JEM

SUPPLEMENTAL MATERIAL

Green blatt et al., http://www.jem.org/cgi/content/full/jem.20092531/DC1

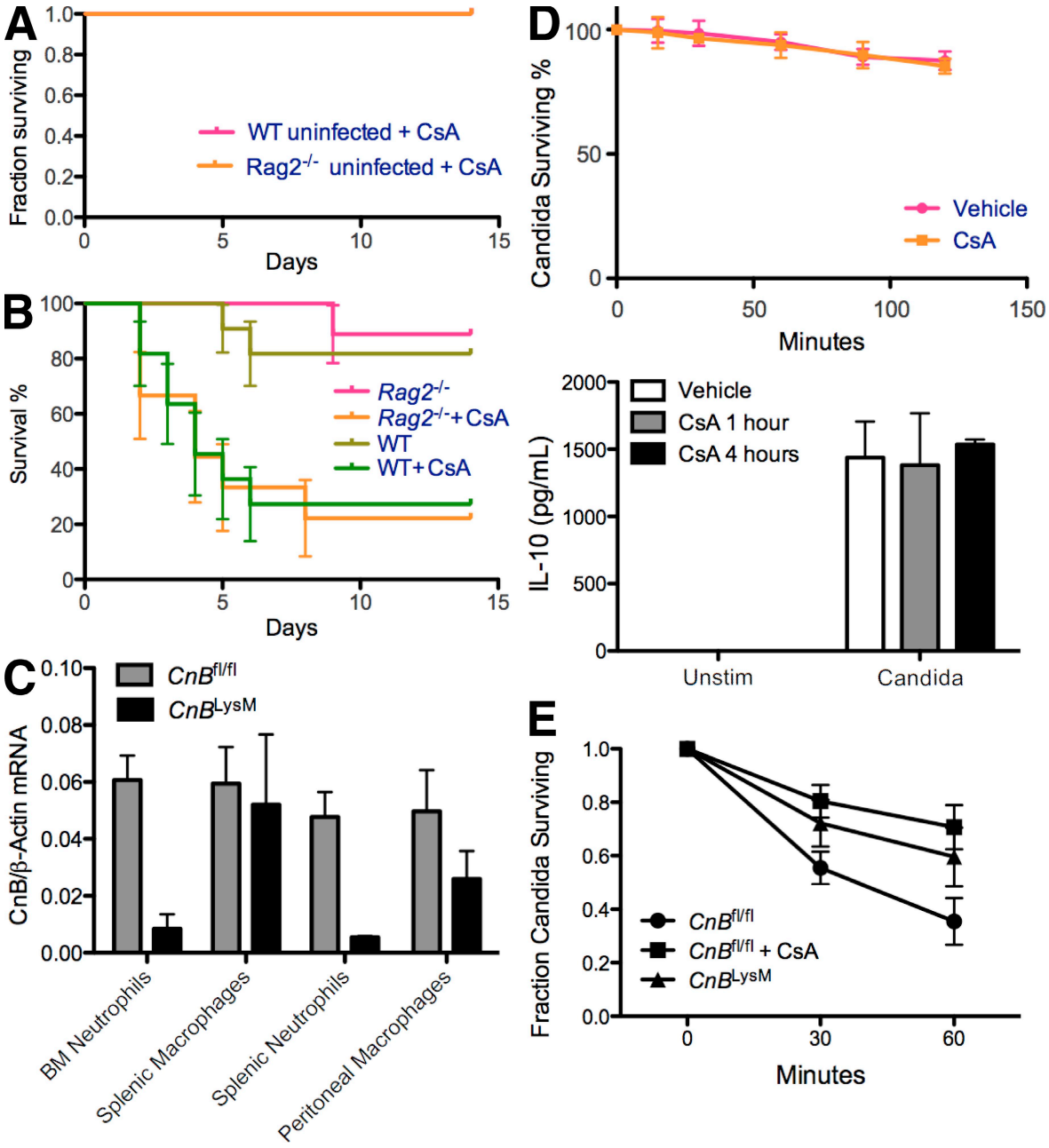

Figure S1. Controls for in vivo and in vitro experiments using *C. albicans*. (A) WT and *Rag2*$^{-/-}$ mice were treated with 200 mg/kg CsA and monitored for survival. $n = 4$ per group. (B) Kaplan-Meier survival curve showing the survival of *C. albicans*–infected mice.*Rag2*$^{-/-}$ or WT control 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. $P \leq 0.005$ 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*^{LysM} and *CnB*$^{fl/fl}$ mice and their expression of CnB 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 ELISA. (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.

