Supplementary Materials and Methods

MDM stimulation

MDMs were treated with MDP (Bachem), Pam3Cys (EMD Millipore), lipid A (Peptides International), poly I:C, flagellin, CLO97, or CpG (Invivogen). Supernatants were assayed for TNF (MAb1, MAb11), IL8 (G265-5, G265-8), IL10 (JES3-9D7, JES3-12G8) (BD Biosciences), IL1β (CRM56, CRM57), IL12 (8.3, 8.6) or IL6 (MQ2-13A5, MQ2-39C3) (Biolegend) by ELISA.

Intestinal lamina propria cell isolation

Intestinal lamina propria cells were isolated from colonic resection specimens from uninvolved intestine in non-IBD patients undergoing surgery for diverticular disease or colon cancer as in (1).

Mouse studies

C57BL/6N mice were maintained in a specific pathogen-free facility. Experiments were performed per Yale University Institutional Animal Care and Use Committee and National Institutes of Health guidelines. 15 μg/mouse ON-TARGETplus siRNA against RNF186 (sequence per J-050361-12) or control (sequence per D-001810-02) with UU overhang/in vivo design (Dharmacon) was administered i.p. in AteloGene Systemic Use (Cosmo Bio USA) and studies were conducted 48h later. Colonic lamina propria cells were isolated as previously described(2) and $CD11b^+$ cells were then purified (Miltenyi Biotec). Supernatants were assayed for: TNF (6B8, MP6-XT22), IL1β (B122), IL12 (C15.6, C17.8), IL10 (JES5-16E3, JES5-2A5) (Biolegend).

mRNA expression analysis

RNA was isolated using Trizol reagent (Thermo Fisher Scientific). Quantitative PCR was performed using All-In-One qPCR Mix (Genecopoeia) with normalization to GAPDH. Primers as per Table S1.

Transfection of siRNAs and DNA vectors in vitro

100nM scrambled, siGENOME or ON-TARGETplus SMARTpool small interfering RNA (siRNA) against RNF186, TRAF6, p40phox, p47phox, p67phox, NOS2 or ATG5 (Dharmacon) (4 pooled siRNAs for each gene) were transfected into MDMs using Amaxa nucleofector. Forty-eight hours later cells were used in experimental studies. RNF186 was amplified from HEK293 cells and subcloned into pcDNA.3 to generate pcDNA.3-WT RNF186 FLAG- or myc-tagged constructs [C-terminus]). The pcDNA3-RNF186 ΔZnF mutant (Cys58Ala, His60Trp, Cys63Ala, Cys66Ala) and pcDNA3-RNF186-Ala64Thr variants were generated through site-directed mutagenesis (Agilent Technologies). RIP2 from pOTB7-hRIPK2 (TranSOMIC technologies) was subcloned into pcDNA3 (pcDNA3-hRIPK2-FLAG [C-terminus]). Plasmids were used expressing HA-WT Ub (Addgene plasmid 17608), HA-Ub K63 (Addgene plasmid 17606; leaves K63 lysine intact), HA-Ub K48 (Addgene plasmid 17605; leaves K48 lysine intact) (deposited by Ted Dawson(3)), ATG5 (Addgene plasmid 24922; deposited by Toren Finkel(4)), NOS2 (gift of Tony Eissa(5)), p47phox (gift of Celine DerMardirossian(6)). For co-immunoprecipitation experiments in HEK293 cells, 1.5 μg HA-Ub, FLAG-RIP2, myc-RNF186 and 1 μg of NOD2 were transfected by Lipofectamine 2000 (Invitrogen) for 48 hours. FLAG-tagged WT or ΔZnF RNF186 (1.5 μg) and ATG5-, NOS2-, p47phox-expressing vectors (2 μg) were transfected into MDMs by Amaxa nucleofector for 48 hours. For luciferase studies, HEK293 cells were transfected with 500 ng of each of the RNF186 constructs or empty vector$\pm$50 ng NOD2 (pcDNA.3)$\pm$50 ng AP-1-luciferase

or NFκB luciferase vectors (Clontech) along with 15 ng pRL-TK (Promega) as a Renilla normalization control.

Luciferase activity

Cells were lysed and assayed for luciferase and Renilla activity according to the manufacturer's instructions (Promega).

Immunoprecipitation and immunoblotting assays

Immunoblotting were performed with antibodies to RNF186 (Aviva Systems Biology and Novus Biologicals) or GAPDH (6C5, EMD Millipore). For co-immunoprecipitation experiments, RIP2, myc-RNF186, FLAG-RIP2 or TRAF6 were immunoprecipitated with anti-RIP2, anti-myc, anti-FLAG or anti-TRAF6 antibodies (Cell Signaling Technology) conjugated to protein A or protein G–agarose. Associated proteins were examined with RIP2 (25/RIG-G, BD Biosciences), NOD2 (Cayman Chemical Company), IRAK1 (D51G7), TRAF6 (D21G3), ERK1/2 (137F5), phospho-ERK1/2 (E10), phospho-p38 (28B10), IκBα, phospho-IκBα (14D4), phospho-JNK1 (G9), myc (9B11), ubiquitin, HA (C29F4), FLAG (9A3), Dectin-1 (E1X3Z), Syk (4D10) (Cell Signaling Technology), p38 (A-12) or JNK1 (D-2) (Santa Cruz Biotechnology).

Flow cytometry staining

Permeabilized MDMs were assessed for protein expression by flow cytometry using antibodies to RNF186 (Aviva Systems Biology), ATG5 (EPR1755, Abcam), NOS2, LC3II (Cell Signaling Technology), p40phox (D-8), p47phox (A-7), or p67phox (D-6) (Santa Cruz Biotechnology). Phospho-proteins were examined using fluorophore-conjugated antibodies to phospho-ERK (E-

10), phospho-p38 (28B10), phospho-JNK (G9) or phospho-IκBα (14D4) (Cell Signaling Technology).

Recombinant protein purification

HEK293 cells were transfected with 5 µg of myc-RNF186 constructs (WT or mutants) or FLAG-RIP2 using lipofectamine 2000. After 72h cells were lysed and myc-RNF186 or FLAG-RIP2 were purified with anti-myc or -FLAG antibodies (Cell Signaling Technology) conjugated with protein G agarose beads, respectively. The proteins were eluted with myc or FLAG peptides (APExBIO), respectively.

***In vitro* ubiquitination assays**

Purified recombinant myc-RNF186 (750ng) was incubated with FLAG-RIP2 (400ng) in a 50µl reaction mixture containing HA-Ubiquitin (5µg), 100nM UBE1, 500nM UBE2N (Ubc13/Uev1a), 2mM $MgCl_2$, 2mM ATP and 1X reaction buffer (Boston Biochem). Reactions mixture was incubated at 30°C for 1h and terminated by the addition of 1X loading dye followed by heating to 95°C for 5min. The samples were probed with antibodies to the HA tag.

Intracellular ROS measurement.

Intracellular ROS was measured by incubating MDMs with 10µM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Life Technologies) for 30 min and assessed by flow cytometry.

Intracellular bacterial clearance.

MDMs were infected in triplicate for 1 hour with adherent invasive *Escherichia coli* (AIEC) strain LF82 (a generous gift from Dr. Emiko Mizoguchi), *Enterococcus faecalis* (*E. faecalis*) or *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) at 10:1 MOI, washed with PBS and incubated in HBSS medium containing 50μg/ml gentamicin for an additional hour. Mouse cells were co-cultured with bacteria for 20 minutes, washed with PBS and incubated in HBSS medium containing 50μg/ml gentamicin for an additional 220 minutes. Cells were washed, lysed with 1% Triton X-100 and plated on LB or MacConkey agar. Colony forming units (CFU) were quantified.

Statistical Analysis

Significance was assessed using two-tailed Student's t-test. A Bonferroni-Holm correction was applied for multiple corrections where appropriate. $P<0.05$ was considered significant.

