All are retained in an inactive state in the cytosol by phosphorylation of serines in an N-terminal Serine-rich region domain. Upon intracellular calcium influx, calmodulin displaces an autoinhibitory loop from the active site of the phosphatase calcineurin (Bram and Crabtree, 1994). Calcineurin then removes the inhibitory phosphates, allowing NFATs to translocate to the nucleus where they collaborate with other transcription factors, such as AP-1, to effect changes in gene transcription (Clipstone and Crabtree, 1992). Although NFATs have been extensively studied in the context of T cells, relatively few studies have examined their function in myeloid lineages (Losa García et al., 1996; Dalmarco et al., 2008; Zanoni et al., 2009).

Despite their effectiveness in blocking T cell–mediated pathology, the use of calcineurin inhibitors is tempered by their many adverse effects (Woo et al., 1997). One such toxicity with particularly high morbidity and mortality is infection with opportunistic fungal pathogens such as *Candida albicans*, *Mucor sp.*, aspergillus, and histoplasma. Indeed, invasive candidal infections seen in CsA-treated patients are not just a general consequence of systemic suppression of adaptive immunity but are, rather, a result of the specific blockade of evolutionarily conserved innate pathways for fungal resistance.

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infection has a mortality of up to 50% (De Rosa et al., 2009). By one estimate, CsA treatment leads to a fourfold increase in the risk for systemic fungal infection as compared with prednisolone- and azathioprine-based immunosuppressive regimens in the first 6 mo after kidney transplant (Tharayil John et al., 2003). CsA has also been observed to promote susceptibility to C. albicans infection in mouse models (Vecchiarelli et al., 2009). Despite the dire clinical outcome of disseminated fungal infections, the mechanism by which CsA influences host-fungal interactions remains largely unexplored.

Recent studies have identified a family of ITAM-containing C-type lectin receptors that is important for the detection and killing of fungi. Dectin-1, the best characterized member of this family, recognizes β(1,3)-glucans in fungal cell walls, triggering phagocytosis, intracellular calcium flux, and cytokine production (Ariizumi et al., 2000b; Brown et al., 2002, 2003; LeibundGut-Landmann et al., 2007; Gross et al., 2009). Dectin-1–deficient mice are highly susceptible to infection with C. albicans (Taylor et al., 2007). Although Syk, CARD9, PLC-γ, and NFATs are all implicated in signaling downstream of dectin-1, the contribution of specific transcription factors to dectin-1–induced transcriptional responses and the physiological contribution of these pathways to antifungal responses remains incompletely characterized (Gross et al., 2006; Hara et al., 2007; LeibundGut-Landmann et al., 2007).

In this paper, we further explore the effects of calcineurin inhibitors on immunity to fungal pathogens and show that increased susceptibility to fungal infections seen with CsA treatment is not a generic effect of inhibiting adaptive immune responses. Rather, it is a consequence of specific inhibition of an innate immune pathway that regulates antifungal resistance in myeloid lineage leukocytes. We demonstrate that calcineurin activity is required for the candidacidal activity of neutrophils, as well as for transcriptional responses through the dectin-1 receptor that regulate inflammatory responses to this pathogen.

RESULTS AND DISCUSSION
CsA acts on the innate immune system to block resistance to C. albicans infection
To determine whether CsA promotes fungal infection through effects on the innate or the adaptive immune system, Rag2−/− mice, which lack an adaptive immune system, and WT mice were used in a model of disseminated fungal infection. Because C. albicans is the most common fungal pathogen in humans, we used a model of disseminated C. albicans infection in mice (Diekema et al., 2002; Schelenz, 2008). Mice of both infection has a mortality of up to 50% (De Rosa et al., 2009). By one estimate, CsA treatment leads to a fourfold increase in the risk for systemic fungal infection as compared with prednisolone- and azathioprine-based immunosuppressive regimens in the first 6 mo after kidney transplant (Tharayil John et al., 2003). CsA has also been observed to promote susceptibility to C. albicans infection in mouse models (Vecchiarelli et al., 2009). Despite the dire clinical outcome of disseminated fungal infections, the mechanism by which CsA influences host-fungal interactions remains largely unexplored.

