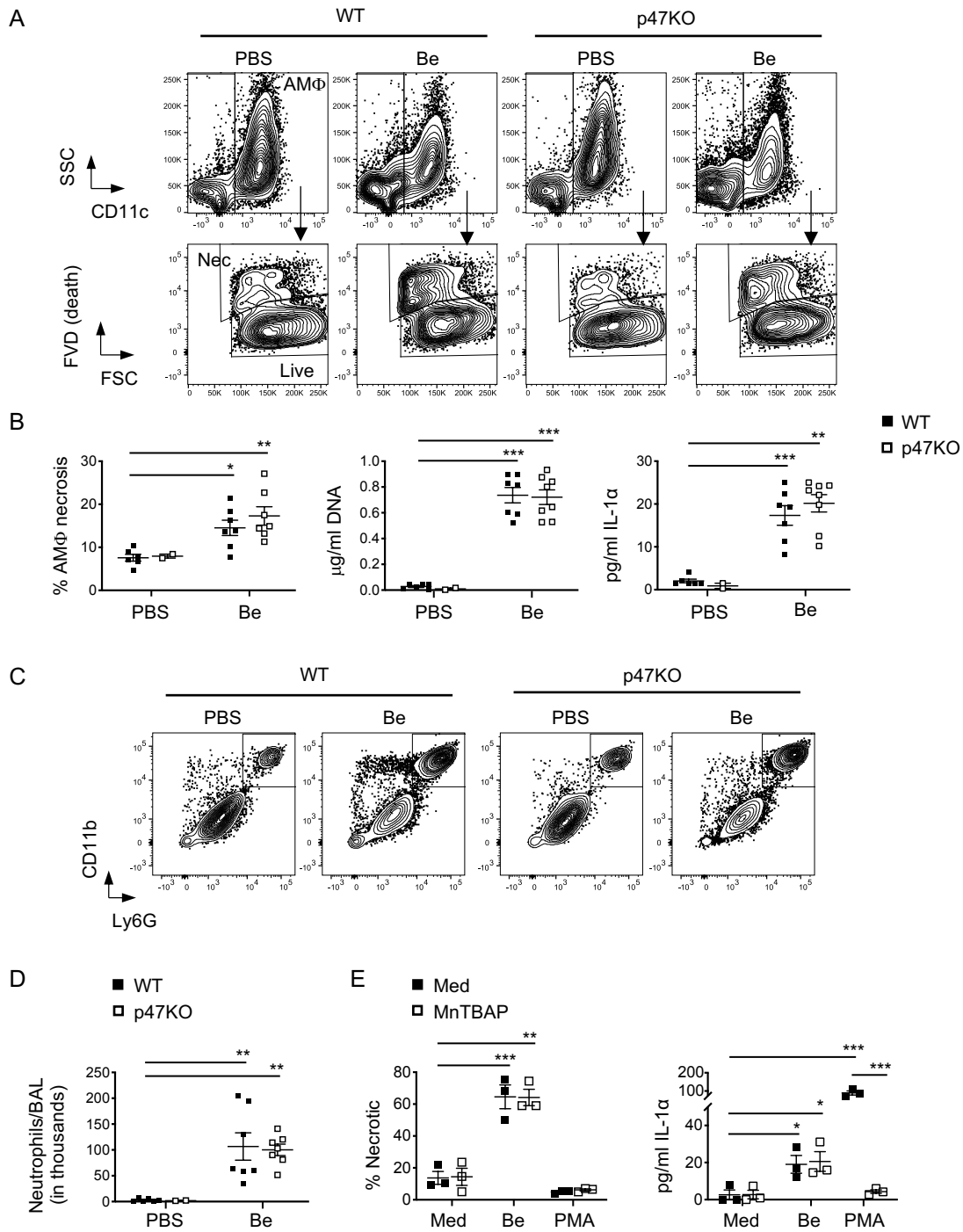
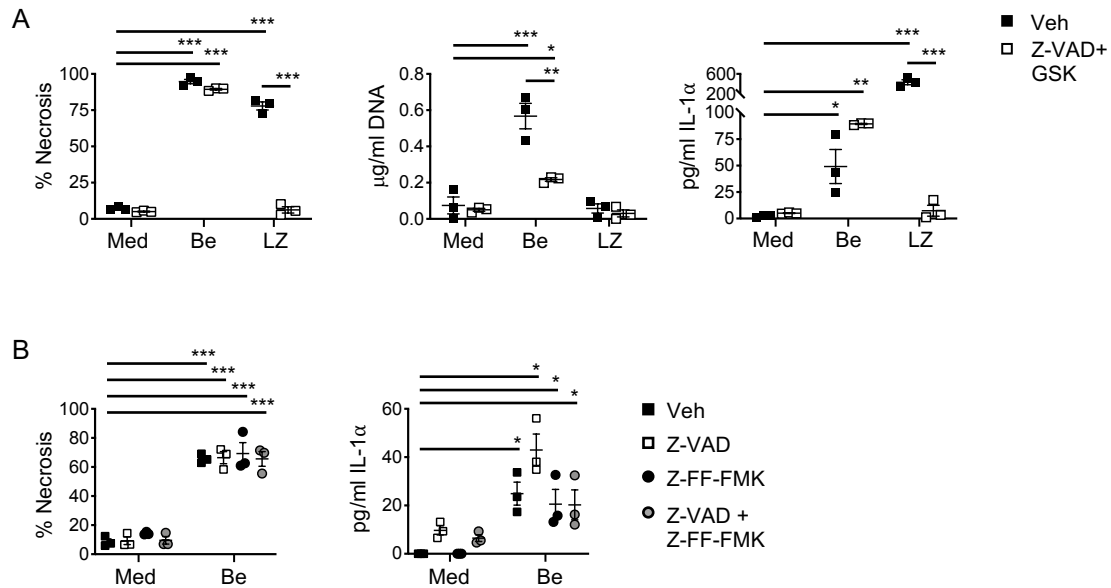
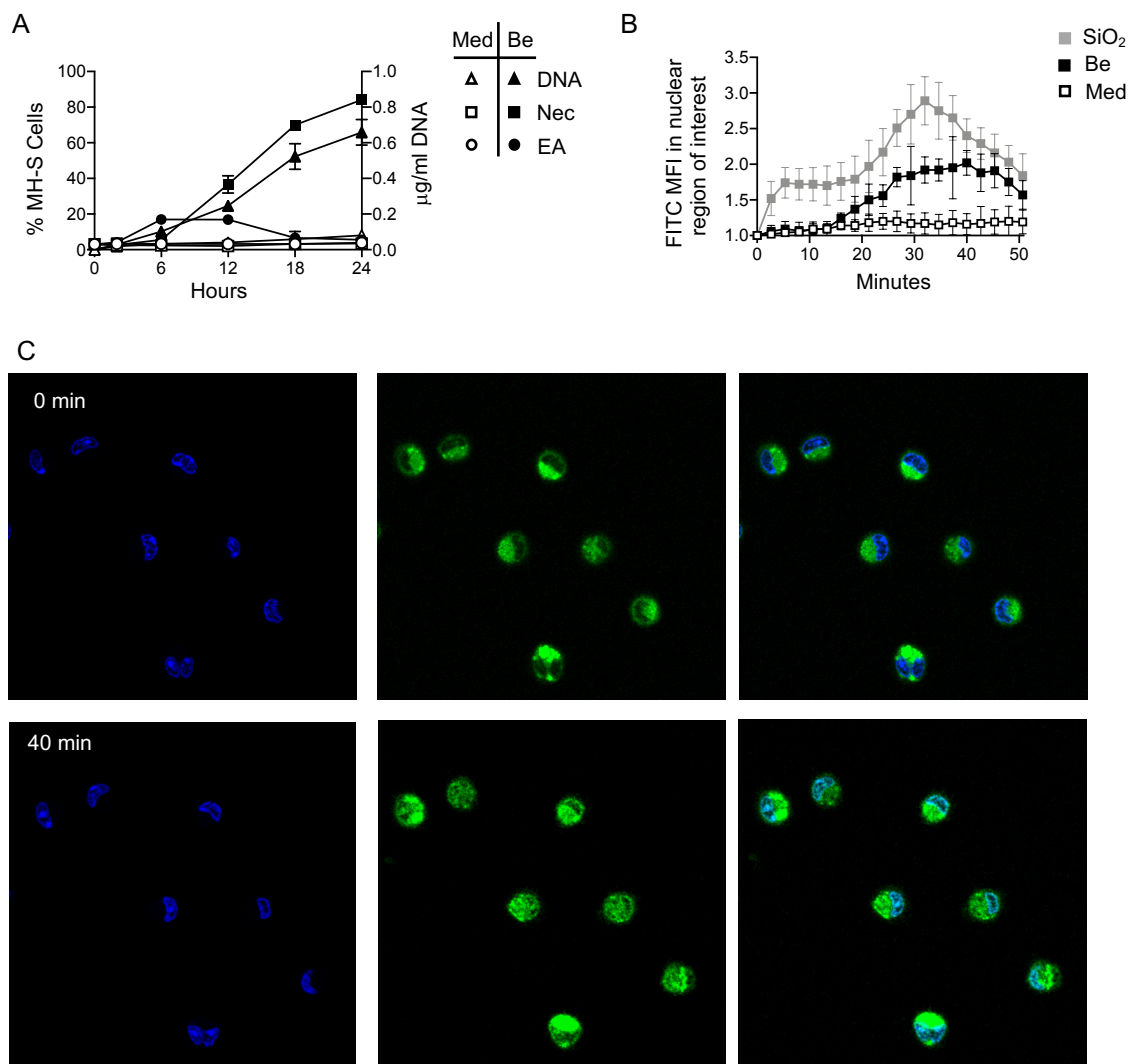




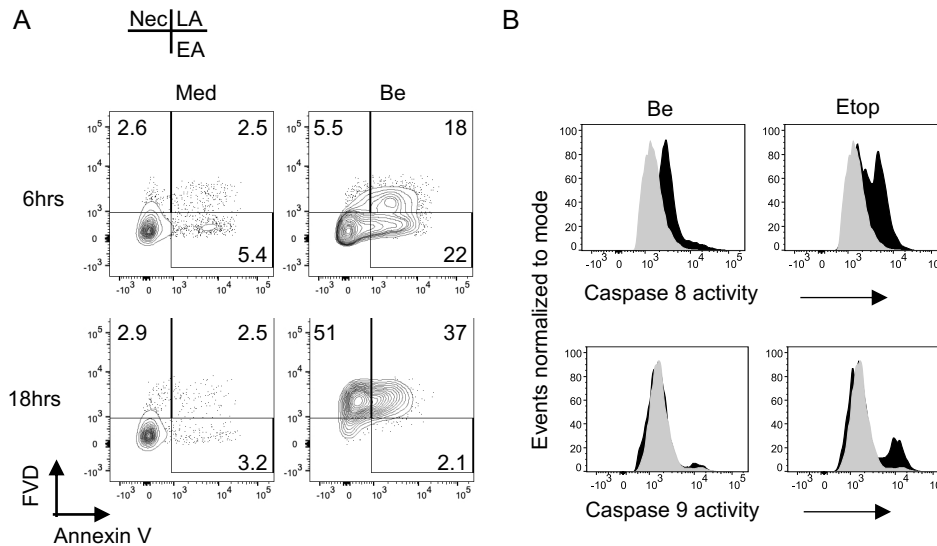
Supplemental Figure 1. ROS are not required for Be-induced AMΦ death or DAMP release. (A-C) B6 WT and *Nadph p47^{-/-}* (p47KO) mice were exposed i.t. to PBS +/- 80 μg Be and sacrificed 18hrs later. **(A)** BAL single cells were analyzed for expression of CD11c and SSC to differentiate AMΦs (upper row). AMΦs were analyzed for live (FVD⁻) and necrotic (Nec, FVD⁺) cells. **(B)** Percent AMΦs that were necrotic and concentrations of DNA and IL-1α in the BALF of PBS- or Be-exposed WT and p47KO mice are shown. The gating strategy **(C)** and total number of neutrophils/BAL **(D)** in PBS- or Be- exposed WT or p47KO mice are shown. **(E)** AMΦs from B6 mice were pretreated with 100 μM MnTBAP for 15 min and then exposed to media (Med) +/- 50 μg/ml Be or 2.5 μM PMA for 18hrs. Percent of AMΦs that were necrotic and concentrations of IL-1α in culture SNs are shown. Data in A,C are from representative samples/treatment group from one of two independent experiments. Data in B,D are combined from two independent experiments (n=7 mice/group). Data in E are combined from three independent experiments (performed in duplicate/experiment with separate cohorts of mice as sources of AMΦs for each experiment). Symbols on graphs are values from individual mice (B,D) or experimental means (E), bars indicate means +/- SEM. A one-way ANOVA was used to test for statistical differences between groups. Statistical p values for selected comparisons are indicated as * P<0.05, ** p<0.01, ***p<0.001.