References

1. Hedl M, Lahiri A, Ning K, Cho J, & Abraham C (2014) Pattern Recognition Receptor Signaling in Human Dendritic Cells is Enhanced by ICOS Ligand and Modulated by the Crohn's Disease ICOSLG Risk Allele. *Immunity* 40:734-746.
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3. Lim KL, *et al.* (2005) Parkin mediates nonclassical, proteasomal-independent ubiquitination of synphilin-1: implications for Lewy body formation. *J Neurosci* 25(8):2002-2009.
4. Lee IH, *et al.* (2008) A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy. *Proc Natl Acad Sci U S A* 105(9):3374-3379.
5. Musial A & Eissa NT (2001) Inducible nitric-oxide synthase is regulated by the proteasome degradation pathway. *J Biol Chem* 276(26):24268-24273.
6. Gianni D, DerMardirossian C, & Bokoch GM (2011) Direct interaction between Tks proteins and the N-terminal proline-rich region (PRR) of NoxA1 mediates Nox1-dependent ROS generation. *Eur J Cell Biol* 90(2-3):164-171.

Figure S1

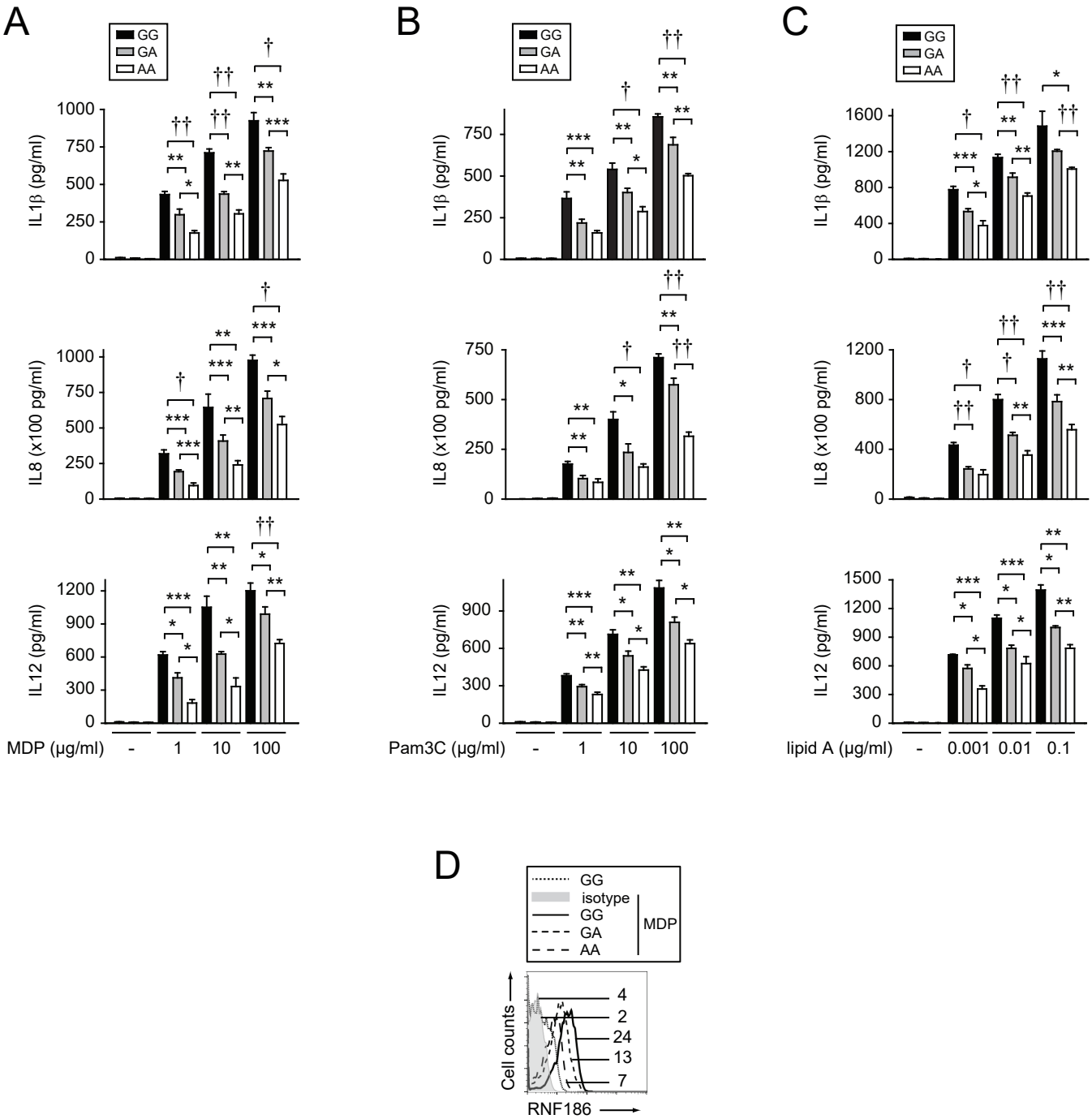

Figure S1. Human MDMs from rs6426833 A risk carriers demonstrate reduced PRR-induced cytokine secretion. MDMs from rs6426833 GG, GA, AA carriers (n=10 donors/genotype) were treated for 24h with: **(A)** 1, 10 or 100μg/ml MDP (recognized by NOD2), **(B)** 1, 10 or 100μg/ml Pam3Cys (recognized by TLR2), or **(C)** 0.001, 0.01 or 0.10 μg/ml lipid A (recognized by TLR4). Mean cytokine secretion + SEM. **(D)** Representative flow cytometry for **Figure 1L**. Isotype control is from MDP-treated GG carriers. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<1\times10^{-4}$; ††, $p<1\times10^{-5}$.

