Figure S1

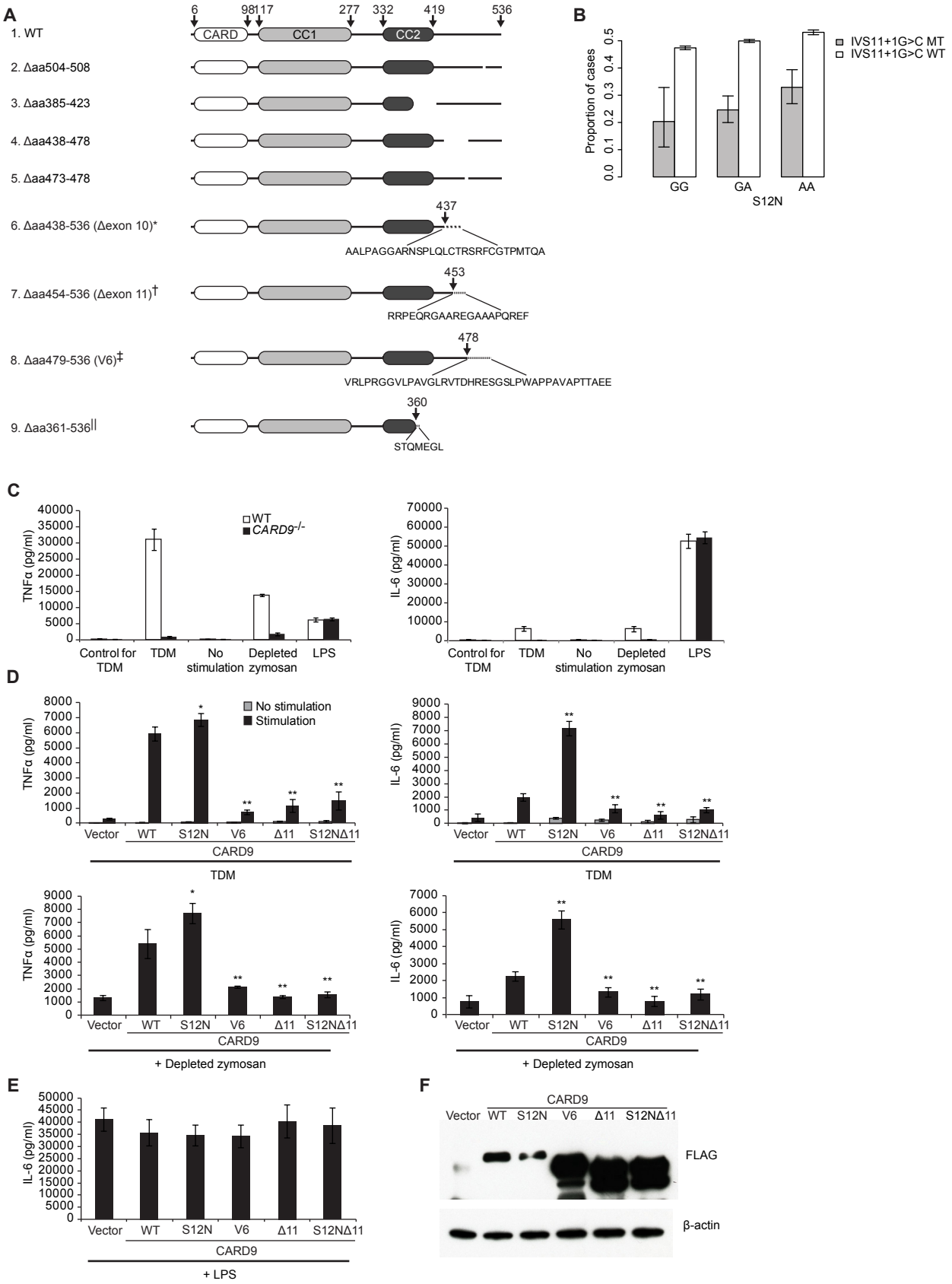
A
1. WT
2. Δaa504-508
3. Δaa385-423
4. Δaa438-478
5. Δaa473-478
6. Δaa438-536 (Δexon 10)*
7. Δaa454-536 (Δexon 11)†
8. Δaa479-536 (V6)‡
9. Δaa361-536||
CARD
CC1
CC2
AALPAGGARNSPLQLCTRSRFCGTPMTQA
RRPEQRGAAREGAAAPQREF
VRLPRGGVLPAVGLRVTDHRESGSLPWAPPAVAPTTAEE
STQMEGL
B
IVS11+1G>C MT
IVS11+1G>C WT
Proportion of cases
GG
GA
AA
S12N
C
WT
CARD9-/-
TNFα (pg/ml)
IL-6 (pg/ml)
Control for TDM
TDM
No stimulation
Depleted zymosan
LPS
D
No stimulation
Stimulation
Vector
WT
S12N
V6
Δ11
S12NΔ11
CARD9
TDM
+ Depleted zymosan
E
+ LPS
F
FLAG
β-actin

Figure S1. CARD9 Variants Show Impaired Cytokine Responses, Related to Figure 1. **(A)** Identification of CARD9 variants: the Clontech human immune cDNA panel was screened by targeting the first or second and last exons of *CARD9* mRNA. Amplicons from cDNA libraries derived from spleen, lymph node, and peripheral blood leukocytes were subsequently cloned and sequenced. These variants were also identified in the presence of the S12N polymorphism. Some identified variants contained combinations of the deletions shown here (e.g., a variant with deletions for aa 385-423 and aa 504-508). *Δaa438-536 (Δexon 10): deletion of exon 10 results in truncation of WT protein at aa437; frameshift results in non-native 29 aa appended at C terminus. †Δaa454-536 (Δexon 11): deletion of exon 11 results in truncation of WT protein at aa453; frameshift results in non-native 20 aa appended at C terminus. ‡Δaa479-536 (V6): insertion of partial or whole intron between exons 11 and 12 results in truncation of WT protein at aa478; translation of intron results in non-native 39 aa appended at C terminus. ||Δaa361-536: insertion of partial intron between exons 7 and 8 results in truncation of WT protein at aa360; translation of intron results in non-native 7 aa appended at C terminus. **(B)** CARD9 Δ11 is a protective variant for IBD. 33,311 IBD cases and 33,938 healthy controls from the International Inflammatory Bowel Disease Genetics Consortium were used for Immunochip analysis. All samples have European ancestry and have gone through standard QC pipelines. 166 cases and 441 controls carry at least one copy of the IV11+1G>C splice variant. The proportion of cases was conditioned on the combination of the splice and the S12N variants. Shaded/white bars show the proportions of cases for samples with/without the splice variant (IVS11+1G>C). On the x-axis, A allele is the S12N variant and the G allele is the wild-type allele. Error bars show the 95% binomial proportion confidence interval. The OR among SS homozygotes is 0.27 [0.13-0.52], and the OR among NN homozygotes is 0.44 [0.33-0.58]. Fisher's exact test *P* values are 2E-5, 5E-20, and 1E-9 respectively for 0/1/2 copies of S12N variants. For samples without the splice variant, the S12N mutation is then unsurprisingly significant associated with the proportion of cases (Armitage chi-square trend test $P < 2E-16$). **(C-F)** CARD9 truncations impair TDM- and depleted zymosan-induced cytokine production. **(C)** WT or *Card9*$^{-/-}$ BMDCs were stimulated with 100 μg/ml depleted zymosan, 1 μg/well TDM, or 100 ng/ml LPS for 24 h and cytokine levels were assessed by ELISA. Data were obtained from four independent experiments performed in triplicate (n = 4). **(D-E)** *Card9*$^{-/-}$ BMDCs transduced with indicated CARD9 variants were stimulated with 100 μg/ml depleted zymosan, 1 μg/well TDM, or 100 ng/ml LPS

and cytokine levels were assessed by ELISA. Data were obtained from four independent experiments performed in duplicate (n = 4). **(F)** Expression of CARD9 variants in total lysates used in (D-E) as detected by Western blot. Bars represent means ± s.d. *$P < 0.05$, ** $P < 0.005$. Comparisons are relative to stimulated CARD9 WT in (D) and (E).

