Calcineurin regulates innate antifungal immunity in neutrophils

Matthew B. Greenblatt,[1] Antonios Aliprantis,[1,2,3] Bella Hu,[1] and Laurie H. Glimcher[1,2,3,4]

[1]Department of Immunology and Infectious Diseases, Harvard School of Public Health, and [2]Department of Medicine and [3]Division of Rheumatology, Allergy and Immunology, Brigham and Women's Hospital, Harvard Medical School, Harvard University, Boston, MA 02115

[4]Ragon Institute of Massachusetts General Hospital, Harvard and MIT, Charlestown, MA 02129

Patients taking immunosuppressive drugs, like cyclosporine A (CsA), that inhibit calcineurin are highly susceptible to disseminated fungal infections, although it is unclear how these drugs suppress resistance to these opportunistic pathogens. We show that in a mouse model of disseminated *Candida albicans* infection, CsA-induced susceptibility to fungal infection maps to the innate immune system. To further define the cell types targeted by CsA, we generated mice with a conditional deletion of calcineurin B (CnB) in neutrophils. These mice displayed markedly decreased resistance to infection with *C. albicans*, and both CnB-deficient and CsA-treated neutrophils showed a defect in the ex vivo killing of *C. albicans*. In response to the fungal-derived pathogen-associated molecular pattern zymosan, neutrophils lacking CnB displayed impaired up-regulation of genes (IL-10, *Cox2*, *Egr1*, and *Egr2*) regulated by nuclear factor of activated T cells, the best characterized CnB substrate. This activity was Myd88 independent and was reproduced by stimulation with the β(1,3) glucan curdlan, indicating that dectin-1, rather than toll-like receptors, is the upstream activator of calcineurin. Our results suggest that disseminated fungal 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.

CORRESPONDENCE
Laurie H. Glimcher:
lglimche@hsph.harvard.edu

Abbreviations used: CnB, calcineurin B; CsA, cyclosporine A; PAMP, pathogen-associated molecule pattern; PAS, periodic acid-Schiff; ROS, reactive oxygen species; TLR, Toll-like receptor.

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 (Macian, 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

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 207 No. 5 923-931
www.jem.org/cgi/doi/10.1084/jem.20092531

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., 1989). 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