Figure S2

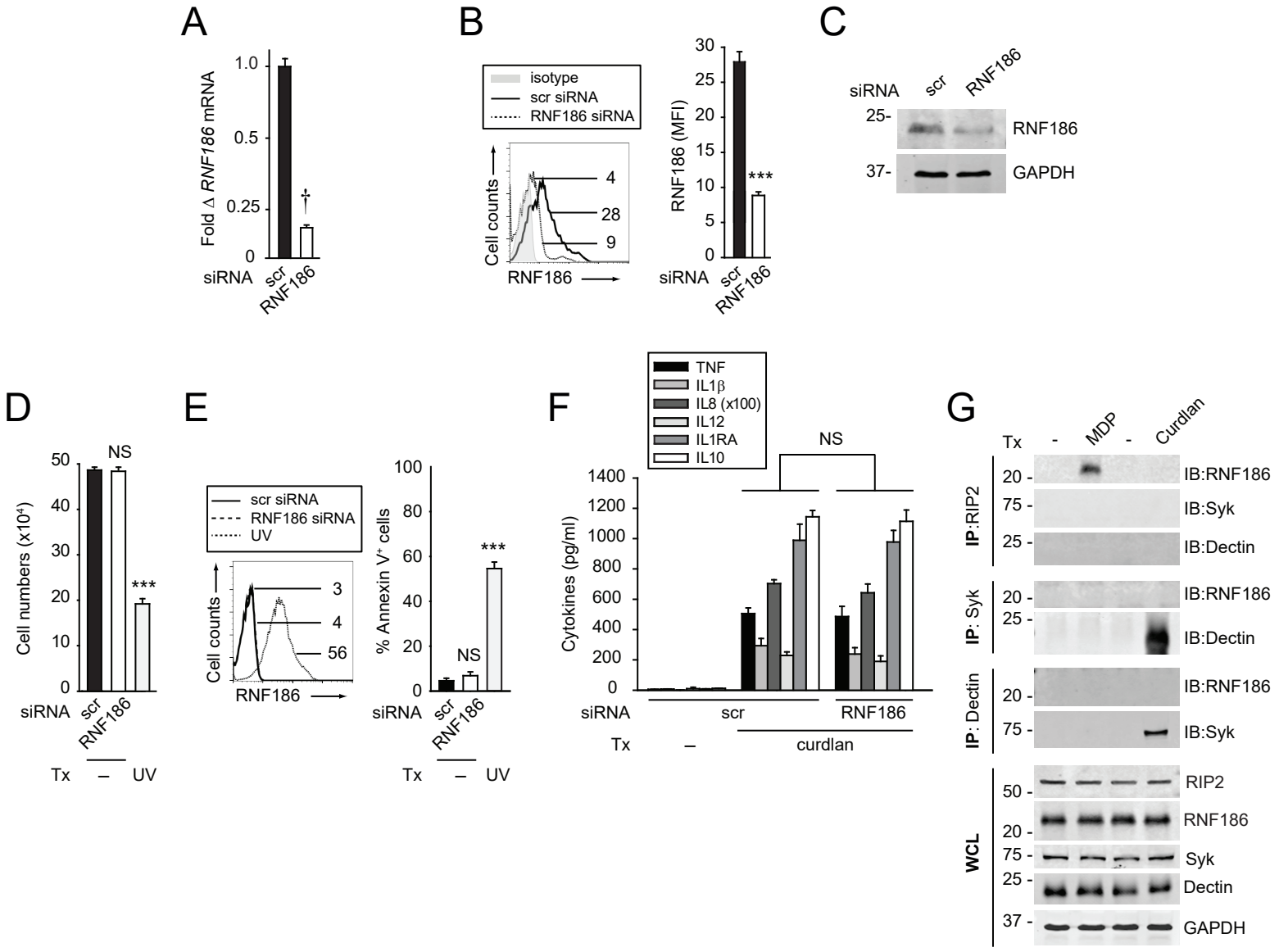

Figure S2. RNF186 expression is effectively reduced with knockdown and cells remain viable and functional. (A-F) Human MDMs were transfected with scrambled or RNF186 siRNA. **(A)** *RNF186* mRNA expression (n=6 donors; similar results in an additional n=6). **(B-C)** RNF186 protein expression (anti-RNF186 antibody per Novus Biologicals) by: **(B)** flow cytometry with representative flow cytometry and summary graph with MFI (n=4 donors) and **(C)** Western blot. **(D)** Cell counts. **(E)** Percent dead cells as assessed by annexin V staining with representative flow cytometry and summary graph of MFI (n=4 donors; similar results in an additional n=4). UV stimulation at 50-100 J/m^2 is shown as a positive control for '**D**' and '**E**'. **(F)** Cells were treated with 100 µg/ml curdlan (MilliporeSigma) for 24h. Cytokine secretion (n=6 donors; similar results in an additional n=6). **(G)** MDMs were treated with 100µg/ml curdlan or 100µg/ml MDP (as a control for RNF186-associated proteins) for 15 min. RIP2, Syk or Dectin-1 (Cell Signaling Technology) was immunoprecipitated (IP) followed by immunoblotting (IB) for the indicated proteins. Expression of the respective proteins and GAPDH in whole cell lysates (WCL) served as loading controls. IP with IgG was also used as a control. Mean + SEM. NS, not significant; scr, scrambled; Tx, treatment. Marker positions are shown (kDa). ***, $p<0.001$; †, $p<1\times10^{-4}$.

Figure S3

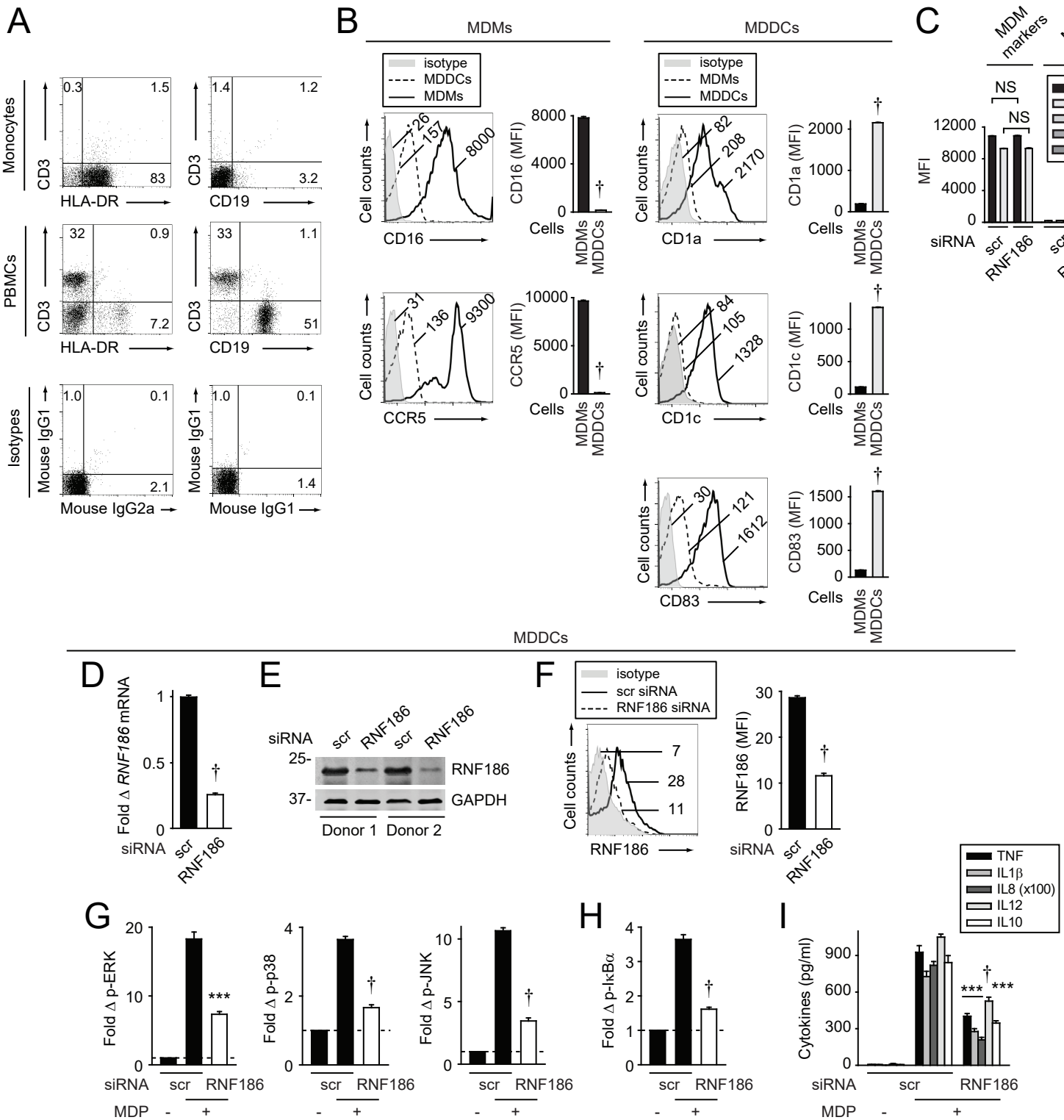

Figure S3. RNF186 promotes PRR-induced signaling and cytokines in MDDCs. (A) Isolated monocytes were stained with HLA-DR (BD Biosciences) along with negative control staining for T cell (CD3) and B cell (CD19) markers (BD Biosciences). PBMCs are used as positive controls for CD3 and CD19. **(B)** Differentiated MDMs (n=6 donors) were stained with macrophage markers (CD16, CCR5; Biolegend). MDDC markers (CD1a, CD83 [Biolegend], CD1c [BD Biosciences]) were included as negative controls. **(C)** MDMs (n=6 donors) were transfected with scrambled or RNF186 siRNA and then assessed for maintenance of macrophage markers (CD16, CCR5). MDDC markers (CD1a, CD1c, CD83) were used as negative controls. **(D-I)** MDDCs (differentiated with 20ng/ml GM-CSF [Shenandoah Biotechnology] and 20ng/ml IL4) were transfected with scrambled or RNF186 siRNA. **(D)** *RNF186* mRNA expression (n=6 donors). RNF186 protein expression by: **(E)** Western blot and **(F)** flow cytometry. MDDCs were treated with 100 μg/ml MDP and assessed for: **(G, H)** fold phospho-protein expression at 15min and **(I)** cytokine secretion at 24h. For '**F-I**' (n=6 donors; similar results in an additional n=6). Mean + SEM. NS, not significant; scr, scrambled. ***, $p<0.001$; †, $p<1\times10^{-4}$.