Figure S2

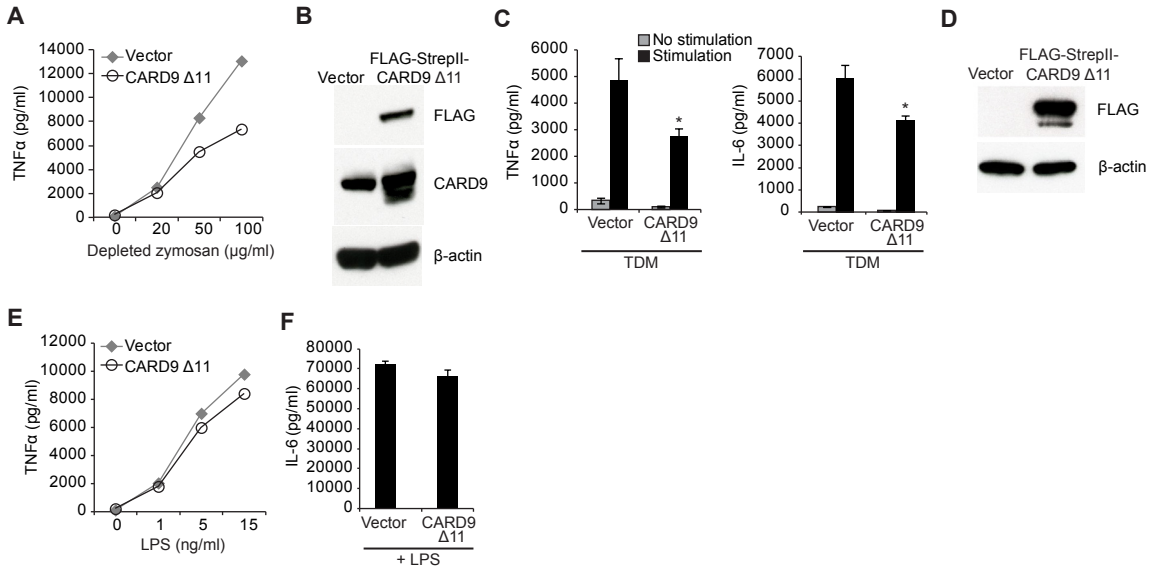
A
Vector
CARD9 Δ11
TNFα (pg/ml)
Depleted zymosan (μg/ml)
B
FLAG-StrepII-
Vector CARD9 Δ11
FLAG
CARD9
β-actin
C
No stimulation
Stimulation
TNFα (pg/ml)
IL-6 (pg/ml)
Vector
CARD9 Δ11
TDM
D
FLAG-StrepII-
Vector CARD9 Δ11
FLAG
β-actin
E
Vector
CARD9 Δ11
TNFα (pg/ml)
LPS (ng/ml)
F
IL-6 (pg/ml)
Vector
CARD9 Δ11
+ LPS

Figure S2. CARD9 Δ11 Acts in a Dominant Negative Manner, Related to Figure 2.
(A) Human MDDCs were transduced, stimulated, and cytokine levels assessed by ELISA. Experiments were performed in triplicate; similar results were obtained from two additional donors. **(B)** CARD9 expression in lysates from (A) and (E) assessed by Western blot. **(C)** WT murine BMDCs were transduced, stimulated, and cytokine levels assessed by ELISA. Data were obtained from three independent experiments performed in triplicate (n = 3). **(D)** CARD9 expression in lysates used in (C) and (F) assessed by Western blot. **(E)** Human MDDCs were transduced, stimulated, and cytokine levels assessed by ELISA as in (A). **(F)** WT murine BMDCs were transduced, stimulated, and cytokine levels assessed by ELISA as in (C). Bars represent means ± s.d. $*P < 0.05$.

Figure S3

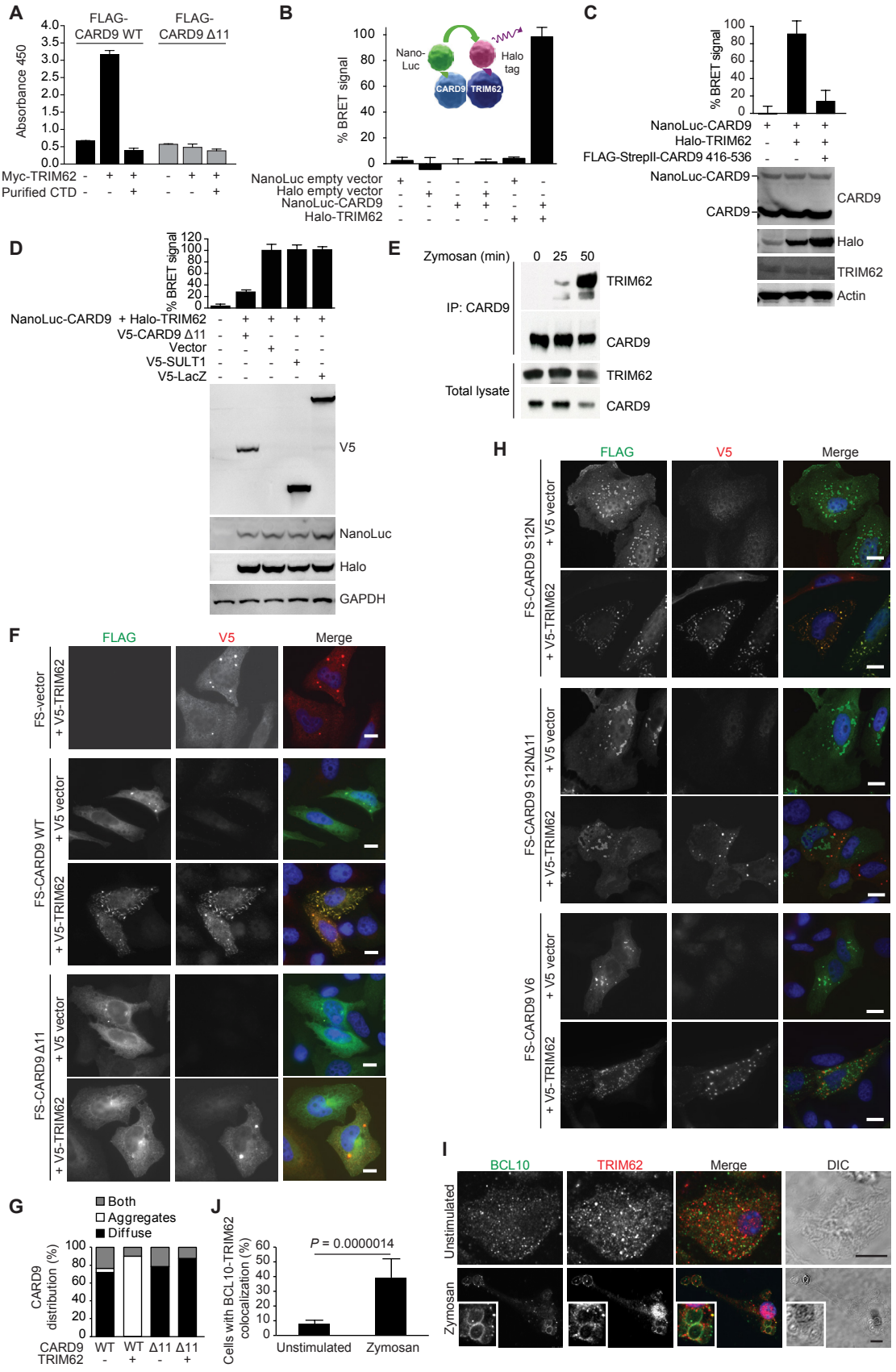
A
FLAG-CARD9 WT
FLAG-CARD9 Δ11
Absorbance 450
Myc-TRIM62
Purified CTD
B
% BRET signal
Nano-Luc
Halo tag
CARD9
TRIM62
NanoLuc empty vector
Halo empty vector
NanoLuc-CARD9
Halo-TRIM62
C
% BRET signal
NanoLuc-CARD9
Halo-TRIM62
FLAG-StrepII-CARD9 416-536
CARD9
Halo
TRIM62
Actin
D
% BRET signal
NanoLuc-CARD9 + Halo-TRIM62
V5-CARD9 Δ11
Vector
V5-SULT1
V5-LacZ
V5
NanoLuc
Halo
GAPDH
E
Zymosan (min)
0 25 50
IP: CARD9
TRIM62
CARD9
Total lysate
TRIM62
CARD9
F
FLAG
V5
Merge
FS-vector
+ V5-TRIM62
FS-CARD9 WT
+ V5 vector
+ V5-TRIM62
FS-CARD9 Δ11
+ V5 vector
+ V5-TRIM62
G
Both
Aggregates
Diffuse
CARD9 distribution (%)
CARD9
TRIM62
WT - WT + Δ11 - Δ11 +
H
FLAG
V5
Merge
FS-CARD9 S12N
+ V5 vector
+ V5-TRIM62
FS-CARD9 S12NΔ11
+ V5 vector
+ V5-TRIM62
FS-CARD9 V6
+ V5 vector
+ V5-TRIM62
I
BCL10
TRIM62
Merge
DIC
Unstimulated
Zymosan
J
P = 0.0000014
Cells with BCL10-TRIM62 colocalization (%)
Unstimulated
Zymosan