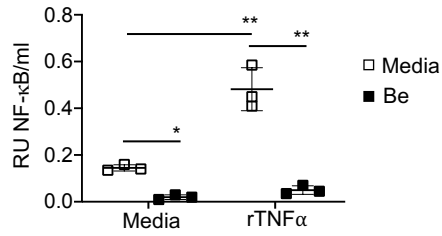
Supplemental Figure 2. Be-induced AM Φ cell death and IL-1 α release are independent of cathepsin B/L, caspase, RIP1K and RIP3K enzyme activity. (A) To determine if simultaneous blockade of both apoptotic/necroptotic programs would rescue Be-induced AM Φ death, we pretreated B6 AM Φ s with 20 μM Z-VAD-FMK (Z-VAD) + 0.5 μM GSK'872 (GSK) for 15 min. Cells were then cultured for 18hrs in media with or without 50 $\mu\text{g/ml}$ Be or 10 $\mu\text{g/ml}$ LPS + 40 μM Z-VAD-FMK (LZ) as a positive control for necroptosis and cell death and DAMP release was analyzed as described in Figure 1. Percent of AM Φ s that underwent necrosis and the concentrations of extracellular DNA and IL-1 α in culture SNs are shown. **(B)** B6 AM Φ s were pretreated with vehicle (Veh), 20 μM Z-VAD-FMK (Z-VAD), 10 μM Z-FF-FMK or Z-VAD + Z-FF-FMK for 15 min and exposed to media (Med) +/- 50 $\mu\text{g/ml}$ Be for 18hrs. Percent of AM Φ s that underwent necrosis and concentrations of IL-1 α in SNs are shown. Data are combined from three independent experiments (performed in duplicate/experiment with separate cohorts of mice as sources of AM Φ s for each experiment). Symbols on graphs indicate the mean value from each experiment, bars indicate the mean of means +/-SEM. A one-way ANOVA was used to test for statistical differences between groups. Statistical p values for selected comparisons are indicated as * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



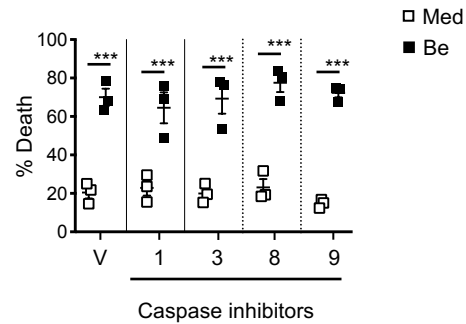
Supplemental Figure 3. Be-exposed MH-S cell death and associated lysosomal leakage. (A) The AMΦ-derived cell line MH-S was exposed to media (Med, white symbols) or Be particles (Be, black symbols) and the kinetics of early apoptosis (EA, circles), necrosis (Nec, squares) and DNA release (DNA, triangles) were analyzed over time as described in Figure 1. Symbols indicate the mean experimental values from two independent experiments (performed in triplicate/experiment) and error bars indicate SD. (B) FITC-dextran was used to load endosomes in MH-S cells and Hoechst was used to label nuclei. Cells were analyzed prior to (0hrs) and after addition of Be or SiO₂ particles by live cell imaging as described in Methods. FITC fluorescence associated with previously FITC negative nuclear regions was quantified using Imaris software at each time point in individual cells (n=10 cells were tracked per treatment group). Symbols show three-point rolling mean in FITC-dextran fluorescence intensity in nuclear regions of interest that were defined at time 0 for each cell. Each data track was normalized to equal 1 at time 0. Error bars indicate SD. Data are representative of one of two independent experiments. (C) Images of MH-S cells at 0 and 40 min after Be exposure for a representative sample are shown and show Hoechst (blue), FITC-Dextran (green) and overlay 60x images.