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genotypes were infected with $10^5$ *C. albicans* yeasts by i.v. injection and then treated daily with 200 mg/kg CsA or vehicle. Both CsA-treated *Rag2"−/−"* and CsA-treated WT mice showed a median survival of 4 d after challenge with the yeasts (Fig. 1 A). In contrast, the majority of both *Rag2"−/−"* and WT mice treated with vehicle survived the 14-d experiment. Treatment with CsA in the absence of infection did not alter mouse survival (Fig. S1 A). Similar results were also obtained using a lower (40 mg/kg) dose of CsA (Fig. S1 B). To confirm that the CsA-treated mice died as a result of failure to control *C. albicans* infection, histological analysis of the kidneys and quantitative assessments of renal *C. albicans* burdens 4 d after infection were performed (Fig. 1, B and C). *C. albicans* was stained using the periodic acid-Schiff (PAS) stain. The kidneys of both *Rag2"−/−"* and WT mice treated with CsA showed fulminant infection with germinating hyphal *C. albicans* forms with no obvious difference in disease severity. In contrast, *C. albicans* levels were substantially lower in the kidneys of vehicle-treated mice. Collectively, these data indicate that CsA acts on the innate immune system to promote susceptibility to infection with *C. albicans*.

**Mice with a conditional deletion of calcineurin B (CnB) in neutrophils fail to control *C. albicans* infection**

To both further define the cell type responsible for suppression of antifungal immunity by CsA and to establish that the suppression is the result of a calcineurin-dependent effect of CsA, we generated mice with a conditional deletion of a CnB floxed allele by cre recombinase expressed under the lysozyme M promoter (hereafter, *CnB"lysm"* mice; Clausen et al., 1999; Neilson et al., 2004). To determine the efficiency of cre-mediated recombination in different myeloid cell lineages, we measured CnB mRNA levels in neutrophils sorted from the BM and spleen as a CD11b"high" Gr-1"high" population (Fig. S1 C). Analysis of CnB expression by real-time PCR showed efficient deletion (~90%) of CnB in neutrophils at both sites. Macrophages were also sorted from the spleen as CD11b"int" F4/80"+" cells. In contrast to the efficient deletion seen in neutrophils, however, splenic macrophages showed no appreciable deletion of CnB. Peritoneal macrophages showed only modest deletion (~50%). Hence, we attribute the effects described in the next paragraph primarily to the function of CnB in neutrophils, although we cannot rule out an additional contribution from CnB in certain macrophage subsets.

**Figure 2.** *CnB"lysm"* mice display increased susceptibility to fungal infection. (A) Kaplan–Meier survival curve showing the survival of *C. albicans*–infected mice. *CnB"flox"* or *CnB"lysm"* mice were infected with $10^5$ *C. albicans* yeasts by i.v. injection ($n = 10–11$ mice per group). Mice were then monitored daily for survival. *P* < 0.0001 by the log-rank test. Results are representative of three independent experiments. (B) *CnB"lysm"* or control *CnB"flox"* mice were infected with *C. albicans* as in A. Homogenates of the kidney were made 4 d after infection and *C. albicans* quantitated by serial dilution and colony counting. Each dot represents the mean of two measurements taken from a single mouse. *P* < 0.0001 by an unpaired Student’s *t* test. Results are representative of three independent experiments. Horizontal bars show the mean of the group. Error bars show SD. (C) Histological analysis of the kidneys of *C. albicans*–infected mice 4 d after challenge. *C. albicans* were visualized by PAS stain (purple color). Histology is representative of four mice per group. Results are representative of three independent experiments. Bars, 100 µm.
Figure 3. Zymosan activates the calcineurin–NFAT pathway. (A) Primary neutrophils were isolated and treated with CsA or vehicle for 15 min before mixing 1:1 with *C. albicans* yeasts, strain SC 5314. At the indicated time points, aliquots were taken and CFUs of *C. albicans* determined by serial dilutions on YPD agar. Relative killing was determined by normalization to *C. albicans* added to medium without neutrophils. Results are representative of three independent experiments. (B) Expression of NFATc1, NFATc2, and NFATc3 was determined in neutrophils and macrophages as indicated by real-time PCR. Results are representative of three independent experiments. (C) Primary neutrophils stimulated with 100 µg/ml zymosan and blotted for expression of NFATc1. Results are representative of three independent assays. (D) Primary neutrophils were stimulated with zymosan for 30 min, and nuclear and cytoplasmic fractions were blotted for NFATc1. Results are representative of three independent experiments. (E) Primary neutrophils were stimulated with the indicated concentrations of zymosan for 12 h, and the supernatant levels of IL-10 and IL-6 were quantified by ELISA. Results are representative of three independent experiments. (F) Primary neutrophils were stimulated with zymosan for 6 h and the expression of Cox2 was determined by real-time PCR. Results are representative of three independent experiments. (G) NFATc1 NFATc3<sup>−/−</sup> neutrophils and neutrophils from double heterozygous NFATc1<sup>−/−</sup> NFATc3<sup>−/−</sup> mice were isolated and stimulated with 100 µg/ml zymosan. 12 h later, supernatants were harvested and IL-10 levels determined by ELISA. Results are representative of three independent experiments. Error bars show SD.
CnBlysM mice and littermate controls were infected with 10^5 C. albicans, as in the previous section, and monitored for survival (Fig. 2 A). CnBlysM mice succumbed to C. albicans infection ~7 d after challenge. As in the experiment presented in Fig. 1, the majority of control mice survived the 14-d experiment. CFU counts measured from kidney tissue 4 d after infection (Fig. 2 B) revealed a significantly higher renal burden of C. albicans in CnBlysM mice compared with controls, indicating death from a failure to control infection. Likewise, histological analysis of the kidneys revealed extensive PAS-positive lesions in CnBlysM mice, indicating death from a failure to control infection.