Figure S4

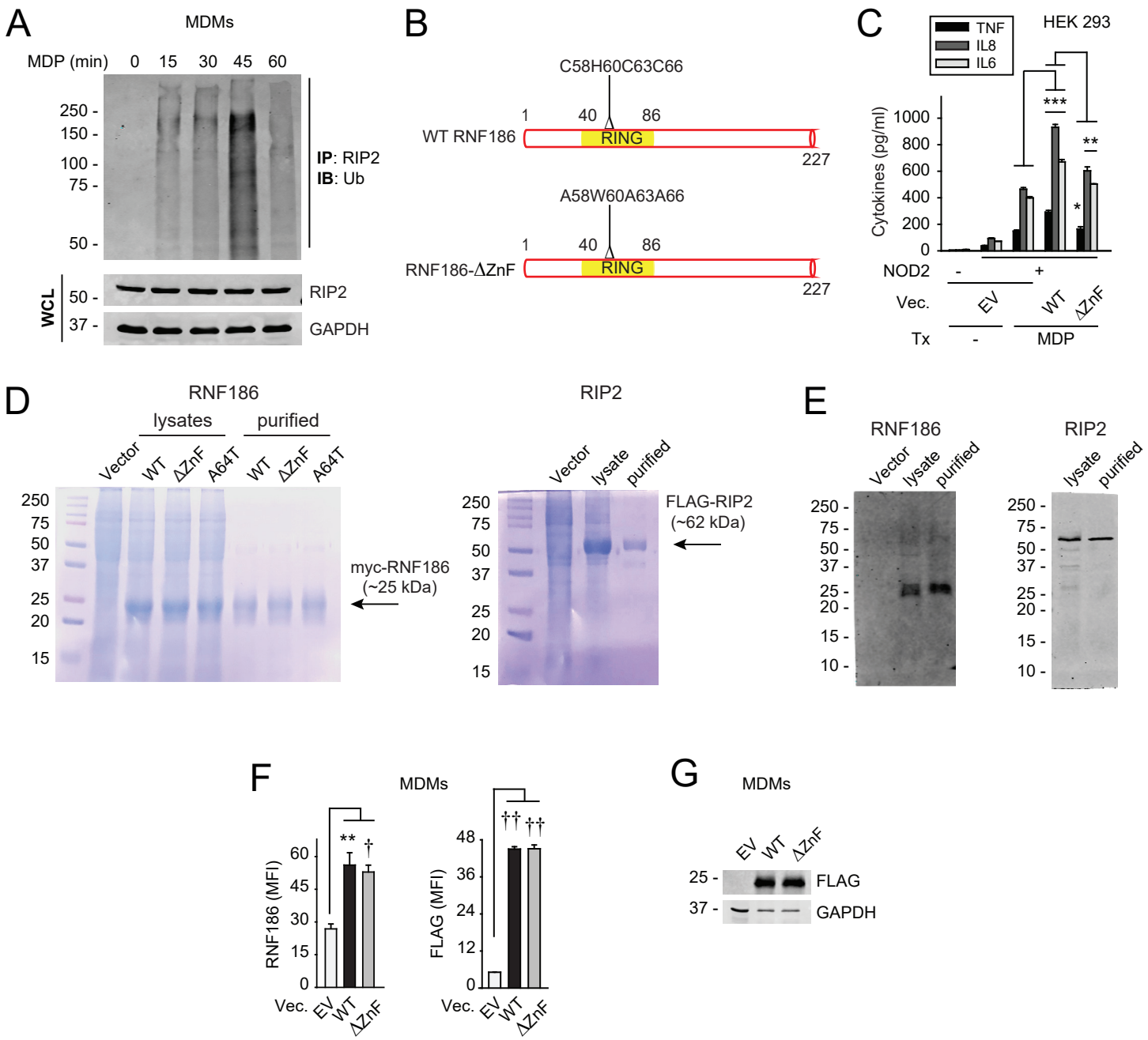

Figure S4. NOD2-induced ubiquitination of the RIP2 complex peaks at 45 minutes. (A) MDMs were treated with 100 µg/ml MDP for the indicated times. RIP2 was immunoprecipitated (IP) and ubiquitinated proteins (Ub) were assessed by Western blot (representative of 2 independent experiments). **(B)** RNF186 protein model representing wild type (WT) and ΔZnF mutants. **(C)** HEK293 cells were transfected with empty vector (EV), WT or ΔZnF RNF186 along with NOD2. Cells were then treated with 100µg/ml MDP. Cytokine secretion at 24h (6 replicates; representative of 2 independent experiments). **(D-E)** Purified proteins of myc-tagged RNF186 (WT and ΔZnF) and FLAG-tagged RIP2 were assessed by: **(D)** Coomassie staining and **(E)** Western blot of myc (RNF186) and FLAG (RIP2). Lysates from HEK293 transfected cells prior to purification are included as controls to show enriched RNF186 band and contrast with singular band after purification. **(F-G)** MDMs from rs6426833 AA risk carriers were transfected with EV, FLAG-tagged WT RNF186, or ΔZnF RNF186 and protein expression as detected by: **(F)** flow cytometry (n=6 donors) and **(G)** Western blot. Mean + SEM. Vec, vector. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<1\times10^{-4}$; ††; $p<1\times10^{-5}$.