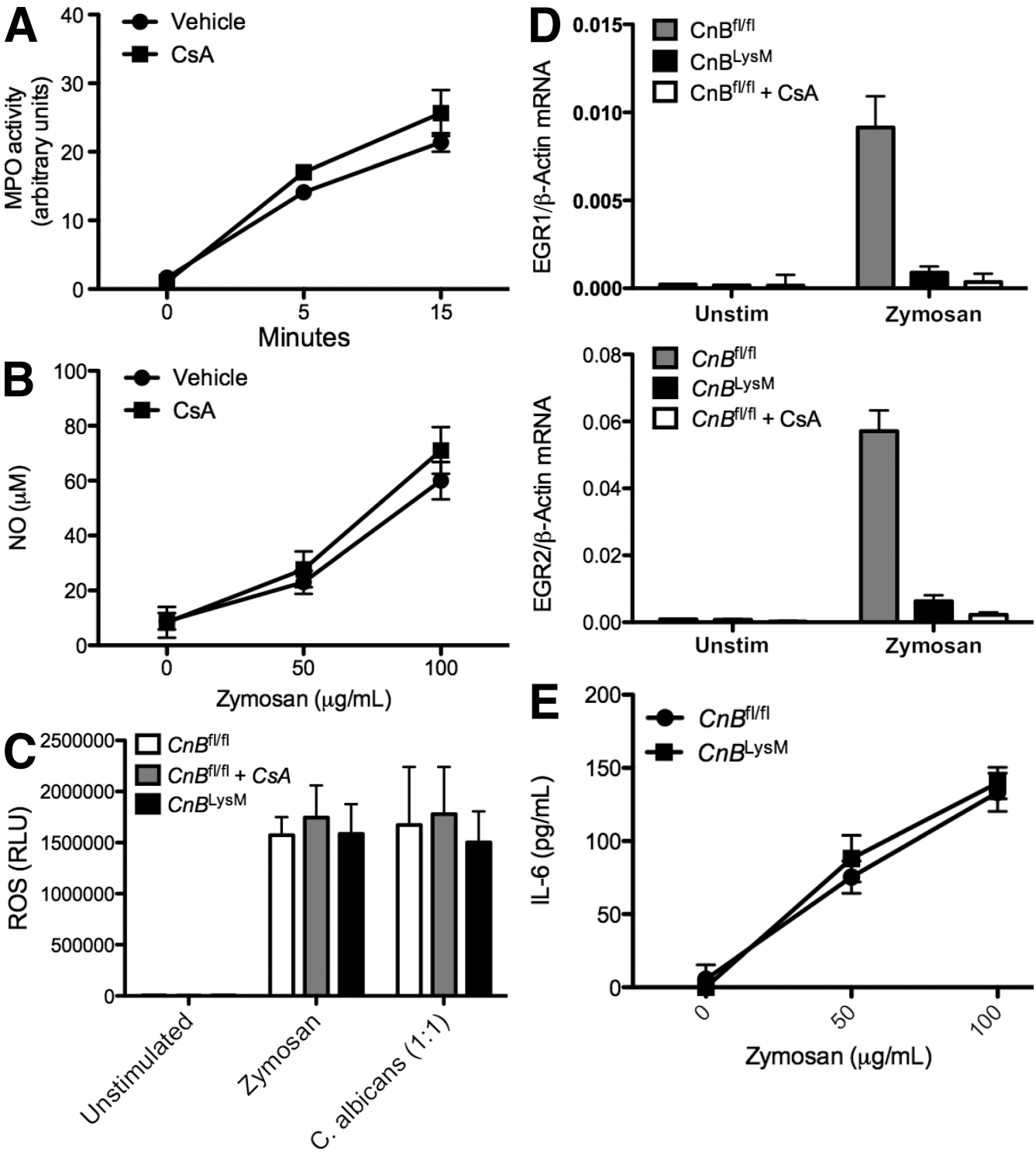

Figure S2. Further characterization of CnB-deficient neutrophils. (A) Primary neutrophils were pretreated with CsA and stimulated with 100 µg/ml zymosan. Degranulation was monitored by detection of supernatant myeloperoxidase (MPO) activity. Supernatant was mixed at a 1:3 ratio with peroxidase substrate (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 H_3PO_4). (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^{LysM} and $CnB^{fl/fl}$ mice and stimulated with 100 µg/ml zymosan. IL-6 secretion was determined 12 h later by ELISA. Error bars show SD.