Figure S3. CARD9 Variants Interact with TRIM62, Related to Figure 3.

(A) ELISA showing the interaction between differentially tagged CARD9 constructs and TRIM62 using lysates containing FLAG-CARD9 or FLAG-CARD9Δ11 and 3x-Myc-TRIM62, individually expressed in HEK293T cells. Interactions were measured with or without purified CTD (C-terminal domain of CARD9, aa 416-536). **(B)** Schematic shows a BRET system in which nano-luciferase-tagged CARD9 (donor) is paired with Halo-tagged TRIM62 (acceptor) to generate BRET signal. Indicated constructs were expressed in HEK293T cells and the normalized BRET signal plotted for each condition. **(C)** Indicated constructs were transfected in HEK293T cells and BRET signal was monitored to detect interactions between CARD9 and TRIM62 in the context of co-expression of FLAG-StrepII-CARD9$_{416-536}$; Western blots show expression levels of endogenous and tagged protein in cells used for the BRET assay. **(D)** Indicated constructs were transfected in HEK293T cells and BRET signal was monitored to detect interactions between CARD9 and TRIM62 in the context of co-expression of CARD9 Δ11 or negative controls (vector alone, V5-SULT1, V5-LacZ); Western blot shows protein levels present in cells used for the BRET assay. **(E)** THP-1 cells were stimulated with 100 μg/ml zymosan for the indicated time periods followed by immunoprecipitation of endogenous CARD9. **(F)** TRIM62 and indicated CARD9 variants were expressed alone or in combination in HeLa cells. FS, FLAG-StrepII. Scale bars, 10 μm. **(G)** Immunostaining of cells shown in (F) was quantified based on CARD9 cellular distribution. Data are represented as mean of three independent experiments (n = 3). **(H)** HeLa cells were co-transfected with CARD9 S12N, S12NΔ11, or V6 and either V5-tagged TRIM62 or V5 vector alone. FS, FLAG-StrepII. Scale bars, 10 μm. **(I)** WT BMDCs were stimulated with depleted zymosan and stained for endogenous TRIM62 and BCL10. **(J)** Immunostaining of cells shown in (I) were quantified based on colocalization of TRIM62 and BCL10.

Figure S4

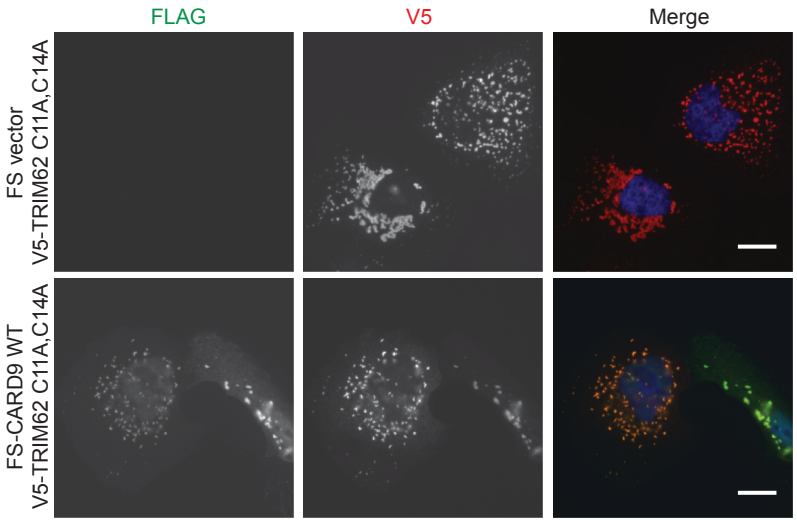
FLAG
V5
Merge
FS vector
V5-TRIM62 C11A,C14A
FS-CARD9 WT
V5-TRIM62 C11A,C14A

Figure S4. E3 Ligase-Dead Mutant TRIM62 C11A,C14A Colocalizes with CARD9, Related to Figure 4. HeLa cells were co-transfected with a TRIM62 ligase-dead variant (C11A,C14A) and either FLAG-StrepII-tagged CARD9 or FLAG-StrepII vector alone. FS, FLAG-StrepII. Scale bars, 10 μm.