Regulation of antifungal responses in neutrophils by calcineurin through NFAT-dependent and -independent pathways

When neutrophils encounter a pathogen, they attempt to limit infection by both directly killing the offending agent and producing molecules that promote an effective inflammatory response, including cytokines and prostanoids. To explain the observation that mice deficient in calcineurin activity in innate immune cells succumb rapidly to C. albicans, the response of calcineurin-deficient neutrophils to this pathogen was further explored in vitro. First, we tested the role of calcineurin in fungal responses. Both CnB-deficient and CsA-treated neutrophils were defective in the ability to kill C. albicans within 30 min (Fig. 3 A). Similar results were obtained with two C. albicans strains, SC5314 (Fig. 3 A) and NCCLS 11 (Fig. S1 E). CsA was not seen to directly affect C. albicans survival in the absence of neutrophils (Fig. S1 D). Likewise, C. albicans that were pretreated with CsA or vehicle, washed thoroughly, and then added to neutrophils were found to induce equivalent IL-10 production, making it unlikely that possible direct effects of CsA on yeast metabolism complicate the interpretation of these results (Fig. S1 D).

Despite this difference in killing, classical effector pathways mediating the fungicidal activity of neutrophils, including degranulation, as monitored by myeloperoxidase release, and the production of nitric oxide, were unaltered by treatment with CsA (Fig. S2, A–C; Aratani et al., 1999). Likewise, CsA treatment or CnB deficiency did not affect reactive oxygen species (ROS) production or phagocytosis in response to zymosan or C. albicans (Fig. S4, E–G). Collectively, these data suggest that calcineurin regulates the ability of neutrophils to kill C. albicans through a novel anti-microbial pathway.

Next, we examined the transcriptional effector mechanisms downstream of CnB in neutrophils leading to the control of inflammatory responses to fungal pathogens. Because NFAT transcription factors are the best characterized targets of calcineurin, we examined the expression of NFATc1, NFATc2, and NFATc3 in neutrophils and macrophages. Although macrophages expressed NFATc1, NFATc2, and NFATc3, neutrophils only expressed NFATc1 and NFATc3 (Fig. 3 B). We then examined if pathogen-associated molecule patterns (PAMPs) from the fungal cell wall can activate the calcineurin–NFAT pathway. Neutrophils were purified from the BM of WT mice and stimulated with zymosan, an extract of yeast cell walls rich in β(1,3)-glucans. A substantial increase in NFATc1 protein was seen after 6 h of stimulation (Fig. 3 C). To directly demonstrate that zymosan is able to activate NFATs, neutrophils were stimulated with zymosan and the activation status of NFATc1 was determined by monitoring nuclear translocation (Fig. 3 D). Nuclear translocation of NFATc1 occurred after 30 min, demonstrating that zymosan activates NFATs in a manner consistent with previous reports in other myeloid cell types (Goodridge et al., 2007).