Figure S5

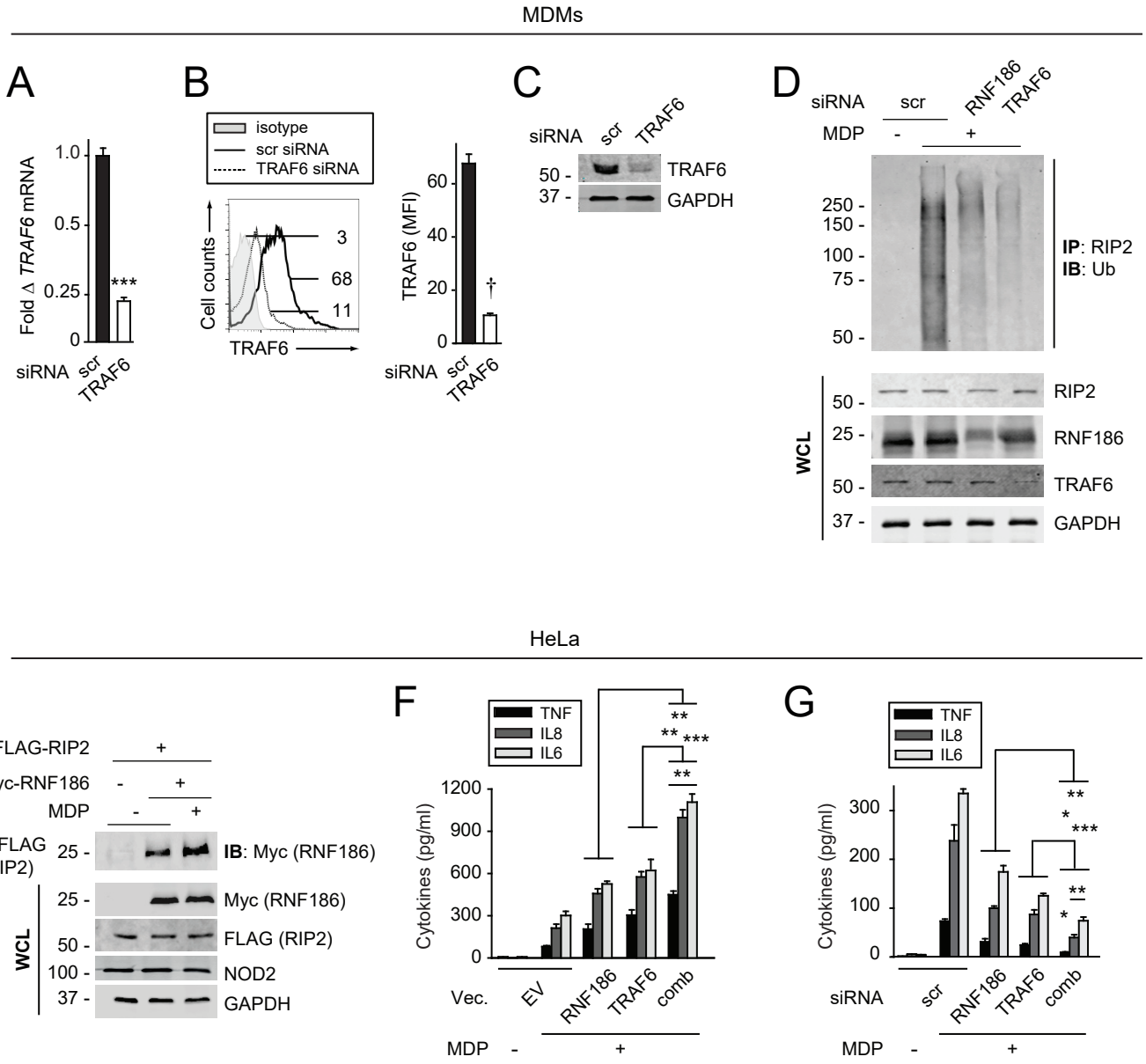

Figure S5. RNF186 and TRAF6 function in a non-redundant and cooperative manner to mediate NOD2-induced signaling and cytokines in both macrophages and epithelial cells. (A-C) MDMs were transfected with scrambled or TRAF6 siRNA. **(A)** *TRAF6* mRNA expression (n=6 donors). **(B)** TRAF6 protein expression with representative flow cytometry and mean fluorescence intensities (MFI) indicated and summary graph (MFI) (n=6 donors). **(C)** TRAF6 protein expression by Western blot. **(D)** MDMs were transfected with scrambled, RNF186 siRNA or TRAF6 siRNA, and then treated with 100 µg/ml MDP for 45 min. RIP2 was immunoprecipitated (IP) and ubiquitinated proteins (Ub) assessed by Western blot. Proteins in whole cell lysates (WCL) served as loading controls. **(E)** HeLa cells were transfected with empty vector (EV) or WT myc-RNF186, FLAG-RIP2 and NOD2. Cells were then treated with 100µg/ml MDP for 15min and FLAG (RIP2) was immunoprecipitated (IP) followed by immunoblotting (IB) for Myc (RNF186). **(F-G)** HeLa cells (6 replicates; representative of 2 independent experiments) were transfected with NOD2 and **(F)** empty vector (EV) or RNF186- or TRAF6-expressing vectors, alone or in combination (comb) or **(G)** scrambled, RNF186 or TRAF6 siRNA, alone or in combination. **(F-G)** Cells were treated with 100 µg/ml MDP and cytokine secretion was assessed at 24h. Mean + SEM. Scr, scrambled; vec., vector. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<1\times10^{-4}$.

Figure S6

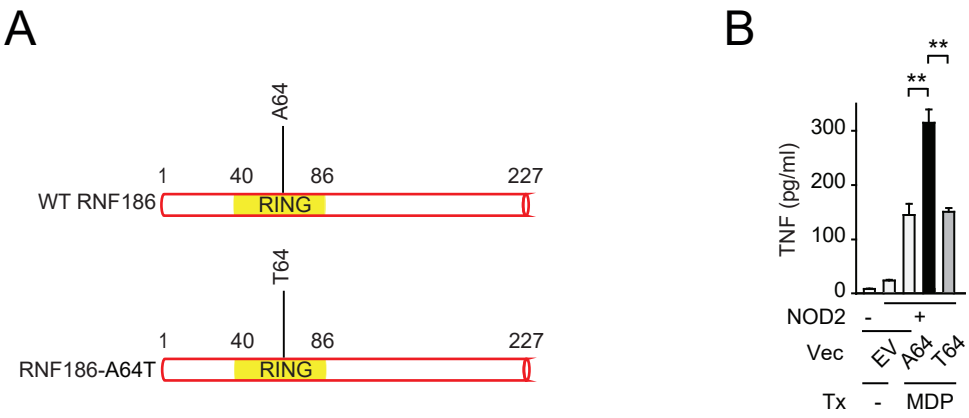

Figure S6. The rare RNF186 T64 IBD-risk variant is located in the RING domain. (A) RNF186 protein model representing wild type (WT) A64 and rare disease-risk T64 variant. **(B)** TNF secretion from studies in **Figure 4C**. Mean + SEM. EV, empty vector; Tx, treatment; Vec, vector. **, $p<0.01$.