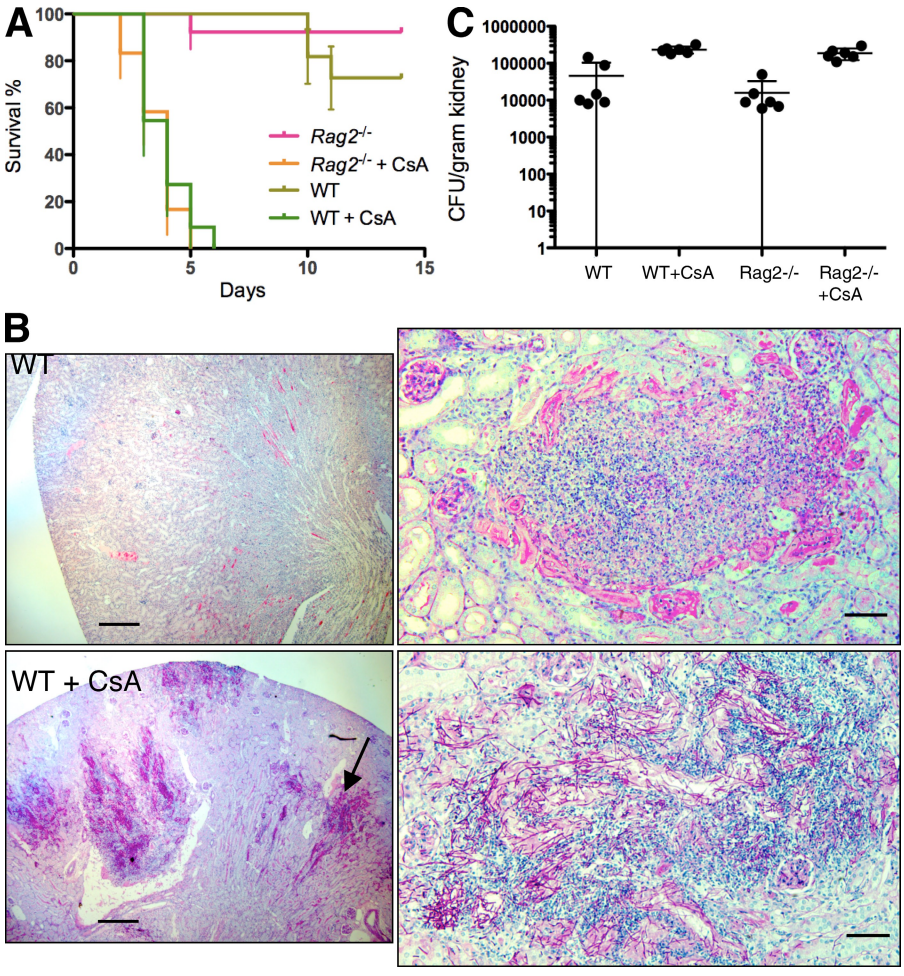

Figure 1. CsA suppresses innate resistance to *C. albicans* infection. (A) Kaplan-Meier survival curve showing the survival of *C. albicans*–infected mice. *Rag2*$^{-/-}$ or WT control mice were infected with 10^5 *C. albicans* yeasts by i.v. injection and then treated daily with 200 mg/kg CsA or vehicle control (n = 11–12 mice per group). Mice were then monitored daily for survival. $P < 0.0001$ by log-rank test comparing CsA-treated to vehicle-treated groups, with no significant difference between either Rag2$^{-/-}$ or WT groups. Results are representative of three independent experiments. (B) Mice were infected as in A and histological analysis of the kidneys was performed 4 d after infection. *C. albicans* were visualized by PAS stain (purple, arrow). Histology is representative of four per group. Results are representative of three independent experiments. Bars: (left) 500 µm; (right) 100 µm. (C) CsA- or vehicle-treated *Rag2*$^{-/-}$ or WT 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 comparing either CsA-treated group to the corresponding vehicle-treated group by an unpaired Student's t test. Results are representative of two independent experiments. Horizontal bars show the mean of the group. Error bars show SD.

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.