Figure S5

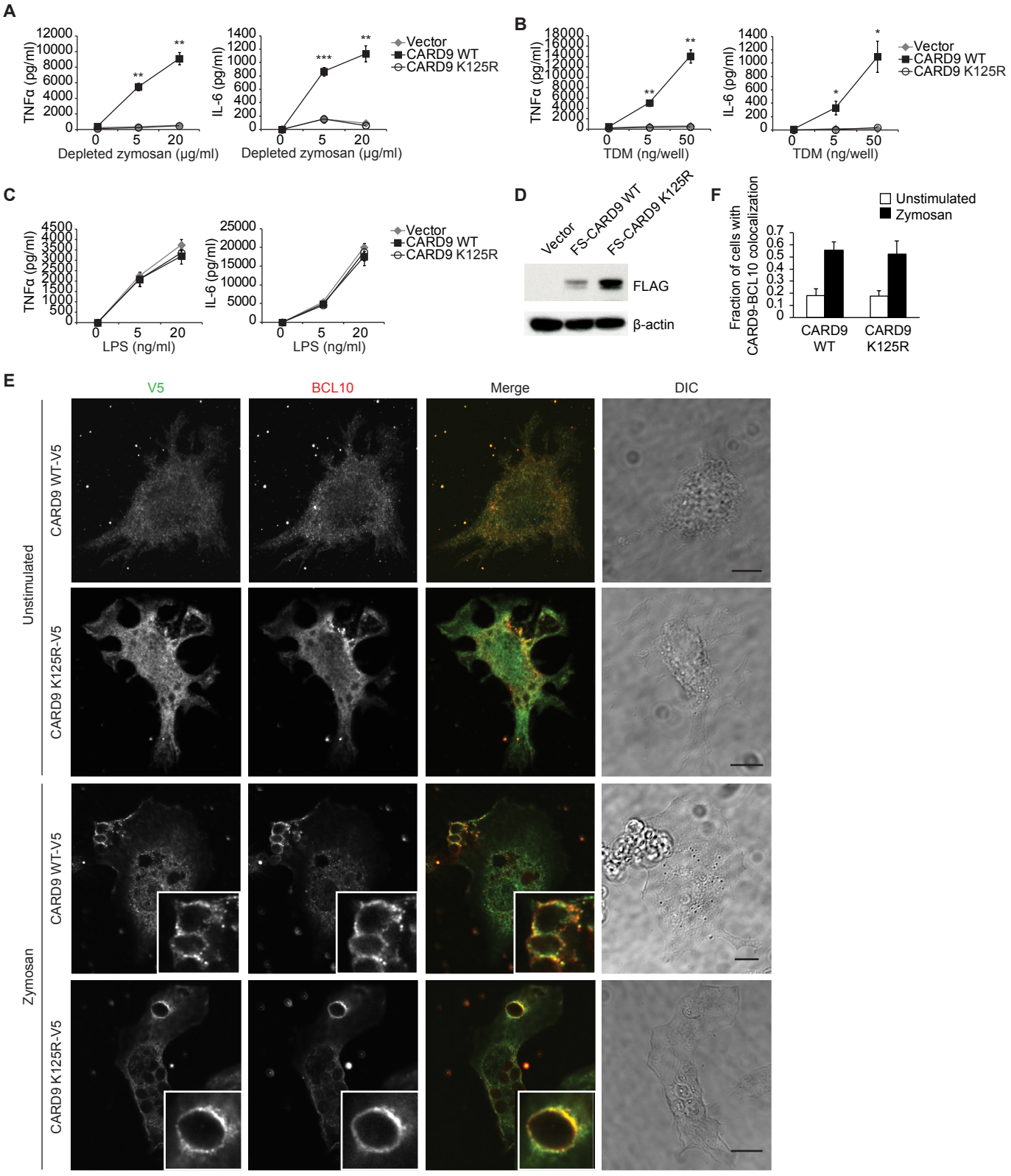
A
TNFα (pg/ml)
IL-6 (pg/ml)
Depleted zymosan (μg/ml)
Vector
CARD9 WT
CARD9 K125R
B
TDM (ng/well)
C
LPS (ng/ml)
D
Vector
FS-CARD9 WT
FS-CARD9 K125R
FLAG
β-actin
F
Unstimulated
Zymosan
Fraction of cells with CARD9-BCL10 colocalization
CARD9 WT
CARD9 K125R
E
V5
BCL10
Merge
DIC
Unstimulated
Zymosan
CARD9 WT-V5
CARD9 K125R-V5

Figure S5. TRIM62-mediated CARD9 Ubiquitination at K125 Is Critical for CARD9 Activation, Related to Figure 5.

(A-C) *Card9*$^{-/-}$ BMDCs were transduced with lentivirus expressing CARD9 WT or CARD9 K125R. Cells were stimulated with (A) depleted zymosan, (B) TDM, or (C) LPS and cytokine levels were assessed by ELISA. Data were obtained from three independent experiments performed in duplicate (n = 3). **(D)** Expression of CARD9 in total lysates from (A-C) as detected by Western blot. **(E)** *Card9*$^{-/-}$ BMDCs were transduced with lentivirus expressing V5-tagged CARD9 WT or CARD9 K125R. Cells were stimulated with depleted zymosan and colocalization was assessed between V5-tagged CARD9 and endogenous BCL10. **(F)** Quantification of the results shown in (E). Bars represent means ± s.d. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Comparisons in (A-C) are relative to stimulated CARD9 WT. FS, FLAG-StrepII.

Figure S6

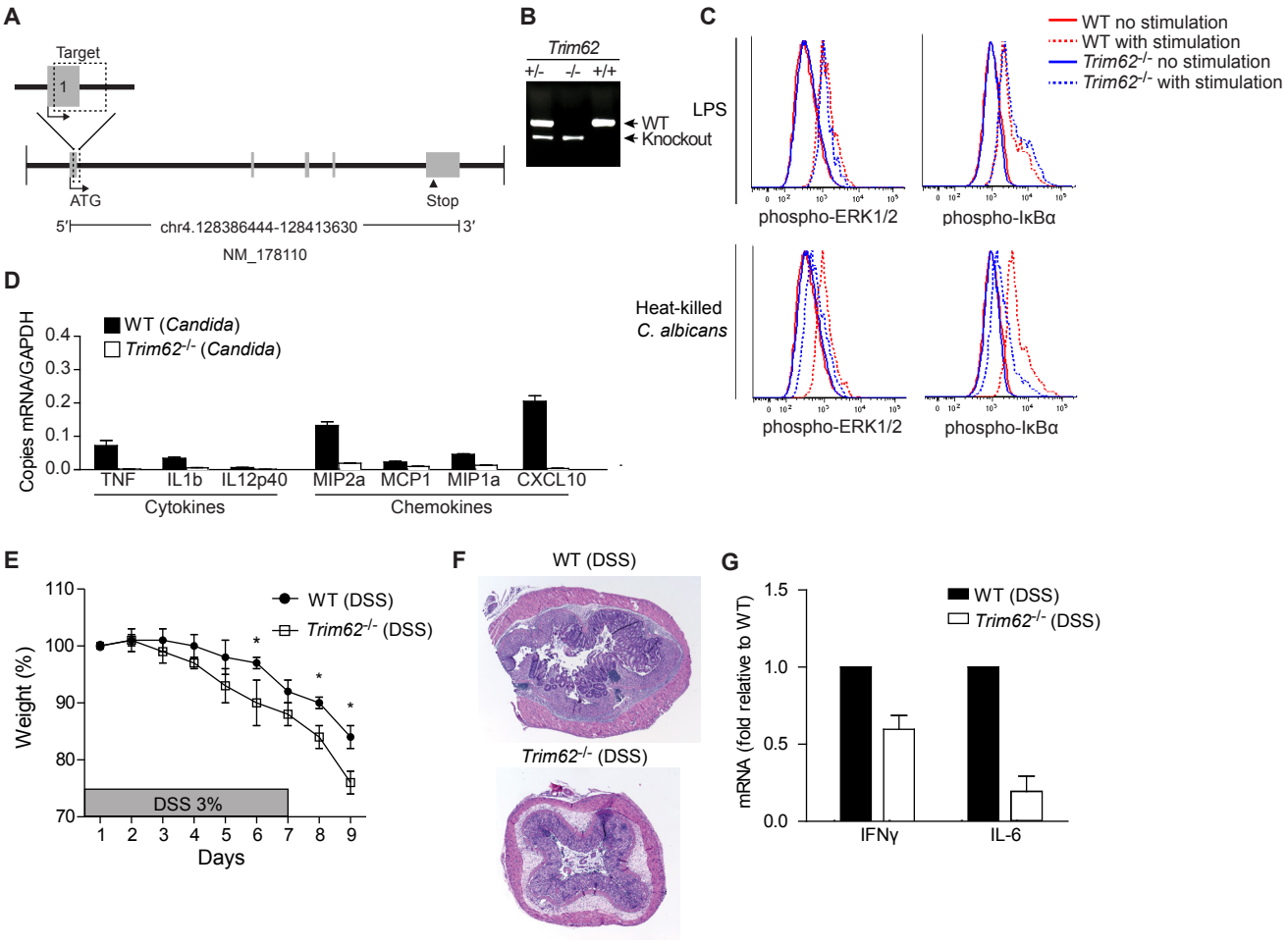
A
Target
1
ATG
Stop
5′
chr4.128386444-128413630
3′
NM_178110
B
Trim62
+/- -/- +/+
WT
Knockout
C
WT no stimulation
WT with stimulation
Trim62-/- no stimulation
Trim62-/- with stimulation
LPS
phospho-ERK1/2
phospho-IκBα
Heat-killed
C. albicans
phospho-ERK1/2
phospho-IκBα
D
WT (Candida)
Trim62-/- (Candida)
Copies mRNA/GAPDH
0.4
0.3
0.2
0.1
0.0
TNF
IL1b
IL12p40
MIP2a
MCP1
MIP1a
CXCL10
Cytokines
Chemokines
E
WT (DSS)
Trim62-/- (DSS)
Weight (%)
110
100
90
80
70
DSS 3%
1 2 3 4 5 6 7 8 9
Days
F
WT (DSS)
Trim62-/- (DSS)
G
WT (DSS)
Trim62-/- (DSS)
mRNA (fold relative to WT)
1.5
1.0
0.5
0.0
IFNγ
IL-6