Figure S7

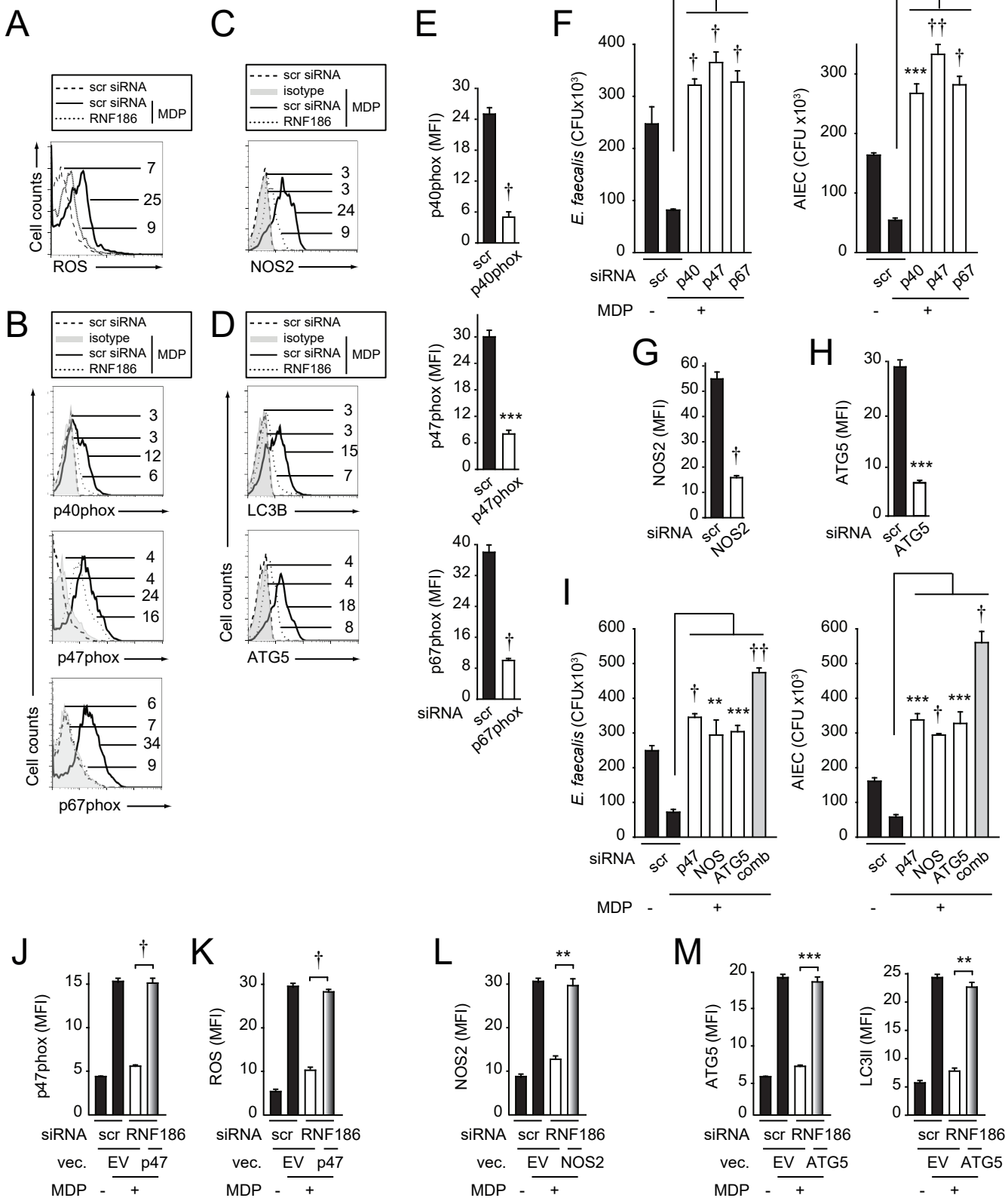

Figure S7. RNF186 promotes multiple NOD2-induced antimicrobial pathways. (A-D) Representative flow plots with mean fluorescence values (MFI) for: **(A) Figure 5B**, **(B) Figure 5C**, **(C) Figure 5D**, and **(D) Figure 5E**. **(E-I)** Human MDMs (n=6) were transfected with scrambled or the indicated siRNA, alone or in combination (comb). **(E, G-H)** Protein expression was assessed by intracellular flow cytometry and summary graph of MFI is shown. **(F & I)** MDMs were treated with 100μg/ml MDP for 48h and then assessed for intracellular bacterial clearance. **(J-M)** MDMs (n=6) were transfected with scrambled or RNF186 siRNA ± p47phox-, NOS2- or ATG5-expressing vectors or empty vector (EV), then left untreated or treated with 100μg/ml MDP for 48h. Expression of the indicated measures by flow cytometry (MFI). Mean + SEM. Scr, scrambled; Vec, vector. **, p<0.01; ***, p<0.001; †, p<1×10^{-4}; ††, p<1×10^{-5}.

Figure S8

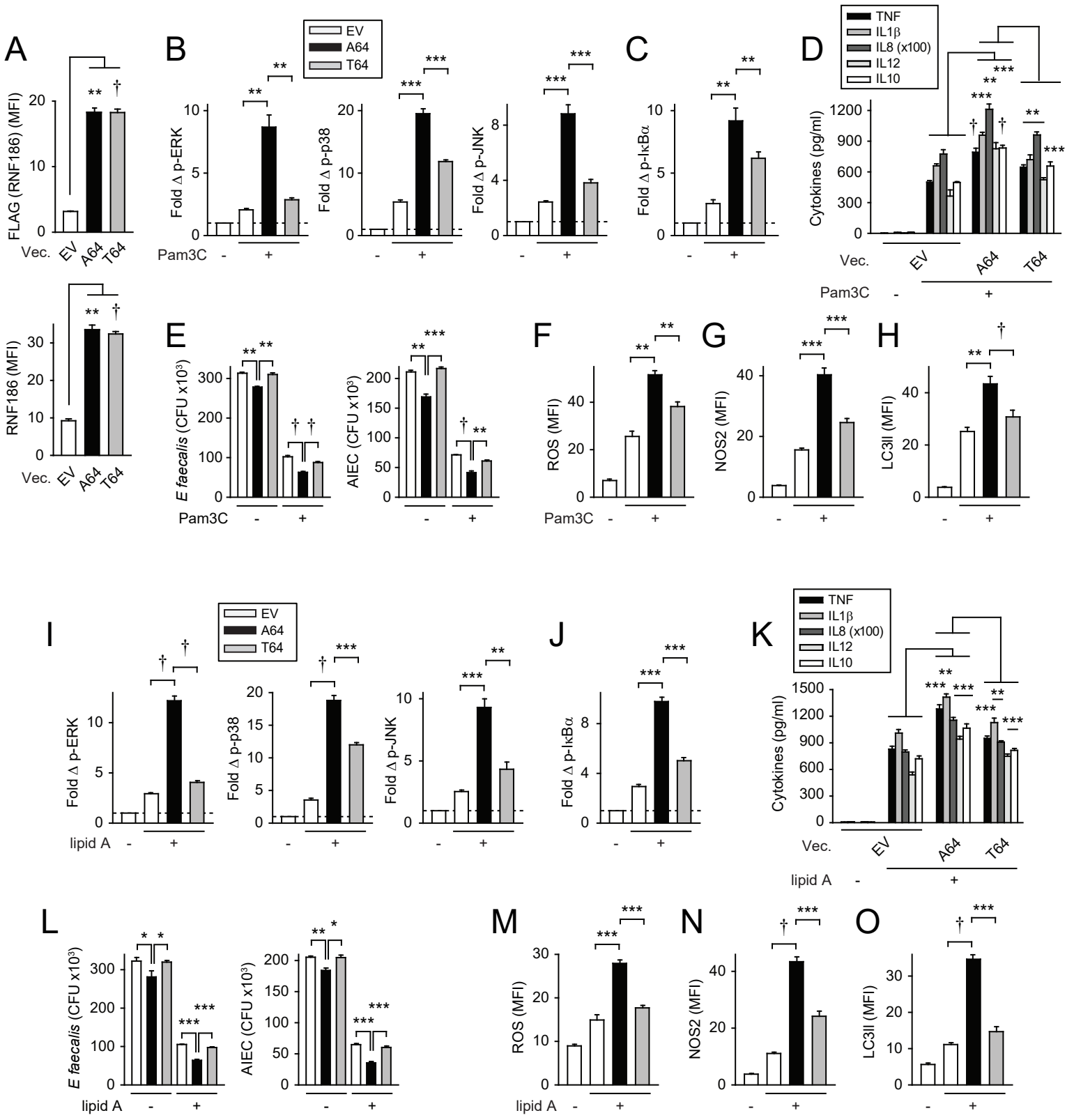

Figure S8. The rare RNF186 A64T IBD-risk variant results in decreased PRR-induced outcomes compared to WT RNF186. MDMs from rs6426833 AA risk carriers (low RNF186 expressors) were transfected with empty vector (EV), FLAG-tagged RNF186-A64 (WT) or RNF186-T64 (risk variant). **(A)** RNF186 protein expression by flow cytometry (n=6 donors). Cells were then treated with: **(B-H)** 10μg/ml Pam3Cys (n=6 donors) or **(I-O)** 0.1 μg/ml lipid A (n=6 donors). **(B-C, I-J)** Fold phospho-proteins at 15min. **(D, K)** Cytokines at 24h. **(E, L)** After 48h intracellular bacterial clearance was assessed. **(F-H, M-O)** Antimicrobial pathways were assessed by flow cytometry. Mean + SEM. Vec, vector. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<1\times10^{-4}$.