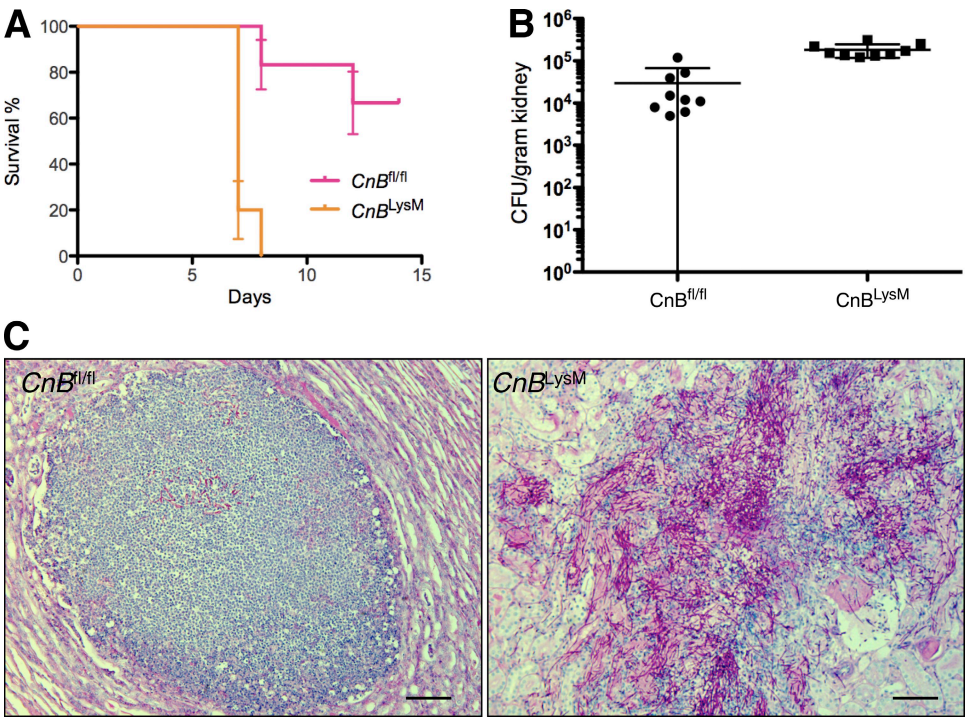

Figure 2. *CnB*^{LysM} mice display increased susceptibility to fungal infection. (A) Kaplan-Meier survival curve showing the survival of *C. albicans*–infected mice. *CnB*$^{fl/fl}$ or *CnB*^{LysM} mice were infected with 10^5 *C. albicans* yeasts by i.v. injection (n = 10–11 mice per group). Mice were 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*$^{fl/fl}$ 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.