Figure S6. Characterization of *Trim62*$^{-/-}$ mice, Related to Figure 6.

(A) Targeting strategy for *Trim62*$^{-/-}$ mice. Targeted locus is indicated by dashed square. Deleted genome sequence in the mouse genomic chromosome 4 is TGAGCCTGGGCTGCGAGCACTACTTCTGCCGCCGCTGCATCACCGAGCACTGGGTGC GGCAGGAGGCTCAGGGCGCCCGCGACTGCCCGGAGTGCCGGCGCACGTTCGCCGAG CCCGCGCTCGCGCCCAGCCTCAAGCTGGCCAACATCGTGGAGCGCTACAGCGCCTTC CCGCTGGACGCCATCCTCAACGCGCGCCGAGCCGCGCGACCCTGCCAGGCTCACGA CAAGGTCAAGCTCTTCTGCCTCACGGACCGCGCACTGCTCTGCTTCTTCTGTGATGA GCCCGCGCTGCACGAGCAACACCAGGTCACTGGCATCGACGATGCCTTCGAGGAGT TGCAGGTGCGAGGCGGACAGGCGGGGCTGTAGGTGGGGTCAGGGTTCCAATAGGGG CAAGGGCCACAGGCCGGCTTAATGCCAAAGGGGCGTGGCTCCTGTGAGGGGCGGGG CAGGGGCGGGTCCCTTGAGCATCACTGTGTCAAATGGGCTTGAGAGAGATCTGGAT GGGAGACAGGTCGAGGTGGGATAGGATGATGGAGAGAGGAAGCTACAGCTGGACC AGATGCTGGCTGAGGCTGAGACAGAATGGGAGACAGCAGCTCCTTCTGGACTTTT. **(B)** Agarose gel of PCR-based genotyping confirming deletion of *Trim62*. The following primers were used for genotyping: F primer, ATGGCGTGCA GCCTCAAGGACG; R primers, TGTGGCCCTTGCCCCTATTG for WT allele and CCCTAGGAATGCTCGTCAAGA for knockout allele. **(C)** CD11c^{+} splenocytes were untreated, stimulated with LPS, or stimulated with heat-killed *C. albicans*, and phosphorylated proteins were assessed via intracellular staining and flow cytometry. Representative histogram overlays are shown for each stimulus and phosphoprotein (n = 4). Red lines represent WT mice and blue lines represent *Trim62*$^{-/-}$ mice; solid lines represent unstimulated cells and dashed lines represent stimulated cells. **(D)** Cytokine and chemokine responses in *Trim62*$^{-/-}$ spleen 6 days following infection with live *C. albicans* (n = 4). **(E)** Daily body weight as assessed during and after treatment with DSS. **(F)** H&E staining of WT and *Trim62*$^{-/-}$ mice at day 9 following DSS. **(G)** IL-6 and IFNγ expression levels in colon of WT and *Trim62*$^{-/-}$ mice at day 9 following DSS. *Trim62*$^{-/-}$ data are shown relative to WT mice with DSS. For (E-G), n = 8 WT and n = 7 *Trim62*$^{-/-}$.

Supplemental Experimental Procedures

CARD9 Variant Cloning

Human CARD9 variants were amplified from the human Clontech cDNA panel, which includes spleen, lymph mode, thymus, tonsil, bone marrow, fetal liver, and peripheral blood leukocyte cDNA libraries. CARD9 isoforms were identified by targeting the first or second exon and last exons of full-length CARD9. The following primers were used: **F1**, AAAAAAAGATCTTCCCTGCAGCCCCGGGCAGCATCTC; **F2**, AAAAAAAGATCTCCTGCCTGCTGAGGCCATGTC; **R1,** AAAAAAGCGGCCGCTGGTCGGGGCCTGCGCTGCTGC; **R2,** AAAAAGCGGCCGCGATGGCGTGTGCATGGGGGTGGTGAGCAC.
PCR amplicons from cDNA libraries derived from spleen, lymph node, and peripheral blood leukocytes were subsequently cloned into a pCMV vector (modified Clontech pCMV-Myc vector with no Myc epitope and modifications to MCS) using BglII and NotI.

Preparation and Infection of BMDCs

Littermates were used between 7 and 10 weeks of age and were age-matched for each experiment. To prepare and infect BMDCs, bone marrow stem cells were cultured for 3 days at 37°C and 5% CO_2 in complete RPMI supplemented with 37.5 ng/mL recombinant murine GM-CSF (PeproTech). Suspension cells were removed on day 3. 1 ml of concentrated virus containing 8 μg/ml polybrene (hexadimethrine bromide, Sigma H9268) was added into each cell culture well and cell culture plates were spun for 90 min at room temperature. After spinning down, the virus media was removed and replaced with fresh RPMI and GM-CSF. Cultures were supplemented with fresh RPMI and GM-CSF with 3 μg/ml puromycin on day 5 for shRNA knockdown. Cells used for protein overexpression were transduced again on day 6. Functional assays were performed on day 9. The purity of BMDC cultures was assessed on day 9 by flow cytometry using CD11c antibody (BD Pharmingen). Cultures were at least 88% $CD11c^+$ on day 9.

Human MDDCs

Human monocytes were purified from buffy coat from the blood bank of Massachusetts

General Hospital using a human monocyte enrichment cocktail following a standard protocol supplied by the manufacturer (Stemcell Technologies). Purified monocytes were incubated on 10-cm cell culture plates at 37°C and 5% CO_2 in RPMI1640 with 10% FBS. Two hours later, non-adherent cells were collected and kept at -80°C for other use and adherent cells were continually cultured in RPMI1640 with 10% FBS containing 50 ng/ml human GM-CSF (Promocell) and 100 ng/ml human IL-4 (Promocell) for 7 days. Medium was changed every 2-3 days with RPMI1640 containing 10% FBS, 50 ng/ml human GM-CSF, and 100 ng/ml human IL-4.

Cytokine ELISAs

Murine BMDCs were transduced with appropriate lentivirus on day 3 and functional assays were performed on day 9 in 48-well or 96-well plates. Human MDDCs were co-transduced with appropriate lentivirus and Vpx-VLPs on day 2 and functional assays were performed on day 7. BMDCs and MDDCs were stimulated with depleted zymosan, pre-coated TDM (1 μg/well for 48-well plates [for BMDCs only], or indicated concentration for 96-well plates), LPS, or corresponding solvent. Twenty-four hours after stimulation, cytokine production in the supernatant was detected using appropriate ELISA kits (BD Biosciences) according to the manufacturer's protocol. Serum IL-6 from immunized mice was quantified using ELISA per the manufacturer's protocol (BD Biosciences).

