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Supplemental Information

Vav Proteins Are Key Regulators of Card9 Signaling for Innate Antifungal Immunity

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Figure S 1

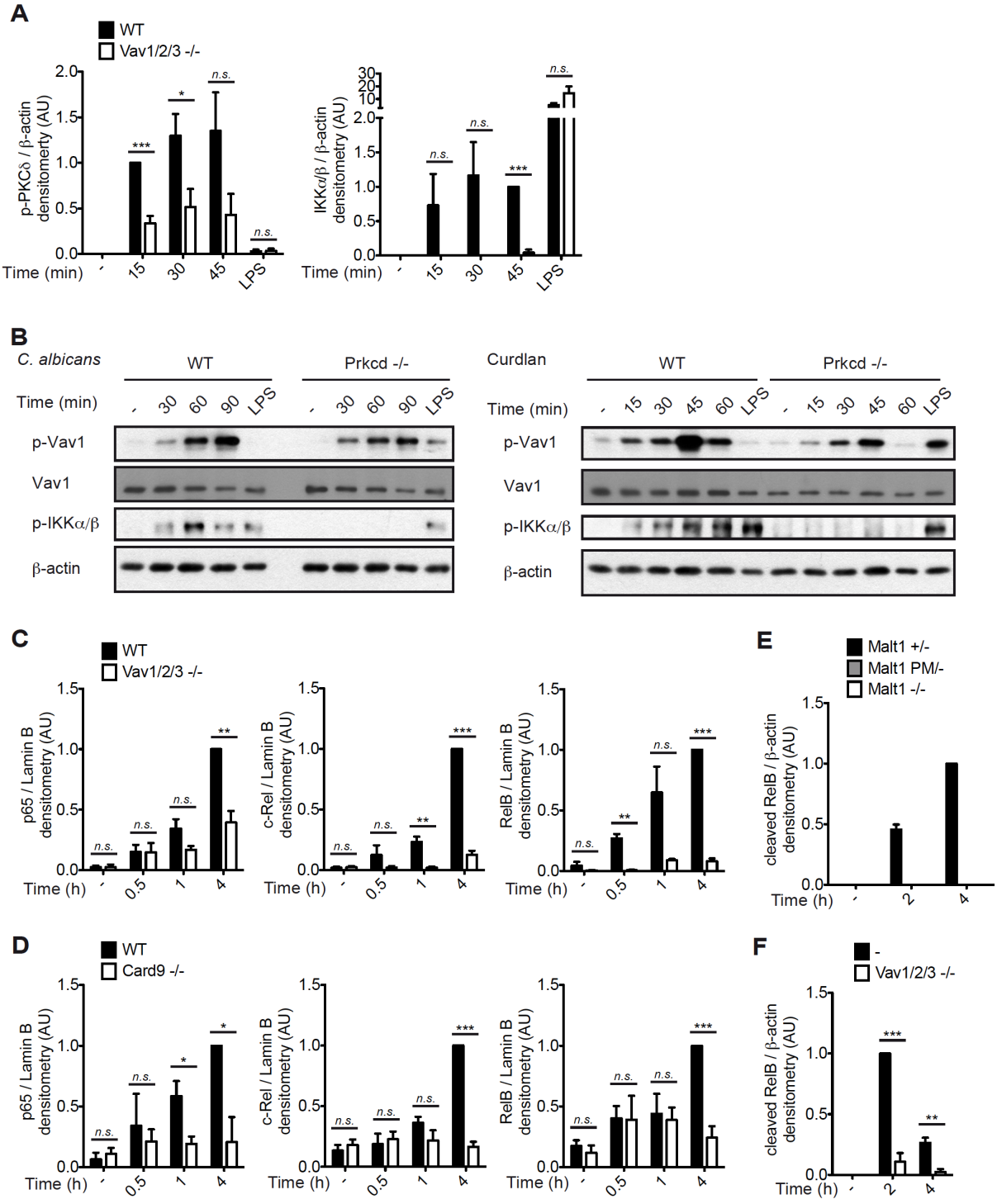

Figure S 1. Vav proteins control CLR-triggered NF-κB activation, Related to Figure 4. (A, C, D) BMDCs from the indicated genotypes were stimulated with *C. albicans* hyphae (MOI 0.5) for various times or with LPS (100 ng/ml) for 45 minutes. (A) Cell lysates were analyzed by immunoblot with anti-phospho-PKCδ and anti-phospho-IKKα/β antibodies. Densitometrical quantification of phospho-PKCδ (left) and phospho-IKKα/β (right) relative to β-actin, normalized to WT BMDCs treated with *C. albicans* for 15 minutes (left) or 45 minutes (right). (B) BMDCs from WT or *Prkcd*$^{-/-}$ mice were stimulated with *C. albicans* hyphae (MOI 0.5) (left) or curdlan (0.5 mg/ml) (right) for various times, or with LPS (100 ng/ml) for 30 minutes. Cell lysates were

analyzed by immunoblot with anti-phospho-Vav1 (Tyr174), anti-Vav1, and anti-phospho-IKKα/β antibodies. β-actin serves as a loading control. Representative data of two independent experiments are shown. (C, D) Nuclear extracts were analyzed by immunoblot with antibodies against the NF-κB subunits p65, c-Rel and RelB. Quantification of nuclear p65, c-Rel and RelB by densitometry, relative to Lamin B, normalized to WT BMDCs treated with *C. albicans* hyphae for 4 hours. (E, F) BMDCs from mice of the indicated genotypes were pretreated with the proteasome inhibitor MG132 for 30 minutes, and then stimulated with curdlan (0.5 mg/ml) (E) or *C. albicans* hyphae (MOI 1) (F) for 2 or 4 hours. Cell lysates were analyzed by immunoblot using antibodies against RelB and β-actin. Densitometrical quantification of cleaved RelB relative to β-actin, normalized to WT BMDCs treated with curdlan for 4 hours (E), or with *C. albicans* for 2 hours (F). (A, C-F) Data of at least three independent experiments are shown as means (±s.e.m.). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, Student's *t*-test. Not significant (*n.s.*). Arbitrary units (AU).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