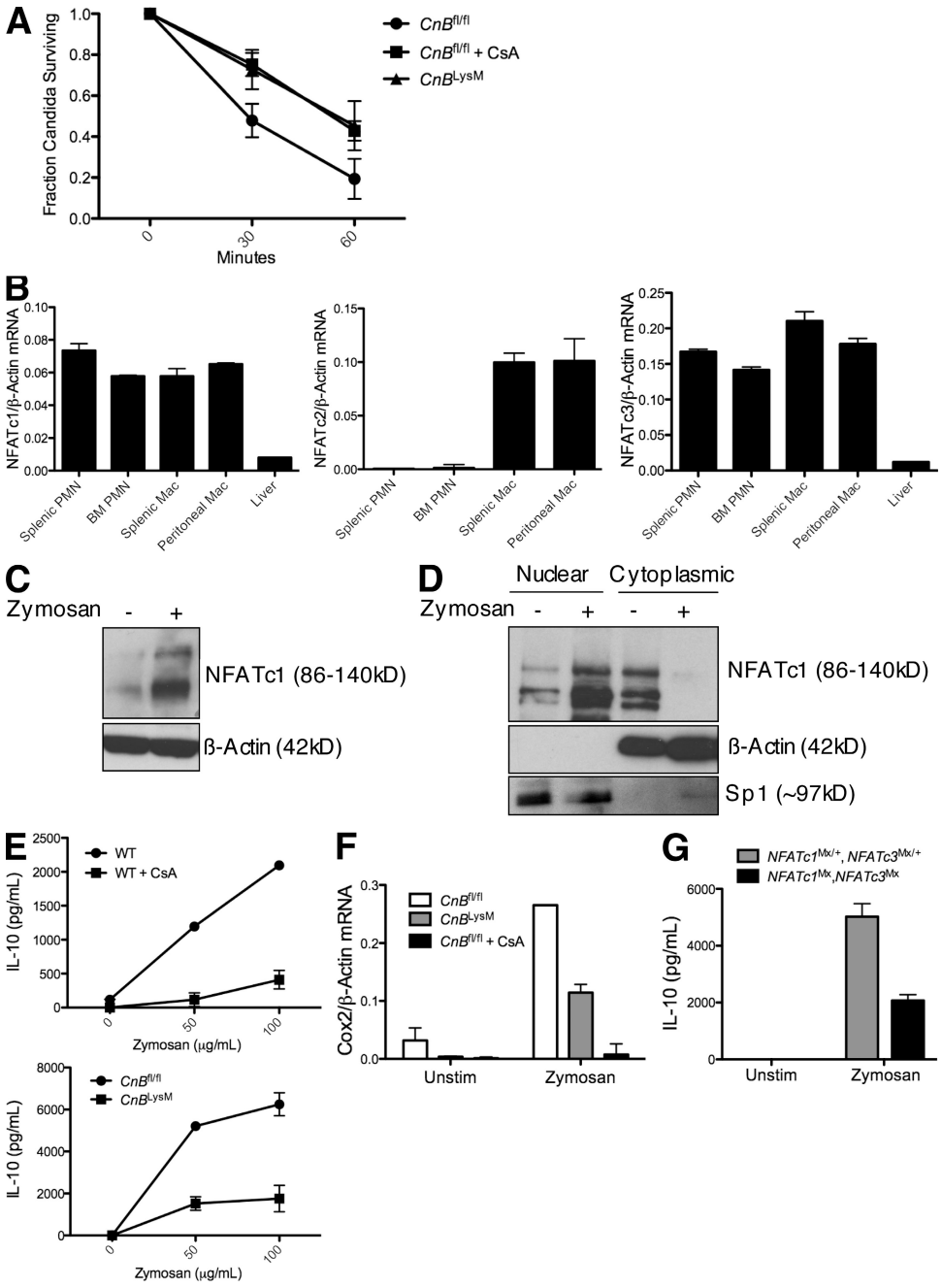

Figure 3. Zymosan activates the calcineurin–NFAT pathway. (A) Primary neutrophils were isolated and treated with CsA or vehicle for 15 min before mixing 1:1 with *C. albicans* yeasts, strain SC 5314. At the indicated time points, aliquots were taken and CFUs of *C. albicans* determined by serial dilutions 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^{Mx} neutrophils and neutrophils from double heterozygous NFATc1$^{Mx/+}$ NFATc3$^{Mx/+}$ 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.

CnB^{lysM} mice and littermate controls were infected with 10^5 *C. albicans*, as in the previous section, and monitored for survival (Fig. 2 A). *CnB*^{lysM} 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 *CnB*^{lysM} mice compared with controls, indicating death from a failure to control infection. Likewise, histological analysis of the kidneys revealed extensive PAS-positive lesions in *CnB*^{lysM} mice containing germinating *C. albicans* hyphae and sheets of tissue-infiltrating neutrophils. In contrast, inflammatory infiltrates were much less frequent in control mice, and, when found, appeared to be nearly devoid of PAS-positive material, indicating effective fungal clearance (Fig. 2 C). Thus, similar to CsA-treated *Rag2*$^{-/-}$ mice, *CnB*^{lysM} mice display a dramatically increased susceptibility to systemic fungal 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 fungicidal 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 dectin-1 dependent (Saijo 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 CnB^{LysM} 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. S3C, 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^{Mx} NFATc3^{Mx}) 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^{Mx} NFATc3^{Mx} neutrophils showed no defect in killing *C. albicans* in vitro (Fig. S4 B). Moreover, NFATc1^{Mx} NFATc3^{Mx} 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 NFATc2$^{-/-}$ mice to the CD11c-cre deleter strain to generate NFATc1^{CD11c} NFATc2$^{-/-}$ 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 this experiment, neutrophils were isolated from *MyD88*$^{-/-}$ 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*$^{-/-}$ 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; Ariizumi et al., 2000a; Wells et al., 2008; Kerrigan et al., 2009; Robinson et al., 2009;

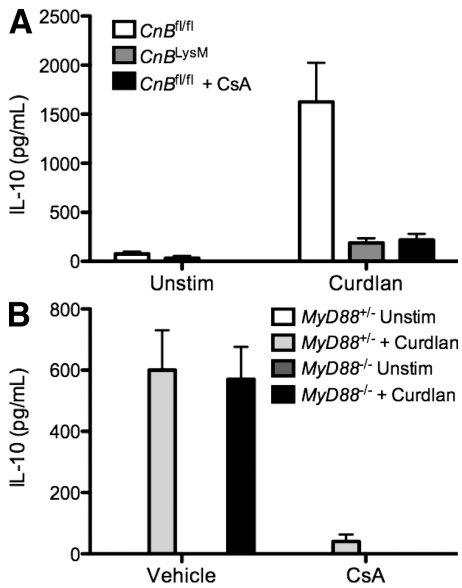

Figure 4. Dectin-1, and not TLRs, are upstream of CnB activation. (A) Primary neutrophils from the indicated genotypes were pretreated with CsA for 15 min and stimulated with 100 µg/ml curdlan. IL-10 secretion was determined after 12 h by ELISA. Results are representative of three independent experiments. (B) Primary neutrophils from the indicated genotypes were pretreated with CsA for 15 min and stimulated with 100 µg/ml curdlan. IL-10 secretion was determined after 12 h by ELISA. Results are representative of two independent experiments. Error bars show SD.