Immunoprecipitation and Western Blotting

BMDCs were collected for Western blot 24 h post stimulation. HEK293T cells were transfected with TransFectin (Bio-Rad) and collected for immunoprecipitation 24 h post transfection. Cells were rinsed in ice-cold PBS and lysed in standard lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% Nonidet P-40, Halt phosphatase inhibitor single-use cocktail (Pierce), and protease inhibitor tablets (Roche)] with rotation for 30 min at 4°C. Insoluble materials were removed by centrifugation.

For Western blots without preceding immunoprecipitation (Figures 1E, 2B, 5D, S1F, S2B, S2D, S5D), equal volumes of cell lysates originating from the same number of cells were loaded.

For immunoprecipitation in HEK293T cells, cell lysates were incubated with StrepII

Sepharose beads (IBA) for 1 h at 4°C. For immunoprecipitation of endogenous CARD9 in THP-1 cells, cell lysates were incubated with anti-CARD9 antibody for 1 h followed by pulldown with Protein A/G PLUS-Agarose (Santa Cruz) by incubation for 1 h. The immunoprecipitates were washed 4 times with standard lysis buffer. Immunoprecipitated proteins and total cell lysates were resolved by SDS-PAGE, transferred to Immobilon-P membranes (Millipore) and immunoblotted with the indicated antibodies. Detection was performed by enhanced chemiluminescence with the Western Lightning Chemiluminescence Reagent (PerkinElmer Life Sciences).

For blots with tagged ubiquitin, cells were lysed in standard lysis buffer with 10 mM N-ethylmaleimide for 30 min. SDS was then added to cell lysates to a final concentration of 1% and lysates were heated at 95°C for 10 min (Bloom and Pagano, 2005). Denatured cell lysates were cooled on ice and diluted with standard lysis buffer to reduce SDS concentration to ≤ 0.1%. Lysates were then incubated for 30 min at 4°C and centrifuged to remove insoluble materials. Cleared cell lysates were incubated with StrepII beads for 90 min at 4°C. The immunoprecipitates were washed exclusively with standard lysis buffer.

To detect endogenous ubiquitination of CARD9, BMDCs from 4 mice were treated on day 8 with 5 μM PR-619 for 30 min, then stimulated with 10 μg/ml depleted zymosan for 1 h with PR-619 remaining in the culture medium. Cells were lysed in 0.75 mL lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% NP-40, 10% glycerol, protease inhibitors (Roche), and 20 mM iodoacetamide) for 10 min at 4°C. Lysate was spun for 10 min at 20,000 rcf. For CARD9 pulldown, 3.75 mg of lysate was used. Supernatants were incubated with 19.6 μg α-CARD9 C-17 (Santa Cruz Biotech, 2 μg/500 μg lysate). Materials were mixed with inversion at 4°C for 2 h. Protein A/G magnetic beads (39.2 μg) were added and incubation continued for 1 h. The beads were then washed 4x with lysis buffer. Protein was eluted by incubation in 100 μL SDS loading buffer at room temperature.

The following antibodies were used: anti-FLAG M2 monoclonal antibody (Sigma); mouse monoclonal anti-β-actin antibody (Sigma); mouse monoclonal anti-hemagglutinin (HA) antibody (Covance); mouse monoclonal anti-Myc antibody (Covance); anti-V5 rabbit polyclonal antibody (Covance); anti-CARD9 (H-90) antibody for immunoprecipitation (Santa Cruz); anti-CARD9 rabbit polyclonal antibody (GeneTex); anti-CARD9 rabbit polyclonal antibody (mouse preferred, Cell Signaling); anti-TRIM62 rabbit polyclonal antibody (Biorbyt); rabbit anti-

CARD9 (H-90, Santa Cruz Biotechnology); and mouse anti-Ub (P4D1, Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies from Dako or Cell Signaling were used as secondary antibodies. To avoid detecting the heavy chain of rabbit IgG, HRP-conjugated IgG fraction monoclonal mouse anti-rabbit IgG (light chain-specific) was used as secondary antibody (Jackson ImmunoResearch).

Tandem Affinity Purification of CARD9

Card9$^{-/-}$ BMDMs were immortalized using J2 virus (Blasi et al., 1985) from AMJ2-C11 cells (ATCC: CRL-2456). Single immortalized *Card9*$^{-/-}$ BMDM cell lines were selected by limiting dilution and cell lines were confirmed by expression of CD11b. Twenty-five 150-mm plates of immortalized *Card9*$^{-/-}$ BMDM cells rescued with either FLAG-StrepII vector control or FLAG-StrepII-tagged CARD9 were grown to 90% confluency. For tandem affinity purification from JAWSII cells, 25 150-mm plates of JAWSII cells rescued with FLAG-StrepII vector control or 50 150-mm plates of JAWSII cells rescued with FLAG-StrepII-tagged CARD9 were grown for 4 days. JAWSII cells rescued with FLAG-StrepII-tagged CARD9 were harvested and split into two equal aliquots and stimulated with or without heat-killed *Candida albicans* strain SC5314 at a multiplicity of infection of 1:2 for 1 h.

Cells were harvested and washed twice with PBS. Cellular extracts were prepared by cell disruption through 15 strokes in a tight-fitting Dounce homogenizer following by two freeze-thaw cycles in standard lysis buffer. The cell extract was incubated with 50 µl anti-FLAG M2 affinity beads (Sigma) for 2-4 h at 4°C. Beads and bound proteins were washed 3 times with lysis buffer and 3 times with StrepII binding buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% glycerol, 0.1% Nonidet P-40, phosphatase inhibitor, and protease inhibitor). Protein complexes were eluted with StrepII binding buffer containing 0.125 mg/ml FLAG peptide (Sigma) for 5 min at 4°C. The elution was repeated once and eluates were pooled. The pooled elution was then incubated with 20-30 µl StrepII Sepharose beads for 1 h at 4°C. StrepII beads were washed 3 times with StrepII binding buffer and eluted with SDS-PAGE loading buffer. Proteins of interest were separated by SDS-PAGE and stained using Colloidal Coomassie GelCode Blue (Pierce). Individual bands were cut from the gel and sent for mass spectrometry analysis at the Taplin Biological Mass Spectrometry Facility of Harvard Medical School.

Shown below (underlined) are fragments of mouse TRIM62 identified by mass

spectrometry from tandem affinity purified-CARD9 from immortalized *Card9*$^{-/-}$ BMDM cells rescued with FLAG-StrepII-tagged CARD9. No fragments of TRIM62 were identified by mass spectrometry from tandem affinity purified-CARD9 from immortalized *Card9*$^{-/-}$ BMDM cells rescued with FLAG-StrepII vector control.