***C. albicans* infections.** Mice were infected with 1×10^5 colony-forming units (c.f.u) of *C. albicans* (strain SC5314) as described (Gross et al., 2006) and monitored daily for health and survival according to institutional guidelines. To determine fungal burden, organ homogenates were plated in dilutions on CHROMagar (BD Biosciences). For histological analyses, kidney sections were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) according to standard protocols. Six hours after infection, serum was collected to analyze cytokine levels by cytometric bead array (CBA; BD Biosciences).

Administration of TDM. Squalane-in-water emulsions containing TDM (Trehalose 6,6′-dimycolate from *Mycobacterium bovis,* Sigma) were prepared by ultrasonic treatment as previously described (Yarkoni and Rapp, 1978). The final concentrations of emulsion components were as follows: 1.5 or 0 mg/ml (vehicle control) TDM, 9% squalane (Sigma), 1% Tween 80 (Sigma), and 90% saline. The TDM emulsions or the squalane-tween-saline vehicle controls were administered to groups of animals via tail vein injection (0.1 ml per mouse). After 24 hours, serum was collected to determine cytokine levels by CBA (BD Biosciences).

Antibodies. Primary antibodies anti-Caspase-1, p10 (sc-514), anti-c-Rel (sc-71), anti-lamin B (sc-6217), anti-phospho-Vav (Tyr 174-R, sc-16408-R), and anti-Vav (sc-132) were from Santa Cruz, and anti-NF-κB p65 (4764), anti-phospho-Erk1/2, (Thr202/Tyr204, 9101), anti-phospho-IKKα/β (Ser176/180, 2697), anti-phospho-JNK (Thr183/Tyr185, 9251), anti-phospho-p38 (Thr180/Tyr182, 4511), anti-phospho-PLCγ2 (Tyr759, 3874), anti-phospho-Syk (Tyr525/526, 2711), anti-phospho-PKCδ (Tyr311, 2055), anti-phospho-tyrosine (9411), and anti-RelB (4922) were purchased from Cell Signaling. Anti-β-actin was purchased from Sigma.