To elucidate the importance of the calcineurin–NFAT pathway on the antifungal transcriptional responses in neutrophils, WT, CnB-deficient, and CsA-treated neutrophils were stimulated with zymosan. Both CnB-deficient and CsA-treated neutrophils were defective in IL-10 production as measured by ELISA in supernatants 12 h after stimulation (Fig. 3 E). Previously, the induction of IL-10 after zymosan stimulation has been noted to be dextrin I-dependent (Saigo et al., 2007). Additional analysis by real-time PCR of neutrophils 6 h after zymosan stimulation showed that other known NFAT target genes, including Cox2, Egr1 and Egr2, were all induced by zymosan in WT cells and that this response was significantly blunted in CnB-deficient and CsA-treated neutrophils (Fig. 3 F and Fig. S2 D; Goodridge et al., 2007; Lazarevic et al., 2009). In contrast, the production of IL-6 was not altered in CnB-deficient or CsA-treated neutrophils (Fig. S2 E).

Notably, although these in vitro studies focus on the functions of CnB in neutrophils, CnB can function similarly in other myeloid lineages. Splenic macrophages isolated from CnBlysM mice displayed improved deletion (~70%) of CnB after 5 d of ex vivo culture in the presence of 20 ng/ml M-CSF (Fig. S3 A). CsA-treated or CnB-deficient macrophages displayed a defect in IL-10 secretion in response to zymosan similar to that seen in neutrophils (Fig. S3 B). Similarly, treatment with CsA blocked IL-10 and IL-12 secretion by primary splenic dendritic cells (Fig. S3 C, D). Because neutrophils were not observed to produce detectable IL-12 in response to zymosan, the effects of CnB/CsA on antifungal responses are likely to be both broad, in that they affect many cell lineages, and specific with respect to their effects in any given lineage.

Because NFATc1 and NFATc3 are the major NFAT family members expressed in neutrophils, we tested whether they were responsible for the production of IL-10 downstream of CnB. NFATc1 and NFATc3 floxed alleles were deleted using the inducible Mx-cre/poly I:C system. The resulting NFATc1 NFATc3 doubly deficient neutrophils (NFATc1^MK1 NFATc3^MK3) showed defective IL-10 production when stimulated with zymosan, which is similar to the defect seen in CnB–deficient neutrophils (Fig. 3 G). Mice with a deletion of only NFATc1 or NFATc3 alone showed little to no defect in IL-10 production (Fig. S4 A). Given that...
NFATc1 and NFATc3 mediate the production of IL-10 downstream of calcineurin, we examined if they might also be involved in promoting candidal killing. Interestingly, in contrast to neutrophils deficient in CnB (Fig. 3 A), NFATc1<sup>−/−</sup> NFATc3<sup>−/−</sup> neutrophils showed no defect in killing C. albicans in vitro (Fig. S4 B). Moreover, NFATc1<sup>−/−</sup> NFATc3<sup>−/−</sup> mice showed no significant alteration in survival in a model of disseminated Candidemia (Fig. S4 C).

To examine if NFATs contribute to cytokine responses to zymosan in other myeloid cells, the expression of NFATc1–c3 was considered in primary splenic dendritic cells. NFATc1–c3 transcripts were present and the expression of NFATc1 and NFATc2 was induced in response to zymosan stimulation. On this basis, we generated dendritic cells deficient for NFATc1 and NFATc2 by breeding NFATc1 floxed allele mice to the CD11c-cre deleter strain to generate NFATc1<sup>−/−</sup>CD11c<sup>−/−</sup> NFATc2<sup>−/−</sup> mice. Splenic dendritic cells from these mice displayed defects in IL-12 and IL-10 production similar those seen with CsA treatment (Fig. S3 D). Dendritic cells lacking only NFATc1 or NFATc2 alone displayed little to no defect in IL-10 secretion (Fig. S3 F). Thus, the specific combination of NFATs responsible for cytokine production in response to zymosan varies from tissue to tissue.

Thus, CnB mediates two distinct pathways in the response to C. albicans. One, which is likely to be NFAT independent, is responsible for the immediate killing of C. albicans, and the other is mediated by NFATc1 and NFATc3 and is involved in transcriptional responses to this pathogen.