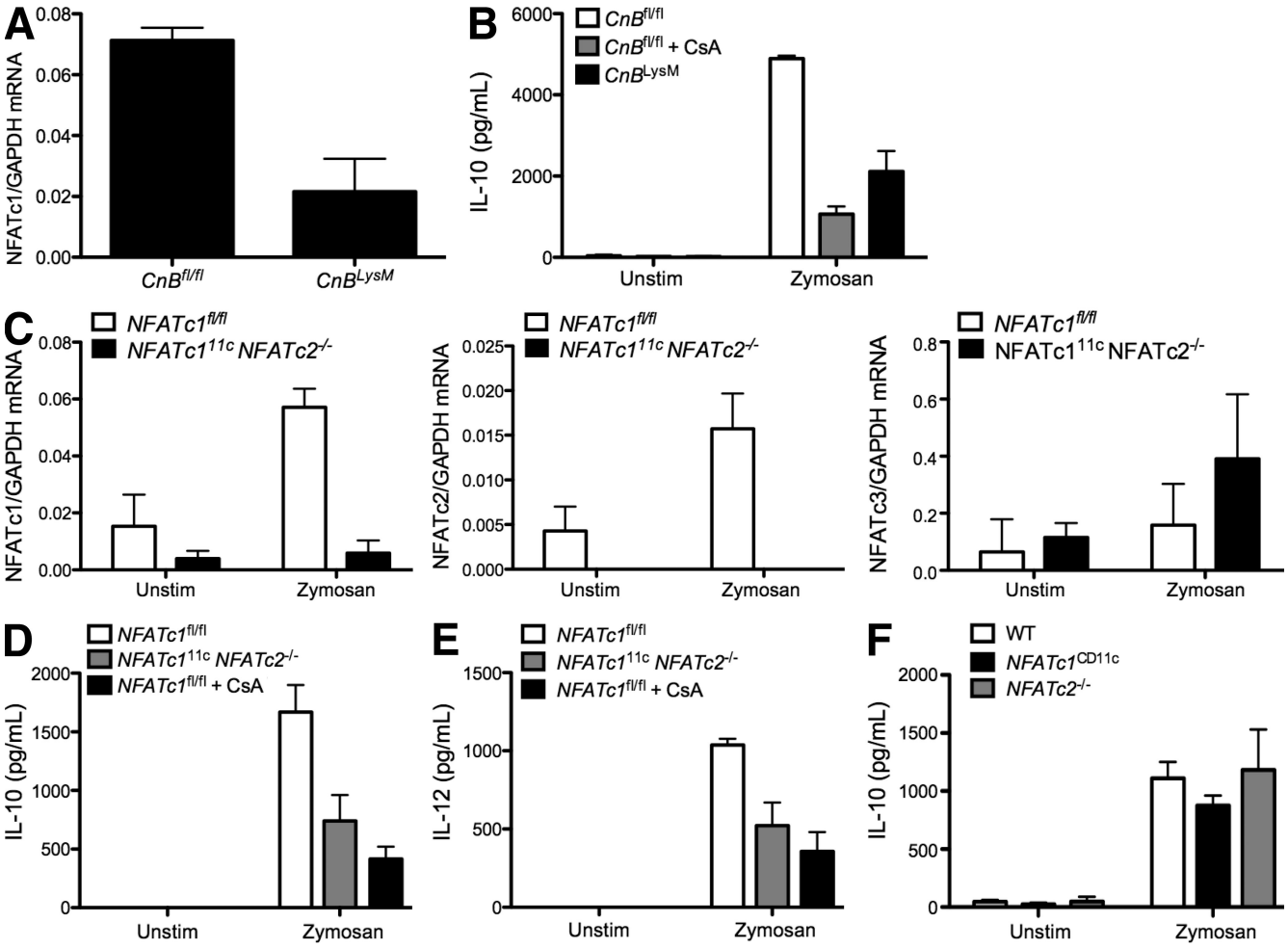

Figure S3. CnB and NFATs function in macrophages and dendritic cells. (A) Splenocytes were isolated from *CnB*^{LysM} and control mice, cultured in 20 ng/mL M-CSF. Nonadherent cells were removed by daily washes. At the end of 5 d, the remaining macrophages were stimulated with 100 µg/ml zymosan and analyzed 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.