Real-time quantitative PCR. Total RNA was isolated and transcribed using standard methods. The specific primer pairs were as follows: IL-1β, 5′-TGTAATGAAAGACGGCACACC-3′ and 5′-TCTTCTTTGGGTATTGCTTGG-3′; β-actin, 5′-AGACCTCTATGCCAACACAG-3′ and 5′-TCGTACTCCTGCTTGCTGAT-3′. The qPCR Core kit for SYBR Green I (Eurogentec) and a LightCycler 480 Real-Time PCR System were used as indicated by the manufacturers. IL-1β mRNA expression was calculated as the ratio of the real-time PCR signal for IL-1β mRNA to that of the β-actin mRNA.

RNA-seq library preparation and data analysis. Total RNA was purified using TRIzol Reagent (Invitrogen) and the RNeasy Mini kit (QIAGEN) with on-column DNase treatment. Purified RNA was submitted to the Genomics & Proteomics Core Facility at the DKFZ (Heidelberg, Germany) where it was subjected to library preparation using the Illumina TruSeq RNA sample preparation kit v2. Libraries were pooled (three samples per lane) and sequenced on an Illumina HiSeq 2000 (50–base pair single-end reads). The raw data quality of all libraries was analyzed using FastQC (v. 0.11.1). Quality trimming and adapter removal was performed using the FASTX-Toolkit (v. 0.0.14). The fastq files were then mapped to the mouse genome downloaded from Ensembl (genome build NCBI37) using TopHat2 (Kim et al., 2013) (v. 2.4.2a). Mapped reads were processed through featureCount (Liao et al., 2014) (v. 1.4.6-p4) at the gene level to account for the number of reads per gene in all samples. This was followed by differential expression analysis using the DESeq2 (Love et al., 2014) (v. 1.8.2) package of R (v. 3.2.0). Count data were normalized using the size factor to estimate the effective library size (Anders and Huber, 2010). After calculating gene dispersion across all samples, a pair-wise comparison of two different conditions resulted in a list of differentially expressed genes for the stimulated wild-type vs. knockout samples. Genes with an adjusted p-value <= 0.1 were defined as differentially expressed and were used for downstream analyses. The p-values were adjusted for multiple testing to reduce the false discovery rate (FDR). Gene set enrichment analysis (GSEA) for NF-κB was performed as previously described (Subramanian et al., 2005). The NF-κB target gene set was taken from earlier studies (Compagno et al., 2009). GSEA was performed on a pre-ranked gene list based on their normalized mean read counts from the DESeq2 analysis. This list was then compared with the list of 120 NF-κB target genes to compute the GSEA enrichment scores. The algorithm implements weighted scores and was run with 1000 permutation tests.

Candidemia patients, control volunteer cohorts, and genotyping. In this study, we included 227 unrelated adult candidemia patients of European genetic background. Patients were enrolled after confirmation of at least one positive blood culture for a *Candida* species. The control cohort of 176 volunteers of European descent consisted of non-*Candida*-infected matched patients from the same clinical departments as the patient cohort. Controls were recruited consecutively from the same hospital wards as infected patients during the study period, with a similar balance of medical, surgical, and oncology patients. Review boards of the involved medical centers approved the study, and patients were enrolled after giving written informed consent (described in detail in (Jaeger et al., 2015)). Patients and control volunteers were genotyped on the Illumina Immunochip SNP array platform, which contains approximately 200,000 SNPs focused on genomic regions known to be involved in immune-mediated diseases (Trynka et al., 2011). After the application of quality control filters, we investigated

whether SNPs in the *VAV* genes influence susceptibility to candidemia in the case-control association. The publicly available SNAP server was used to plot the SNP of interest (rs4914950)(Johnson et al., 2008; Saxena et al., 2007).

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