**The dectin–1 receptor, and not toll-like receptors (TLRs), is upstream of CnB/CsA**

Zymosan has been shown to activate both the dectin–1 receptor through β(1,3)-glucans and TLRs, particularly TLR2 (Dillon et al., 2006). Previous studies indicate that activation of dectin–1, but not TLRs, promotes the sustained elevations in intracellular calcium that are necessary to activate calcineurin (Xu et al., 2009). To directly test whether the activation of dectin–1, and not TLRs, is upstream of the effects of CsA, WT or CnB-deficient neutrophils were stimulated with curdlan, which contains dectin–1–stimulatory β(1,3)-glucans but lacks TLR-stimulatory motifs (Gringhuis et al., 2009). Similar to the results obtained with zymosan, both CsA-treated and CnB-deficient neutrophils showed a defect in IL–10 production after stimulation with curdlan (Fig. 4 A). As expected, CsA did not alter the production of IL–10, TNF, or IL–6 in neutrophils stimulated with LPS, a pure TLR4 agonist (Fig. S4 D). To confirm that contaminants with TLR-stimulating capacity in the curdlan preparation were not confounding the interpretation of these experiments, neutrophils were isolated from MyD88<sup>−/−</sup> mice (Fig. 4 B). In the absence of MyD88, all TLR signaling, except for the activation of the TRIF pathway by TLR4 and TLR3, which are not targeted by fungal pathogens, is defective (Kawai et al., 1999; Hoebe et al., 2003; Yamamoto et al., 2003). WT and MyD88<sup>−/−</sup> neutrophils were equally sensitive to the ability of CsA to block IL–10 production downstream of curdlan (Fig. 4 B).

These results make it unlikely that TLR-stimulatory contaminants confound the interpretation of curdlan as a selective activator of dectin–1 and not TLRs.

In summary, CsA acts on the innate immune system to suppress resistance in a model of disseminated fungal infections. This activity maps to the function of CnB in neutrophils, where it contributes to both immediate killing of C. albicans yeasts and transcriptional responses downstream of dectin–1.

Although calcineurin inhibitors are some of the most potent immunosuppressants available, significant toxicity limits their clinical use (Farkas et al., 2009). Understanding the biology of these adverse effects is critical for the rational design of immunosuppressants with greater clinical utility. In particular, patients taking calcineurin inhibitors are susceptible to infection with a range of opportunistic fungal pathogens, the most common of which is C. albicans (Diekema et al., 2002; Schelenz, 2008). In this paper, we demonstrate that CsA promotes fungal infection through suppression of innate immune antifungal responses, a function which is separable from its action to prevent T cell–mediated allograft rejection. Furthermore, these actions likely reflect inhibition of responses to fungal PAMPs through the dectin–1 receptor, although recent work has identified several related receptors, such as dectin–2, Mincle, and CLEC–2, that use similar signaling pathways and may likewise function upstream of CnB (Matsumoto et al., 1999; Arizumi et al., 2000a; Wells et al., 2008; Kerrigan et al., 2009; Robinson et al., 2009;
Yamasaki et al., 2009). Thus, the disseminated fungal infections observed in CsA-treated patients are not a consequence of broad inhibition of T cell responses but rather reflect specific blockade of evolutionarily conserved innate pathways for sensing fungal pathogens. Our results also help explain the clinical observation that disseminated fungal infections occur more frequently in patients with neutropenia than those with congenital defects in lymphocyte development (De Rosa et al., 2009). Moreover, this study highlights a broader role for calcineurin and NFATs in immune responses beyond antigen receptor signaling because this pathway regulates responses in the myeloid compartment.

Despite the defect in the ex vivo killing of C. albicans by CsA-treated or CnB-deficient neutrophils, these mutant neutrophils did not display significant defects in any of several effector processes, such as phagocytosis, ROS production, myeloperoxidase degranulation, or NO production, suggesting that CnB instigates an as yet uncharacterized candidacidal effector mechanism. The dissociation between transcriptional responses to yeast/zymosan and immediate C. albicans killing seen in NFATc1 NFATc3-deficient neutrophils suggests that CnB mediates two pathways involved in the response to fungal pathogens.

In investigating the transcriptional pathways downstream of CnB, the production of IL-10 in particular was dependent on CnB and NFATc1/c3. Interestingly, although IL-10 is most often cited as an antiinflammatory cytokine and, indeed, directly suppresses the phagocytic and candidacidal activities of neutrophils in vitro, the role of IL-10 in C. albicans resistance in vivo is less clear. Recent studies have correlated high IL-10 production with reduced renal C. albicans burden and tissue damage in i.v. models of C. albicans infection (Rolides et al., 2000; Tavares et al., 2000; MacCallum et al., 2009). Further work is needed to define the balance between pro- and antiinflammatory cytokines that provides for efficient protection from invasive fungal infection. Moreover, it is possible that both the nature and relative importance of the transcriptional response may vary depending on the route and kinetics of fungal exposure.