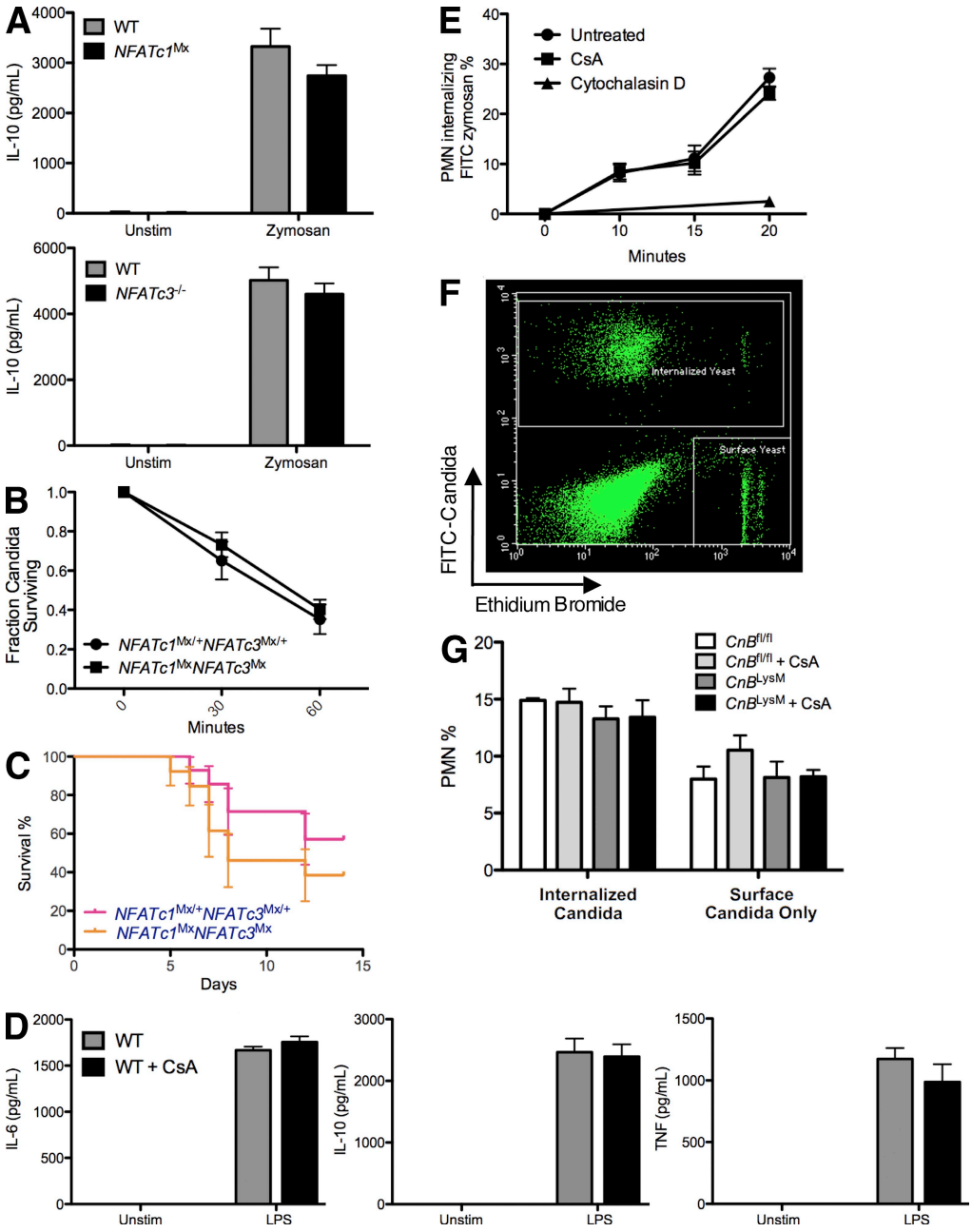

Figure S4. Contribution of NFATc1 and NFATc3 to antifungal responses by neutrophils, effect of CsA on phagocytosis, and responses to LPS. (A) Primary neutrophils were isolated from mice with the indicated genotypes and stimulated with 100 µg/ml zymosan. 12 h later, supernatants were harvested and IL-10 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 10^5 *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 on the supernatant. (E) Neutrophils were pretreated with CsA for 15 min and incubated with FITC-zymosan. At the indicated times, 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.