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 disassociation 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 (Roilides 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 cytokine 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 lysozyme M–cre strain was purchased from The Jackson Laboratory. The CD11c-cre strain was a gift from B. Reizis (Columbia University, New York, NY). *CalcineurinB* and *Nfatc3* floxed allele mice were a gift from G. Crabtree (Stanford University, Palo Alto, CA; Neilson et al., 2004; Canté-Barrett et al., 2007). *Rag2*$^{-/-}$ mice were maintained on the BALB/c background. CnB^{LysM} mice were maintained on a mixed C57BL/6 × 129 background. For NFATc1 NFATc3^{Mx} 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 tibial 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-1^{hi} CD11b^{hi} 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/castor 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 10^6/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 microscopically 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 CO_2 narcosis, and kidneys 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 iScript kit (Bio-Rad Laboratories), and real-time PCR was performed using SYBR green mix (Applied Biosystems). Primers for NFATc1, NFATc2, NFATc3, EGR1, 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 Candia 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% $CD11c^{hi}$ dendritic cells by FACS analysis.

For macrophage cultures, total splenocytes were cultured in 24-well tissue culture plates in the presence of 20 ng/ml M-CSF for 5 d. Nonadherent cells were removed by daily washing. The resulting cells were confirmed to by >95% $CD11b^+$ macrophages by FACS.

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

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 fungal infections in *NFATc1*^{Mx} *NFATc3*^{Mx} mice, that CsA does not alter cytokine secretion after LPS stimulation, and unaltered phagocytosis after treatment of neutrophils with CsA. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20092531/DC1.

The authors thank Gerald Crabtree and Boris Reizis for generously sharing the CnB floxed allele and CD11c-cre lines, Antony Segal, for helpful discussion, Kelly Tsang, Arnold Park, Zachary Swartz, and Shivesh Punit for technical assistance, Dorothy Zhang for assistance with histology, and Marc Wein, Vania Lazarevic, and Fabio Martinon for their thoughtful review of the manuscript.

This work was supported by National Institutes of Health grants AI43953 and AI31541 (L.H. Glimcher), Burroughs Wellcome Fund CAMS award (A. Aliprantis), and K08AR054859 (A. Aliprantis).

L.H. Glimcher declares that she is a Member of the Board of Directors of and holds equity in Bristol-Myers Squibb. The authors have no other conflicting financial interests.

Submitted: 24 November 2009
Accepted: 18 March 2010

REFERENCES

Aliprantis, A.O., Y. Ueki, R. Sulyanto, A. Park, K.S. Sigrist, S.M. Sharma, M.C. Ostrowski, B.R. Olsen, and L.H. Glimcher. 2008. NFATc1 in mice represses osteoprotegerin during osteoclastogenesis and dissociates systemic osteopenia from inflammation in cherubism. *J. Clin. Invest.* 118:3775–3789. doi:10.1172/JCI35711

Aratani, Y., H. Koyama, S. Nyui, K. Suzuki, F. Kura, and N. Maeda. 1999. Severe impairment in early host defense against *Candida albicans* in mice deficient in myeloperoxidase. *Infect. Immun.* 67:1828–1836.

Ariizumi, K., G.L. Shen, S. Shikano, R. Ritter III, P. Zukas, D. Edelbaum, A. Morita, and A. Takashima. 2000a. Cloning of a second dendritic cell-associated C-type lectin (dectin-2) and its alternatively spliced isoforms. *J. Biol. Chem.* 275:11957–11963. doi:10.1074/jbc.275.16.11957

Ariizumi, K., G.L. Shen, S. Shikano, S. Xu, R. Ritter III, T. Kumamoto, D. Edelbaum, A. Morita, P.R. Bergstresser, and A. Takashima. 2000b. Identification of a novel, dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning. *J. Biol. Chem.* 275:20157–20167. doi:10.1074/jbc.M909512199