In summary, the results presented in this paper implicate the calcineurin signaling axis as critical for fungal responses in myeloid cells and specifically implicate the innate immune system as the target of CsA most relevant to the pathogenesis of disseminated fungal infections. In vitro, CnB contributes both to the immediate killing of C. albicans yeasts and to cyto kinase responses, particularly IL-10 production, to fungal PAMPs such as zymosan and curdlan. We propose that the combined defect in candidacidal activity and IL-10 production by calcineurin inhibition leads to both increased pathogen burden and a robust unchecked inflammatory response that promotes excessive tissue damage and lethality. These results refocus inquiry into the mechanism of disseminated fungal infections in CsA-treated patients away from the effects of CsA to suppress adaptive immune responses and toward a specific examination of dectin-1-dependent responses in myeloid cells.

MATERIALS AND METHODS

Mice. The generation of NFATc1 floxed allele mice was previously described (Aliprantis et al., 2008; Horsley et al., 2008). The lysosome M–cre strain was purchased from The Jackson Laboratory. The CD11c–cre strain was a gift from B. Reizis (Columbia University, New York, NY), CalcinewidR and NFATc3 floxed allele mice were a gift from G. Crabtree (Stanford University, Palo Alto, CA; Neilon et al., 2004; Canté-Barrett et al., 2007). Rag2–/– mice were maintained on the BALB/c background. CnB+/- mice were maintained on a mixed C57BL/6 × 129 background. For NFATc1 NFATc3M– mice, Mx-cre was induced by three injections every 2 d of 500 µg poly I:C (Kühn et al., 1995). All mice were housed at the Harvard School of Public Health, and experimental protocols were approved by the Harvard Institutional Animal Care and Use Committee.

Isolation and stimulation of neutrophils. Neutrophils were isolated by flushing femoral and tailial BM and lysing red blood cells in ACK buffer. The remaining cells were then layered over a three-part percoll density gradient (55, 65, and 75% isotonic percoll/PBS) and centrifuged at 500 g for 30 min at room temperature. The interface between the 65 and 75% layers was collected as the neutrophil-enriched fraction. This fraction was determined to be ~95% Gr-1hi CD11b+ B220− CD4+ CD8+ neutrophils by FACS analysis. Microscopic analysis of nuclear morphology confirmed the FACS determination of purity. For stimulation and in vitro killing assays, neutrophils were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, glutamine, penicillin, streptomycin, and 2-mercaptoethanol. Neutrophils were stimulated with 100 µg/ml zymosan (from Saccharomyces cerevisiae; Sigma-Aldrich) or 100 µg/ml curdlan (from Alcaligenes faecalis; Sigma-Aldrich) as indicated. Where CsA was used, cells were pretreated for 15 min with CsA before stimulation. For in vitro studies, CsA was dissolved in ethanol and diluted 1,000× to obtain the indicated concentration. For in vivo studies CsA was prepared as an emulsion in 15% (volume/volume) ethanol/caster oil.

In vivo infection with C. albicans. C. albicans SC5314 was grown for 16 h in YPD broth and washed three times with PBS. Yeasts were then counted on a hemocytometer and suspended at a concentration of 109/ml and 100 µl was injected into 8-wk-old mice of the indicated genotypes via i.v. tail vein injection. C. albicans was observed macroscopically before injection and found to be >99% yeast/hyphal forms. Mice were monitored daily for 14 d. For determination of candidal burdens, mice were euthanized 4 d after injection by CO2 narcosis, and kidneys and livers were harvested and homogenized by mechanical disruption in PBS. Serial dilutions of this homogenate were plated on YPD agar and colonies enumerated to determine quantitative fungal CFU counts. For histological analysis of the kidney, animals were euthanized, and kidneys were harvested and fixed in 4% paraformaldehyde. They were then dehydrated through an ethanol/xylene series and embedded in paraffin. Sections were cut and stained with the PAS stain or hematoxylin and eosin.