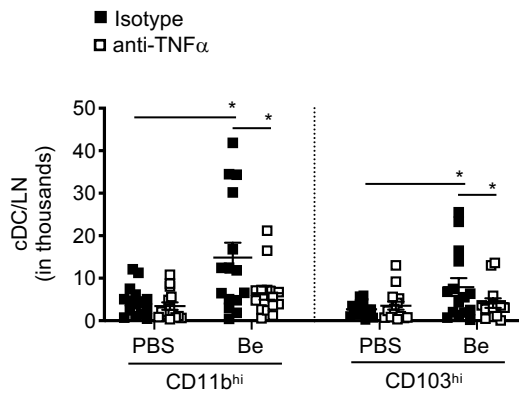
Supplemental Figure 4. Analysis of cell death and caspase activity in Be-exposed AMΦs. (A) B6 AMΦs were cultured in Media (Med) +/- 50 μg/ml Be. Gates used to identify early and late apoptotic cells (EA, LA) vs necrotic (Nec) cells are shown for representative samples exposed for 6 or 18hr. (B) Histograms show the fluorescence shift in FITC indicating caspase 8 or caspase 9 activity in AMΦs exposed for 10hrs to Be or etoposide (Etop) (solid black histograms) vs media controls (solid grey histograms). The 10hr time point was chosen because caspase 8 activity could be observed in both Be-exposed cells and Etop positive controls at this time. Data are representative examples from one of three independent experiments. Numbers on plots are percentages of total cells that are in each gate for the sample shown.



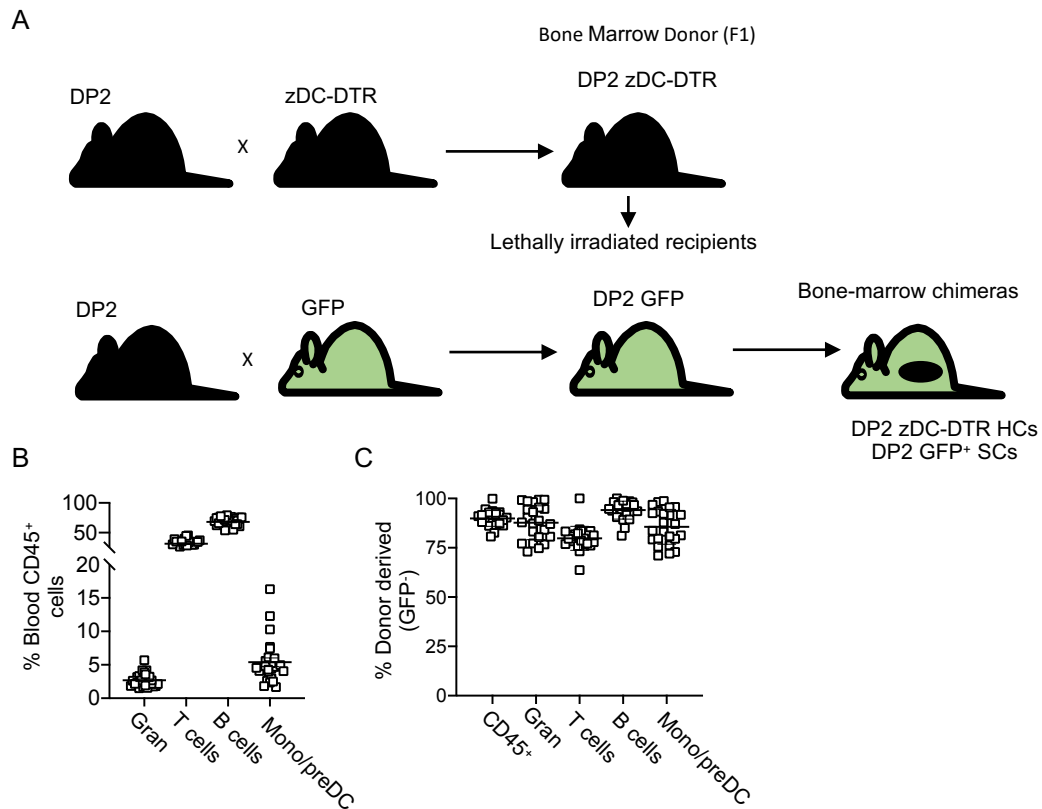
Supplemental Figure 5. Pre-treatment of macrophage cell lines with Be inhibits of TNF α -mediated activation of NF- κ B. RawBlue™ NF- κ B reporter cells were incubated with 50 μ g/ml Be or media for 2hrs, washed and incubated with media +/- 1 μ g/ml rTNF α overnight. The SNs were harvested and tested for the presence of the NF- κ B reporter enzyme as described in Methods. RU NF- κ B activity/ml SN are shown for indicated treatment groups. Symbols on graph indicate triplicate values, bars indicate means and error bars standard deviation. A one-way ANOVA was used to test for statistical differences between groups. Statistical p values for selected comparisons are indicated as * P<0.05, ** p<0.01.