Bram, R.J., and G.R. Crabtree. 1994. Calcium signalling in T cells stimulated by a cyclophilin B-binding protein. *Nature.* 371:355–358. doi:10.1038/371355a0

Brown, G.D., P.R. Taylor, D.M. Reid, J.A. Willment, D.L. Williams, L. Martinez-Pomares, S.Y. Wong, and S. Gordon. 2002. Dectin-1 is a major β-glucan receptor on macrophages. *J. Exp. Med.* 196:407–412. doi:10.1084/jem.20020470

Brown, G.D., J. Herre, D.L. Williams, J.A. Willment, A.S. Marshall, and S. Gordon. 2003. Dectin-1 mediates the biological effects of β-glucans. *J. Exp. Med.* 197:1119–1124. doi:10.1084/jem.20021890

Canté-Barrett, K., M.M. Winslow, and G.R. Crabtree. 2007. Selective role of NFATc3 in positive selection of thymocytes. *J. Immunol.* 179: 103–110.

Clausen, B.E., C. Burkhardt, W. Reith, R. Renkawitz, and I. Förster. 1999. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res.* 8:265–277. doi:10.1023/A:1008942828960

Clipstone, N.A., and G.R. Crabtree. 1992. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature.* 357:695–697. doi:10.1038/357695a0

Dalmarco, E.M., Y.S. Medeiros, and T.S. Fröde. 2008. Cyclosporin A inhibits CD11a/CD18 adhesion molecules due to inhibition of TNFalpha and IL-1 beta levels in the mouse model of pleurisy induced by carrageenan. *Cell Adh Migr.* 2:231–235.

De Rosa, F.G., S. Garazzino, D. Pasero, G. Di Perri, and V.M. Ranieri. 2009. Invasive candidiasis and candidemia: new guidelines. *Minerva Anestesiol.* 75:453–458.

Diekema, D.J., S.A. Messer, A.B. Brueggemann, S.L. Coffman, G.V. Doern, L.A. Herwaldt, and M.A. Pfaller. 2002. Epidemiology of candidemia: 3-year results from the emerging infections and the epidemiology of Iowa organisms study. *J. Clin. Microbiol.* 40:1298–1302. doi:10.1128/JCM.40.4.1298-1302.2002

Dillon, S., S. Agrawal, K. Banerjee, J. Letterio, T.L. Denning, K. Oswald-Richter, D.J. Kasprowicz, K. Kellar, J. Pare, T. van Dyke, et al. 2006. Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. *J. Clin. Invest.* 116:916–928. doi:10.1172/JCI27203

Farkas, S.A., A.A. Schnitzbauer, G. Kirchner, A. Obed, B. Banas, and H.J. Schlitt. 2009. Calcineurin inhibitor minimization protocols in liver transplantation. *Transpl. Int.* 22:49–60. doi:10.1111/j.1432-2277.2008.00796.x

Goodridge, H.S., R.M. Simmons, and D.M. Underhill. 2007. Dectin-1 stimulation by *Candida albicans* yeast or zymosan triggers NFAT activation in macrophages and dendritic cells. *J. Immunol.* 178:3107–3115.

Gringhuis, S.I., J. den Dunnen, M. Litjens, M. van der Vlist, B. Wevers, S.C. Bruijns, and T.B. Geijtenbeek. 2009. Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-kappaB activation through Raf-1 and Syk. *Nat. Immunol.* 10:203–213. doi:10.1038/ni.1692

Gross, O., A. Gewies, K. Finger, M. Schäfer, T. Sparwasser, C. Peschel, I. Förster, and J. Ruland. 2006. Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature.* 442:651–656. doi:10.1038/nature04926

Gross, O., H. Poeck, M. Bscheider, C. Dostert, N. Hannesschläger, S. Endres, G. Hartmann, A. Tardivel, E. Schweighoffer, V. Tybulewicz, et al. 2009. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature.* 459:433–436. doi:10.1038/nature07965