Figure S9

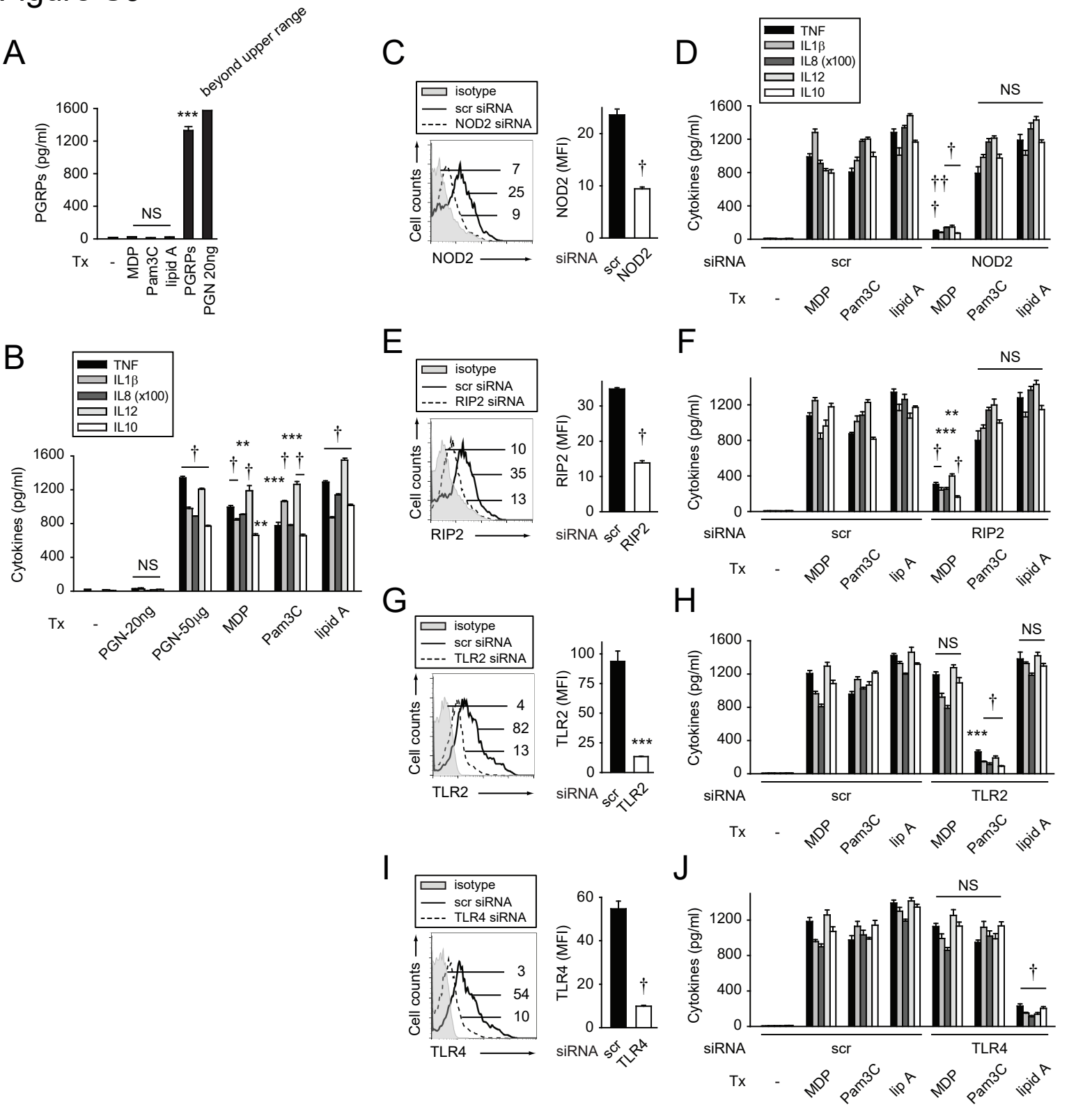

Figure S9. PRRs are appropriately stimulated by their respective ligands. (A-B) MDMs (n=4) were left untreated or treated for 24h with 100μg/ml MDP, 10μg/ml Pam3Cys or 0.1μg/ml lipid A or the indicated doses of PGN. Supernatants were assessed for: **(A)** PGRPs/PGLYRP1 (ThermoFisher). PGRPs and 20ng/ml PGN are shown as controls. **(B)** Cytokines. Note that with 20ng/ml PGN treatment of MDMs the PGRPs level in media is far beyond the upper limit of detection and yet this PGN concentration does not induce cytokine secretion from MDMs, whereas 50μg/ml PGN (~2500-fold greater concentration) induces cytokine levels comparable to other PRR ligands. **(C-J)** MDMs (n=6 donors, similar results in additional 6 donors) were transfected with scrambled or the indicated siRNA. **(C, E, G, I)** PRR expression per flow cytometry. **(D, F, H, J)** Cells were treated with 100μg/ml MDP, 10μg/ml Pam3Cys or 0.1μg/ml lipid A and cytokine secretion was assessed. Significance is compared to the respective scrambled siRNA-transfected condition. NS, not significant; scr, scrambled; Tx, treatment. **, $p<0.01$; ***, $p<0.001$; †, $p<1\times10^{-4}$; ††, $p<1\times10^{-5}$.

Figure S10

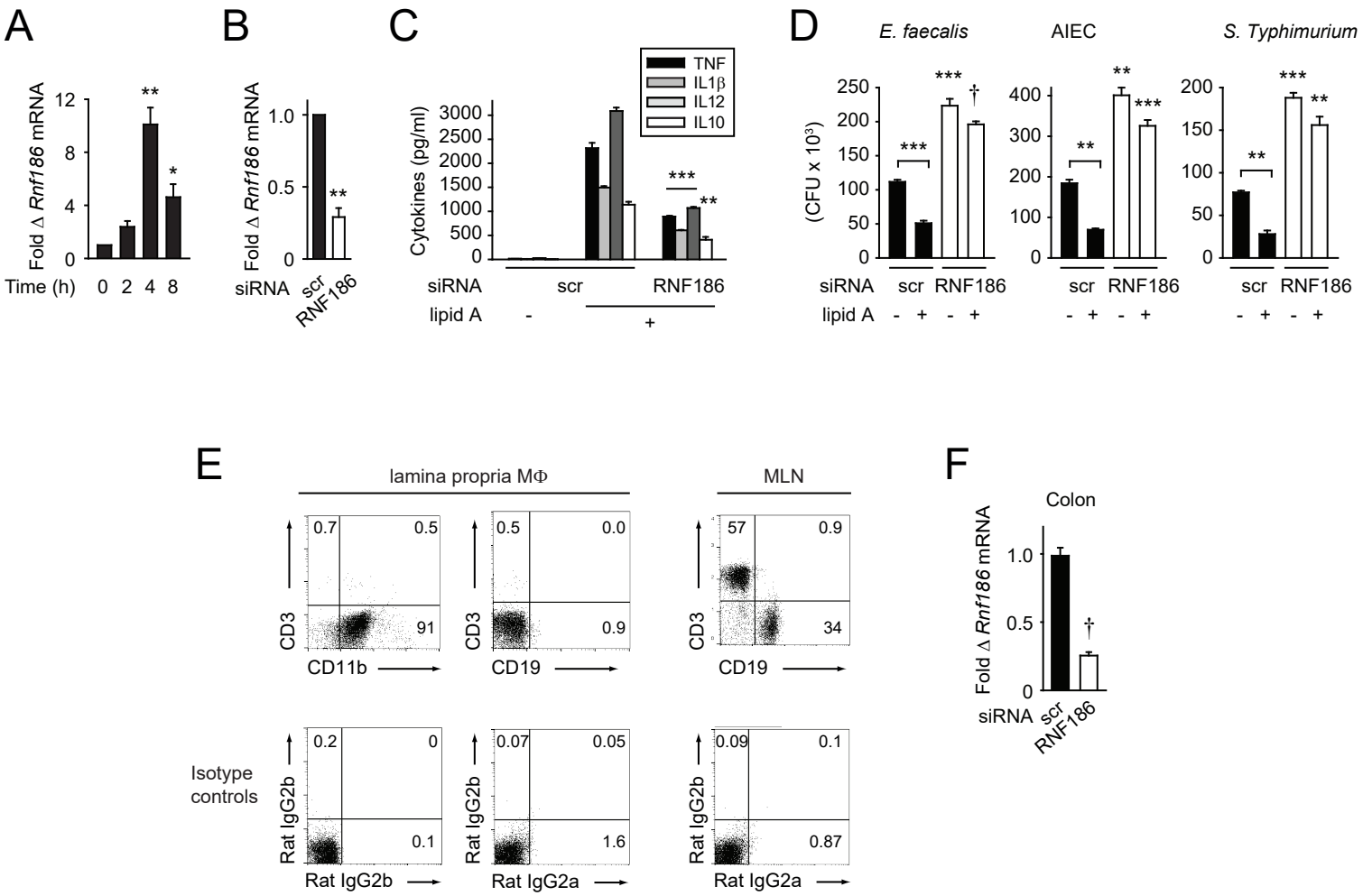

Figure S10. RNF186 promotes PRR-induced outcomes in mouse BMMs and intestinal macrophages. **(A)** Mouse BMMs were treated with 0.1 μg/ml lipid A for the indicated times and *Rnf186* mRNA was assessed (4 replicates, representative of 2 independent experiments). **(B-D)** BMMs were transfected with scrambled or RNF186 siRNA. **(B)** *Rnf186* mRNA expression (4 replicates, representative of 2 independent experiments). **(C-D)** Cells were treated with 0.1 μg/ml lipid A. **(C)** Cytokine secretion at 24h (4 replicates, representative of 2 independent experiments). **(D)** After 48h intracellular bacterial clearance was assessed (5 replicates, representative of 2 independent experiments). **(E)** CD11b+ colonic lamina propria macrophages (MΦ) were isolated from WT mice and purity of colonic macrophages was assessed (anti-CD11b antibody [ThermoFisher Scientific]). MLN is shown as positive control for CD3 (T cell) and CD19 (B cell) markers (Biolegend). **(F)** Mice were injected with scrambled or RNF186 siRNA (n=5; n=6 in additional independent dose response experiment). Colon *Rnf186* mRNA expression. Mean + SEM. Scr, scrambled. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<1\times10^{-4}$.