Quantitative determination of expression by real-time PCR and ELISA. For mRNA expression studies, RNA was isolated using trizol (Invitrogen). cDNA was synthesized using the Superscript kit (Bio-Rad Laboratories), and real-time PCR was performed using SYBR green mix (Applied Biosystems). Primers for NFATc1, NFATc2, NFATc3, EGR3, and EGR2 were used as previously described (Lazarevic et al., 2009).

Nuclear/cytoplasmic fractionation and Western blotting. Nuclear/cytoplasmic fractionation was performed using the NE-PER kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. The subsequent fractions were run on SDS-PAGE and blotted using anti-NFATc1 (clone 7A6; BD), Sp1 (PEP2; Santa Cruz Biotechnology, Inc.), and β-actin (13E5; Cell Signaling Technology).

In vitro C. albicans killing assay. C. albicans strain NCCLS 11 was obtained from American Type Culture Collection. Primary neutrophils were mixed 1:1 with C. albicans yeasts with gentle agitation at 37°C. At the indicated times, 10-µl
aliquots were taken, neutrophils lysed by resuspending in 1 ml of water, and *C. albicans* CFU determined by serial dilution and colony counting on YPD agar.

**ROS production.** Primary neutrophils were cultured at a density of 2 × 10^5 cells/well in a 96-well plate. At the indicated times after stimulation with 100 µg/ml zymosan or a 1:1 addition of live *Candida* yeasts, aliquots were taken and mixed 1:1 with a solution of 2 mM luminol, and chemiluminescence was measured on a luminometer (2010; Monolight).

**Splenic dendritic cell isolation.** Dendritic cells were purified from the single cell suspensions of splenocytes using positive selection with anti-CD11c magnetic beads (Miltenyi Biotec), according to the manufacturer’s instructions. The resulting cells were confirmed to be >85% CD11c+ dendritic cells by FACS analysis.

**Online supplemental material.** Fig. S1. provides controls for in vivo and in vitro studies using *C. albicans*. Fig. S2 shows that IL-6 production, ROS production, and neutrophil degranulation do not require CnB. Fig. S3 shows the contribution of CnB/NFATs to cytokine secretion in dendritic cells and macrophages. Fig. S4 shows no appreciable difference in susceptibility to the contribution of CnB/NFATs to cytokine secretion in dendritic cells and production, and neutrophil degranulation do not require CnB. Fig. S3 shows 10^5 cells/well in a 96-well plate. At the indicated times after stimulation with ROS production.

**Statistics.** All statistical tests were calculated using Prism. For survival curves, error bars represent standard error. All other values graphed are mean ± SD.