Hara, H., C. Ishihara, A. Takeuchi, T. Imanishi, L. Xue, S.W. Morris, M. Inui, T. Takai, A. Shibuya, S. Saijo, et al. 2007. The adaptor protein CARD9 is essential for the activation of myeloid cells through ITAM-associated and Toll-like receptors. *Nat. Immunol.* 8:619–629. doi:10.1038/ni1466

Hoebe, K., X. Du, P. Georgel, E. Janssen, K. Tabeta, S.O. Kim, J. Goode, P. Lin, N. Mann, S. Mudd, et al. 2003. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature.* 424:743–748. doi:10.1038/nature01889

Hogan, P.G., L. Chen, J. Nardone, and A. Rao. 2003. Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev.* 17:2205–2232. doi:10.1101/gad.1102703

Horsley, V., A.O. Aliprantis, L. Polak, L.H. Glimcher, and E. Fuchs. 2008. NFATc1 balances quiescence and proliferation of skin stem cells. *Cell.* 132:299–310. doi:10.1016/j.cell.2007.11.047

Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity*. 11:115–122. doi:10.1016/S1074-7613(00)80086-2

Kerrigan, A.M., K.M. Dennehy, D. Mourão-Sá, I. Faro-Trindade, J.A. Willment, P.R. Taylor, J.A. Eble, C. Reis e Sousa, and G.D. Brown. 2009. CLEC-2 is a phagocytic activation receptor expressed on murine peripheral blood neutrophils. *J. Immunol.* 182:4150–4157. doi:10.4049/jimmunol.0802808

Kühn, R., F. Schwenk, M. Aguet, and K. Rajewsky. 1995. Inducible gene targeting in mice. *Science*. 269:1427–1429. doi:10.1126/science.7660125

Lazarevic, V., A.J. Zullo, M.N. Schweitzer, T.L. Staton, E.M. Gallo, G.R. Crabtree, and L.H. Glimcher. 2009. The gene encoding early growth response 2, a target of the transcription factor NFAT, is required for the development and maturation of natural killer T cells. *Nat. Immunol.* 10:306–313. doi:10.1038/ni.1696

LeibundGut-Landmann, S., O. Gross, M.J. Robinson, F. Osorio, E.C. Slack, S.V. Tsoni, E. Schweighoffer, V. Tybulewicz, G.D. Brown, J. Ruland, and C. Reis e Sousa. 2007. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat. Immunol.* 8:630–638. doi:10.1038/ni1460

Losa García, J.E., F. Mateos Rodríguez, A. Jiménez López, and J.L. Pérez Arellano. 1996. Action of cyclosporin A on mononuclear phagocytes. *J. Investig. Allergol. Clin. Immunol.* 6:222–231.

MacCallum, D.M., L. Castillo, A.J. Brown, N.A. Gow, and F.C. Odds. 2009. Early-expressed chemokines predict kidney immunopathology in experimental disseminated *Candida albicans* infections. *PLoS One*. 4:e6420. doi:10.1371/journal.pone.0006420

Macian, F. 2005. NFAT proteins: key regulators of T-cell development and function. *Nat. Rev. Immunol.* 5:472–484. doi:10.1038/nri1632

Matsumoto, M., T. Tanaka, T. Kaisho, H. Sanjo, N.G. Copeland, D.J. Gilbert, N.A. Jenkins, and S. Akira. 1999. A novel LPS-inducible C-type lectin is a transcriptional target of NF-IL6 in macrophages. *J. Immunol.* 163:5039–5048.

Neilson, J.R., M.M. Winslow, E.M. Hur, and G.R. Crabtree. 2004. Calcineurin B1 is essential for positive but not negative selection during thymocyte development. *Immunity*. 20:255–266. doi:10.1016/S1074-7613(04)00052-4

Northrop, J.P., S.N. Ho, L. Chen, D.J. Thomas, L.A. Timmerman, G.P. Nolan, A. Admon, and G.R. Crabtree. 1994. NF-AT components define a family of transcription factors targeted in T-cell activation. *Nature*. 369:497–502. doi:10.1038/369497a0

Robinson, M.J., F. Osorio, M. Rosas, R.P. Freitas, E. Schweighoffer, O. Gross, J.S. Verbeek, J. Ruland, V. Tybulewicz, G.D. Brown, et al. 2009. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J. Exp. Med.* 206:2037–2051. doi:10.1084/jem.20082818