Figure S11

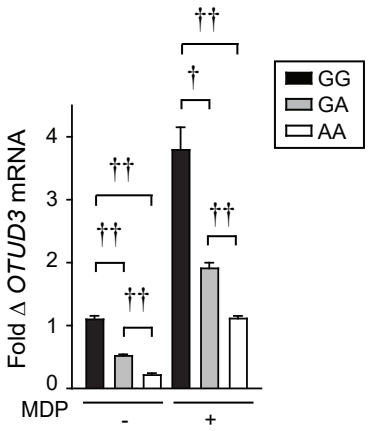

Figure S11. *OTUD3* expression in MDMs is regulated by rs6426833 genotype. MDMs from rs6426833 GG, GA or AA carriers (n=10 donors/genotype) were treated for 4h with 100μg/ml MDP. *OTUD3* mRNA expression**.** †, $p<1\times10^{-4}$**;** ††, $p<1\times10^{-5}$.

Figure S12

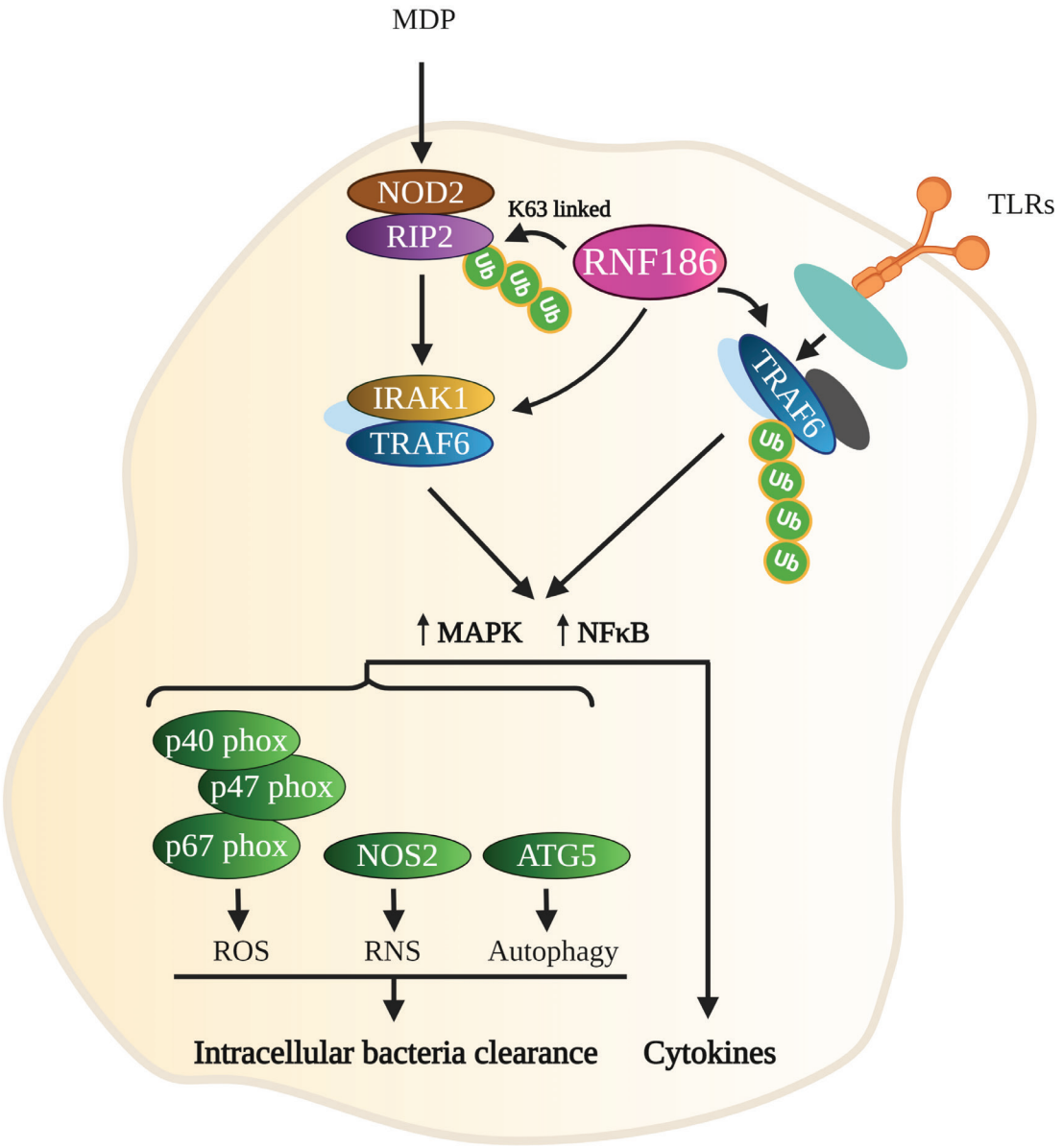

Figure S12. Proposed model of RNF186 mechanisms regulating PRR-initiated outcomes. Upon PRR stimulation in MDMs, RNF186 associates with and is required for assembly of the proximal signaling complex, including IRAK1 and TRAF6. RNF186 then promotes the ubiquitination of the signaling complex associated molecules followed by activation of NFκB and MAPK signaling, cytokine secretion, and the induction of multiple anti-microbial pathways, including ROS, RNS and autophagy, which then promote intracellular bacterial clearance. Importantly, RNF186 regulates outcomes downstream of multiple PRRs. RNF186 E3 ubiquitin ligase activity is required for the ability of RNF186 to optimally promote the PRR-induced outcomes above. Both the rare RNF186 A64T IBD risk variant and the common rs64268331 A IBD risk variant in the *RNF186* region demonstrate a loss-of-function in PRR-induced complex ubiquitination, NFκB and MAPK activation, cytokine secretion, and anti-microbial pathways. RNF186 also regulates live bacteria-induced cytokines and intracellular bacterial clearance in intestinal macrophages.

Table S1

GENE	PRIMER SEQUENCE
Human	
RNF186	FWD: 5’ GAGGATGGACAGGATGAAGTAAG REV: 5’ AGACACCCGGGTAGATGAA
OTUD3	FWD: 5’ GCAGATTCGTGGTACAGAGAAA REV: 5’ GGTGCCTCTGAGTTGTCATT
TRAF6	FWD: 5’ CAGGCTGTTCATAGTTTGAGC REV: 5’ CTGCTGTGCTTCGATTTCAG
GAPDH	FWD: 5’ GGCATGGACTGTGGTCATGAG REV: 5’ TGCACCACCAACTGCTTAGC
Mouse	
Rnf186	FWD: 5’ CCTGGAATGCTTGGTGTGCCG REV: 5’ GGATGGACCAGGTGTCTTCCT
Gapdh	FWD: 5’ CCACTCACGGCAAATTCAAC REV: 5’ CTCCACGACATACTCAGCAC

Table S1. Primer Table.