MACSLKDELL CSICLSIYQD PVSLGCEHYF CRRCITEHWV RQEAQGARDC
PECRRTFAEP ALAPSLKLAN IVERYSAFPL DAILNARRAA RPCQAHDKVK
LFCLTDRALL CFFCDEPALH EQHQVTGIDD AFEELQRELK EQLQALQDSE
REHTEALQLL KRQLAETKSS TKSLRTTIGE AFERLHRLLR ERQKAMLEEL
EADTARTLTD IEQKVQRYSQ QLRKVQEGAQ ILQERLAETD RHTFLAGVAS
LSERLKGKIH ETNLTYEDFP TSKYTGPLQY TIWKSLFQDI HPVPAALTMD
PGTAHQRLIL SDDCTIVAYG NLHPQPLQDS PKRFDVEVSV LGSEAFSSGV
HYWEVVVAEK TQWVIGLAHE AASRKGSIQI QPSRGFYCIV MHDGNQYSAC
TEPWTRLNVR DKLDKVGVFL DYDQGLLIFY NADDMSWLYT FREKFPGKLC
SYFSPGQSHA NGKNVQPLRI NTVRI

Immunostaining

For BMDCs, cells were adhered to Poly-L-lysine-coated coverslips overnight. For THP-1 cells, cells were treated with 50ng/ml PMA for 24 hours. PMA was then removed and the treated cells were kept in culture for another two days without PMA. Cells were adhered to coverslips overnight. All cell types were washed post-stimulation in PBS and fixed in 4% formaldehyde solution in PBS for 15 min. Cells were then permeabilized in 0.2% saponin in PBS for 5 min (HeLa) or 10 min (BMDCs, THP-1), blocked with 10% donkey serum for 15 min, and stained using appropriate antibodies for 1 h. DNA was labeled with Hoechst 33342 (Invitrogen). Following staining, coverslips were washed three times in PBS and mounted in ProLong Gold Antifade Reagent (Invitrogen). Cells were imaged using a Zeiss Axioplan widefield microscope (HeLa) or a Leica SP5 AOBS confocal microscope (BMDCs, THP-1). For analysis of colocalization in BMDCs and THP-1 cells, proteins were quantified by examining overlapping immunofluorescence signals in the presence or absence of depleted zymosan stimulation. For all experiments, results were obtained from three experiments and > 40 cells were counted per condition for each experiment. Statistical significance was calculated using Student's T-test.

For HeLa cells, primary antibodies were anti-FLAG (M2, Sigma-Aldrich) and anti-V5

(Covance). For BMDCs, primary antibodies were goat anti-CARD9 (Santa Cruz Biotechnology), rabbit anti-TRIM62 (Thermo Scientific), mouse anti-FLAG (M2, Sigma-Aldrich), and goat anti-BCL10 (Santa Cruz Biotechnology). For THP-1 cells, primary antibodies were mouse anti-FLAG (M2, Sigma-Aldrich) and rabbit anti-TRIM62 (Thermo Scientific). Secondary antibodies were Alexa488 conjugated with donkey anti-rabbit, Alexa488 conjugated with donkey anti-mouse, Alexa594 conjugated with donkey anti-goat, Alexa594 conjugated with donkey anti-mouse, Alexa594 conjugated with donkey anti-rabbit, or Alexa647 conjugated with donkey anti-goat (Jackson ImmunoResearch).

Generation of CRISPR-Based *CARD9*$^{-/-}$ THP-1 Cell Line and NF-κB Luciferase Assay

The *CARD9*$^{-/-}$ THP-1 cell line was generated by a CRISPR-based approach using a CARD9 sgRNA (ATCGTTCTCGTAGTCCGACA) inserted into the lentivirus-based CRISPR vector pHKO23 (gift from Feng Zhang, Broad institute) as previously described (Cong and Zhang, 2015). Briefly, THP-1 cells were infected with lentivirus carrying CARD9 sgRNA. Two days post-infection, cells were fed with RPMI medium and 1 μg/ml puromycin for 10 days. To isolate single cell lines, cells were diluted and plated into wells at a ratio of 0.3 cells per well in 96-well plates in RPMI without puromycin. *CARD9*$^{-/-}$ cell lines were validated by Western blot. A second-round isolation was performed for *CARD9*$^{-/-}$ cell line #31, which was used for the NF-κB luciferase assay. For the NF-κB luciferase assay, *CARD9*$^{-/-}$ #31 was transduced with Dectin-1 (NM_022570) and a lentivirus-based NF-κB luciferase reporter. *CARD9*$^{-/-}$ cells overexpressing Dectin-1 and the NF-κB luciferase reporter were rescued with indicated CARD9 constructs. Cells with re-expressed CARD9 were expanded for at least four passages prior to luciferase assay. Reporter luciferase activity was examined by according to the manufacturer's protocol (Steadylite Plus, PerkinElmer).

ELISA to Detect CARD9 Interactions

For CARD9-CARD9 interactions, ELISAs were performed using 96-well anti-GST coated plates (Pierce). GST-CARD9 was produced in Sf9 cells using baculovirus and pDEST20 starting backbone (Bac-to-Bac system, Invitrogen) and purified by glutathione affinity chromatography. GST-CARD9 protein was eluted with 20 mM glutathione (Sigma) and buffer was exchanged into 50 mM Tris, 150 mM NaCl, pH 7.5. FLAG-CARD9 or FLAG-CARD9 Δ11

in pcDNA3.2 were overexpressed in HEK293FT (Life Technologies) and affinity-purified on StrepTactin beads (IBA) following the manufacturer's protocol. For all proteins, purity was assessed by SDS-PAGE followed by Coomassie staining; protein was quantified by UV absorbance. Anti-GST coated plates were blocked with 5% non-fat milk (Bio-Rad) in PBS, incubated with 0.2 mg/ml GST-CARD9 for 1 h at room temperature, followed by three washes with milk and incubation with purified FLAG-tagged CARD9 or CARD9 Δ11 at 0.1 mg/ml for 1 h at room temperature. After three washes with ELISA wash buffer (0.05% Tween-20 in PBS), anti-FLAG antibody-HRP conjugate (Sigma) was added in 3% BSA in PBS and incubated for 1 h at 37°C. Following three washes with ELISA wash buffer, 1-step Ultra TMB substrate (Thermo) was added for 20 min, then quenched with 2 M sulfuric acid, and read on a Spectramax plate reader at 450 nm.

CARD9-TRIM62 interactions were measured by a similar protocol using 3x-Myc-TRIM62 (pCMV) and FLAG-CARD9 (pcDNA3.2) individually overexpressed in HEK293T cells. 96-well anti-FLAG coated plates (Sigma) were blocked with 5% milk in PBS, then incubated with FLAG-CARD9 lysate from HEK293T cells (2 x 10^6 cells/ml) for 1 h at room temperature, followed by three washes with milk. Where indicated, purified CTD was added to the final concentration of 40 μM. 3x-Myc-TRIM62 lysate was added for 1 h at room temperature, followed by three washes with ELISA wash buffer. Anti-myc antibody-HRP conjugate (9E10, Abcam) was added in 3% BSA in PBS for 1 h at 37°C, and after three washes with ELISA wash buffer, TMB substrate was added and plates were processed as described above.

BRET Assays

BRET signal was measured by overexpressing NanoLuc-CARD9 (in pFN(NLuc/CMV/Neo)) and Halo-TRIM62 (in pFN28A). For each well of a 6-well plate with 1 x 10^6 HEK293T cells plated overnight, 1500 ng of Halo-TRIM62 DNA, 15 ng of NLuc-CARD9 DNA, and 1500 ng of empty vector control (not containing NLuc or Halo tags) or the indicated protein plasmids were added. The ratio of donor to acceptor plasmid was 1:100 in the final DNA mixtures, as suggested by the manufacturer; the total amount of transfected DNA was kept identical across experimental conditions. Transfectin (Bio-Rad) was used as a transfection agent, according to the manufacturer's protocol. 24 h post transfection, cells were trypsinized and

replated in 96- or 384-well format in phenol red free media (DMEM, Invitrogen). Halo substrate from the NanoBRET detection system (NanoBRET 618, Promega) was added at the time of plating. 24 h after replating, NanoLuc substrate (without any lysis buffer) was added to the live cells in the plates, which were read after 15 min of incubation on an Envision instrument using 486 nm for the NLuc channel and 595 nm for the Halo channel. Halo channel representing acceptor fluorescence was normalized to NLuc channel; signal from NLuc-CARD9 alone (with vector control in place of Halo-tagged partner) was subtracted as background for data analysis.