Roilides, E., H. Katsifa, S. Tsaparidou, T. Stergiopoulou, C. Panteliadis, and T.J. Walsh. 2000. Interleukin 10 suppresses phagocytic and antihyphal activities of human neutrophils. *Cytokine*. 12:379–387. doi:10.1006/cyto.1999.0567

Saijo, S., N. Fujikado, T. Furuta, S.H. Chung, H. Kotaki, K. Seki, K. Sudo, S. Akira, Y. Adachi, N. Ohno, et al. 2007. Dectin-1 is required for host defense against *Pneumocystis carinii* but not against *Candida albicans*. *Nat. Immunol.* 8:39–46. doi:10.1038/ni1425

Schelenz, S. 2008. Management of candidiasis in the intensive care unit. *J. Antimicrob. Chemother.* 61:i31–i34. doi:10.1093/jac/dkm430

Tavares, D., P. Ferreira, and M. Arala-Chaves. 2000. Increased resistance to systemic candidiasis in athymic or interleukin-10-depleted mice. *J. Infect. Dis.* 182:266–273. doi:10.1086/315674

Taylor, P.R., S.V. Tsoni, J.A. Willment, K.M. Dennehy, M. Rosas, H. Findon, K. Haynes, C. Steele, M. Botto, S. Gordon, and G.D. Brown. 2007. Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat. Immunol.* 8:31–38. doi:10.1038/ni1408

Tharayil John, G., V. Shankar, G. Talaulikar, M.S. Mathews, M. Abraham Abraham, P. Punnakuzhathil Thomas, and C. Korula Jacob. 2003. Epidemiology of systemic mycoses among renal-transplant recipients in India. *Transplantation*. 75:1544–1551. doi:10.1097/01.TP.0000061610.34110.04

Vecchiarelli, A., E. Cenci, P. Marconi, R. Rossi, C. Riccardi, and F. Bistoni. 1989. Immunosuppressive effect of cyclosporin A on resistance to systemic infection with *Candida albicans*. *J. Med. Microbiol.* 30:183–192. doi:10.1099/00222615-30-3-183

Wells, C.A., J.A. Salvage-Jones, X. Li, K. Hitchens, S. Butcher, R.Z. Murray, A.G. Beckhouse, Y.L. Lo, S. Manzanero, C. Cobbold, et al. 2008. The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to *Candida albicans*. *J. Immunol.* 180:7404–7413.

Woo, M., D. Przepiorka, C. Ippoliti, D. Warkentin, I. Khouri, H. Fritsche, and M. Körbling. 1997. Toxicities of tacrolimus and cyclosporin A after allogeneic blood stem cell transplantation. *Bone Marrow Transplant.* 20:1095–1098. doi:10.1038/sj.bmt.1701027

Xu, S., J. Huo, K.G. Lee, T. Kurosaki, and K.P. Lam. 2009. Phospholipase Cgamma2 is critical for Dectin-1-mediated Ca2+ flux and cytokine production in dendritic cells. *J. Biol. Chem.* 284:7038–7046. doi:10.1074/jbc.M806650200

Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science*. 301:640–643. doi:10.1126/science.1087262

Yamasaki, S., M. Matsumoto, O. Takeuchi, T. Matsuzawa, E. Ishikawa, M. Sakuma, H. Tateno, J. Uno, J. Hirabayashi, Y. Mikami, et al. 2009. C-type lectin Mincle is an activating receptor for pathogenic fungus, Malassezia. *Proc. Natl. Acad. Sci. USA*. 106:1897–1902. doi:10.1073/pnas.0805177106

Zanoni, I., R. Ostuni, G. Capuano, M. Collini, M. Caccia, A.E. Ronchi, M. Rocchetti, F. Mingozzi, M. Foti, G. Chirico, et al. 2009. CD14 regulates the dendritic cell life cycle after LPS exposure through NFAT activation. *Nature*. 460:264–268. doi:10.1038/nature08118