SUPPLEMENTAL MATERIAL

Green blatt et al., http://www.jem.org/cgi/content/full/jem.20092531/DC1
Figure S1. Controls for in vivo and in vitro experiments using C. albicans. (A) WT and Rag2^{-/-} mice were treated with 200 mg/kg CsA and monitored for survival. n = 4 per group. (B) Kaplan-Meier survival curve showing the survival of C. albicans-infected mice. Rag2^{-/-} or WT control mice were infected with 10^5 C. albicans yeasts by i.v. injection and then treated daily with 40 mg/kg CsA or vehicle control (n = 8–10 mice per group). Mice were then monitored daily for survival. P ≤ 0.005 by log-rank test comparing CsA-treated to vehicle-treated groups, with no significant difference between either Rag2^{-/-} or WT groups. (C) Neutrophils and macrophages were isolated by FACS from CnB^{WT} and CnB^{LYS} mice and their expression of ChB determined by real-time PCR. (D, Top) C. albicans yeasts were incubated in the presence of CsA or vehicle. At the indicated time points, aliquots were taken and CFUs of C. albicans determined by serial dilutions on YPD agar. (D, Bottom) C. albicans yeast were incubated with CsA for the indicated length of time during culture in YPD broth. Afterward, they were washed thoroughly with PBS to remove all CsA and added to neutrophils. After coincubation overnight, the levels of supernatant IL-10 were determined by EUSA. (E) Primary neutrophils were isolated and treated with CsA or vehicle for 15 minutes before mixing 1:1 with C. albicans yeasts, strain NCCLS 11. At the indicated time points, aliquots were taken and CFUs of C. albicans determined by serial dilutions on YPD agar. Relative killing was determined by normalization to C. albicans added to medium without neutrophils. Error bars show SD.
Figure S2. Further characterization of CnB-deficient neutrophils. (A) Primary neutrophils were pretreated with CsA and stimulated with 100 µg/ml zymosan. Degranulation was monitored by detection of supernatant myeloperoxidase (MPO) activity. Supernatant was mixed at a 1:3 ratio with peroxidase substrate (Sigma-Aldrich), and the relative MPO activity was determined by colorimetric assay. (B) Primary neutrophils were pretreated with CsA and stimulated with 100 µg/ml zymosan. After 8 h, NO levels were measured using the Greiss Reagent (1% sulfanilamide, 0.1% naphthylethylene-diamine-dihydrochloride, and 2.5 M H₃PO₄). (C) Primary neutrophils were stimulated with zymosan or a 1:1 addition of yeast, and ROS production was determined 10 min later by luminol chemiluminescence. (D) Primary neutrophils were stimulated with zymosan for 6 h, and the expression of Egr1 and Egr2 was determined by real-time PCR. (E) Primary neutrophils were isolated from CnB^+/+ and CnB^−/− mice and stimulated with 100 µg/ml zymosan. IL-6 secretion was determined 12 h later by ELISA. Error bars show SD.
Figure S3. CnB and NFATs function in macrophages and dendritic cells. (A) Splenocytes were isolated from CnB<sup>LysM</sup> and control mice, cultured in 20 ng/mL M-CSF. Nonadherent cells were removed by daily washes. At the end of 5 d, the remaining macrophages were stimulated with 100 µg/ml zymosan and analyzed for expression of CnB by quantitative PCR. (B) Macrophages were prepared as in A, treated with CsA, and stimulated with 10 µg/ml zymosan. 12 h later, supernatant IL-10 levels were analyzed by ELISA. (C) Dendritic cells were isolated from the spleens of mice with the indicated genotypes by MACS bead purification. 6 h after stimulation with 100 µg/ml zymosan, RNA was isolated and expression of the indicated genes determined by quantitative PCR. (D–F) Dendritic cells were isolated from the indicated genotypes by MACS bead purification and stimulated with 100 µg/ml zymosan for 12 h, and supernatant levels of the indicated cytokines were examined. Error bars show SD.
Figure S4. Contribution of NFATc1 and NFATc3 to antifungal responses by neutrophils, effect of CsA on phagocytosis, and responses to LPS. (A) Primary neutrophils were isolated from mice with the indicated genotypes and stimulated with 100 µg/ml zymosan. 12 h later, supernatants were harvested and IL-10 levels determined by ELISA. (B) Primary neutrophils were isolated and treated with CsA or vehicle for 15 min before mixing 1:1 with C. albicans yeasts. At the indicated time points, aliquots were taken and CFUs of C. albicans determined by serial dilutions on YPD agar. Relative killing was determined by normalization to C. albicans added to medium without neutrophils. (C) Kaplan-Meier survival curve showing the survival of C. albicans–infected mice. Rag2−/− or WT control mice were infected with 10⁵ C. albicans yeasts by i.v. injection and then treated daily with 200 mg/kg CsA or vehicle control (n = 12 mice per group). Mice were then monitored daily for survival. P = 0.24 by the log-rank test. (D) Primary neutrophils were pretreated for 15 min with CsA or vehicle and then stimulated with LPS for 12 h. Secretion of IL-6 (left), IL-10 (middle), and TNF (right) was determined by ELISA. (E) NeutrophilswerepretreatedwithCsAfor15minandincubatedwithFITC-zymosan.Attheindicatedtimes,fluorescence from cell surface–bound FITC-zymosan was quenched by the addition of toluidine blue and the percentage of neutrophils with internalized FITC-zymosan determined by FACS. (F and G) C. albicans yeasts were cultured for 16 h in YPD broth and then labeled with FITC by incubation with 0.1 mg/ml FITC in 0.5 M carbonate/bicarbonate buffer at 37°C for 30 min. Primary neutrophils from the indicated genotypes were pretreated for 15 min with CsA or vehicle and then the FITC-labeled C. albicans added with gentle shaking. After 30 min, ethidium bromide was titrated into the sample to quench the fluorescence from cell surface–bound C. albicans. A sample FACS plot demonstrating neutrophils containing internalized C. albicans and cell surface–bound C. albicans is shown (F). Percentages of the indicated gates are displayed for the various genotypes and treatments (G). Error bars show SD.