Production of Purified CARD9 CTD

The C-terminal domain of CARD9 (CTD, aa 416-536) was expressed as a His_8 tag fusion in pET28a vector in Rosetta BL21 cells; a linker sequence and a TEV cleavage site were incorporated between the His_8 tag and the protein sequence. Expression was induced by 1mM IPTG overnight at 18°C. Bacteria were lysed using BugBuster lysis buffer, and CTD protein was purified by gravity-flow Ni affinity chromatography (Ni-NTA, Qiagen) and eluted in in buffer A (50mM Tris, 150 mM NaCl, 2 mM TCEP, pH 7.5) supplemented with 250 mM imidazole, followed by buffer exchange into buffer A. TEV (His_6-tagged) was used to cleave the tag from the N terminus of the protein, and subtraction Ni column was used to isolate cleaved CTD, which was then subjected to FPLC (Akta, GE Life Sciences) in buffer A. CTD purity was confirmed by mass spectroscopy and SDS-PAGE with Coomassie Blue staining; protein was quantified by UV absorbance at 280 nm.

***In Vitro* Ubiquitination Assays**

To generate purified proteins, FLAG-StrepII-CARD9 and Strep-His_6-HaloTag-TEV-Myc-TRIM62 were inserted into pcDNA3.4. Proteins were expressed in Freestyle 293-F cells using 1 mg DNA/L media and an equal amount of PEI. After 72 h, cells were lysed in buffer A (50 mM HEPES, pH 8.0, 150 mM NaCl, 10% glycerol, 1 mM DTT) with 1% NP-40 and complete EDTA-free protease inhibitors. Lysates were cleared by centrifugation and supernatants applied to a StrepTactin column (1.5 mL settle resin/L culture). The resin was washed twice with 10 column volumes of buffer A. Protein was eluted with 3 x 5 column volumes of buffer A with 15 mM desthiobiotin. Protein was then concentrated with centrifugal concentrators. To remove the tagged fusion proteins from TRIM62, protein was incubated with

His7-TEV protease overnight at 4°C. The cleaved fusion and His7-TEV were removed by incubation with Ni-NTA agarose.

In vitro ubiquitination reactions contained 50 mM Tris, 5 mM $MgCl_2$, 1 mM DTT, 5 mM ATP, 25 nM E1 (LifeSensors), 340 nM UbcH5B (LifeSensors), 2 μg HA-ubiquitin (Boston Biochem), 2 μg CARD9 (or derivative) and 5 nM TRIM62 (or C11A, C14A mutant) in a final volume of 50 μL. Reactions were incubated at 37°C for 30 min and then quenched with an equal volume of 2x SDS loading buffer.

Detection of Conserved Lysines of CARD9 Among Mammals

Human CARD9 was compared with 16 other mammalian species [*Ailuropoda melanoleuca* (Giant panda, UniProtKB entry ID D2HQC1)**,** *Bos taurus* (Bovine, F6RIH1**),** *Callithrix jacchus* (White-tufted-ear marmoset, F6Z8Q9), *Canis familiaris* (Dog, E2R0V1), *Cavia porcellus* (Guinea pig, H0VSP5)**,** *Equus caballus* (Horse, F6UIA3**),** *Gorilla gorilla gorilla* (Western lowland gorilla, G3R5M4), *Loxodonta africana* (African elephant**,** G3TPZ5), *Macaca mulatta* (Rhesus macaque, F7GMY0), *Monodelphis domestica* (Gray short-tailed opossum, F7BSP6), *Mus musculus* (Mouse, A2AIV8), *Nomascus leucogenys* (Northern white-cheeked gibbon, G1R9R3), *Rattus norvegicus* (Rat, Q9EPY0), *Sarcophilus harrisii* (Tasmanian devil, G3W222)**,** *Cricetulus griseus* (Chinese hamster, GenBank accession number XP_003510901), and *Mustela putorius furo* (Domestic ferret, GenBank accession number AER95454)]. Human CARD9 has 17 lysines absolutely conserved with the other 16 mammalian species. Within human CARD9, these lysines are at amino acid positions 58, 60, 93, 97, 125, 150, 156, 179, 233, 306, 340, 383, 391, 490, 496, 505, and 510. CARD9 K179R was not included in final experiments as expression of this mutant was not detectable in HEK293T cells under our experimental conditions.

Assessment of *C. albicans* Internalization in BMDMs

C. albicans were labeled with pHrodo Green dye according to the manufacturer's protocol (Life Technologies). Bone marrow-derived macrophages were prepared by culturing the bone marrow isolated from the femur and tibia of mice in M-CSF (10 ng/mL). On day 4, cells were washed and placed in fresh media with macrophage colony stimulating factor (M-CSF). On day 7, adherent macrophages were detached, counted, and passed into the appropriate size dish

for *in vitro* experiments. BM-derived macrophages isolated from *Trim62*$^{-/-}$ and littermate controls were plated in 24-well plates at 2 ×10^5 cells/well in 1 ml DMEM supplemented with 10% fetal calf serum and incubated overnight. The cells were then incubated with pHrodo-labeled *C. albicans* at an organism/macrophage ratio of 10:1 for 2 hours at 37°C. Phagocytosis was stopped by addition of cold PBS. Unbound yeast were removed by extensive washing and the cells detached in divalent cation-free buffer, then fixed in 2% paraformaldehyde and the intracellular fluorescence of phagocytosed *C. albicans* was determined by flow cytometry using a FACSCalibur (Becton Dickinson).

DSS Colitis

Trim62$^{-/-}$ mice and littermate controls were fed 3% (w/v) dextran sulfate sodium (DSS, MP Biomedicals; MW=36,000-50,000) dissolved in sterile, distilled drinking water *ad libitum*. Mice were treated with 3% DSS for 7 days, followed by 2 days of regular drinking water. Animals were monitored daily for weight loss. Colon tissue was harvested on day 9 for histology and RNA analyses.

Histology

Colon tissue was fixed in 10% buffered formalin and embedded in paraffin. 5 μm sections were cut and stained with H&E.

Real-Time Quantitative PCR (DSS)

Colon tissues were harvested on day 9 after DSS colitis and stored in RNA*later* (Ambion, Austin, TX) per the manufacturer's protocol prior to RNA isolation. RNA was extracted from homogenized tissues using the RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. All RNA samples were reverse-transcribed using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). Using the iQ SYBR Green Supermix (Bio-Rad) for quantitative PCR, mRNA levels were determined using the iCycler with iQ5 Multicolor Real-time PCR Detection System (Bio-Rad). The reaction conditions consisted of 37 cycles of PCR with an annealing temperature of 59°C. Primer sequences: **IFN-γ Fwd:** ATGAACGCTACACACTGCATC, **IFN-γ Rev:** CCATCCTTTTGCCAGTTCCTC; **IL-6 Fwd:** GTAGCTATGGTACTCCAGAAGAC, **IL-6 Rev:** ACGATGATGCACTTGCAGAA. The

threshold cycle (C_T) for each sample was determined for each gene and was normalized to the C_T value of the endogenous housekeeping gene GAPDH. Data were calculated using the $2^{-\Delta\Delta C(T)}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

Real-Time Quantitative PCR (*C. albicans*)

Spleen tissue was harvested 6 days post-live *C. albicans* infection for cytokine and chemokine analyses, as previously described (Means et al., 2009).

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