Supplemental Figure 6. Caspase 1, 3, 8 and 9 activity are not required for Be-induced AM Φ cell death. AM Φ s from B6 mice were pretreated with caspase inhibitors and stimulated with media (Med) or Be for 18hrs as described in Figure 4D. The percent of AM Φ s that were necrotic are shown. Symbols on graph indicate experimental means from three independent experiments (performed in duplicate/experiment with separate cohorts of mice as sources of AM Φ s for each experiment), bars indicate the mean of means $\pm$ SEM. A one-way ANOVA was used to test for statistical differences between groups. Statistical p values for selected comparisons are indicated as ***p<0.001.

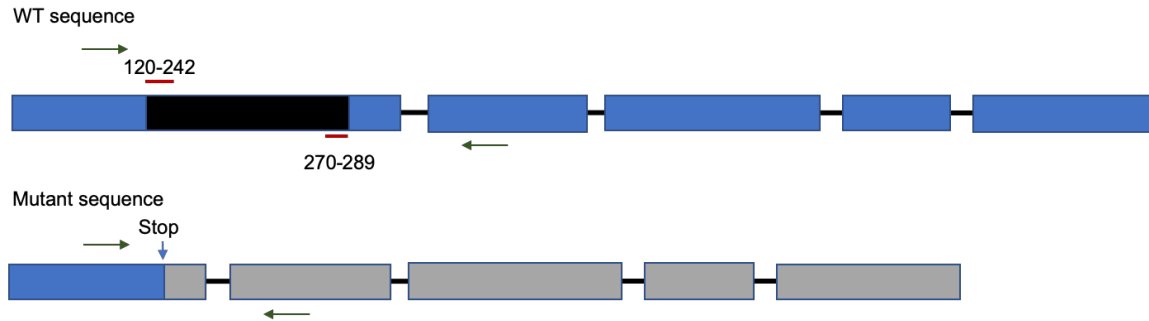


Supplemental Figure 7. Analysis of the effect of anti-TNF α treatment on recruitment of pulmonary DC subsets to the LDLNs of Be-exposed mice. B6 mice (n=15 per group) were treated i.p. with isotype control or anti-TNF α antibodies and exposed i.t. to PBS +/- 50 μ g Be. 24 hrs later cDC in the LDLNs were gated as described in Figure 6C and analyzed for CD11b⁺ or CD103⁺. Total numbers of each subset per LDLN are shown. Data are combined from three independent experiments. Symbols indicate values from individual mice. Bars on graphs indicate means and error bars SEM. A one-way ANOVA was used to test for statistical differences between indicated groups. Statistical p values for selected comparisons are indicated as * p<0.05.

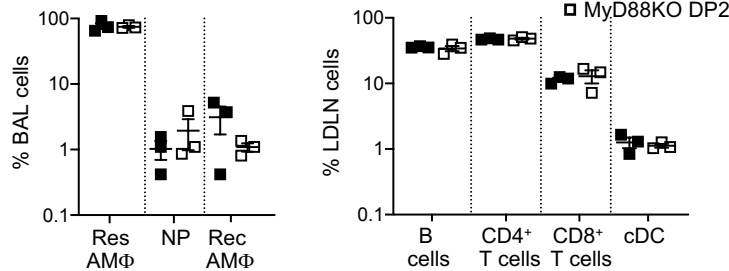


Supplemental Figure 8. Generation of DP2 zDC-DTR bone marrow chimeras for analysis of cDC function in a mouse model of CBD. (A) B6 DP2 zDC-DTR bone marrow donors and B6 DP2 GFP recipients were generated by performing F1 crosses between the indicated homozygous strains. Reconstitution of irradiated DP2 GFP mice with bone marrow from DP2 zDC-DTR mice resulted in DP2 zDC-DTR hematopoietic cells (HCs) and GFP⁺ DP2 stromal cells (SCs). A similar strategy was used to track chimerism in WT and MyD88KO chimeras described in Figure 8C **(B-C)** Mice were bled 8 weeks after bone marrow transplant and CD45⁺ cells were analyzed by flow cytometry. **(B)** Analysis of granulocytes (SSC^{hi}CD11b⁺, Gran), T cells (CD3⁺), B cells (CD19⁺) and monocytes/DC precursors (CD11b⁺/SSC^{lo}, mono/preDC) are shown. **(C)** The percent chimerism of indicated cell populations are shown and expressed as % of each cell population that was GFP⁺. Data are combined from two independent experiments (n=26 mice). Symbols on graphs indicate individual mice, bars indicate means.

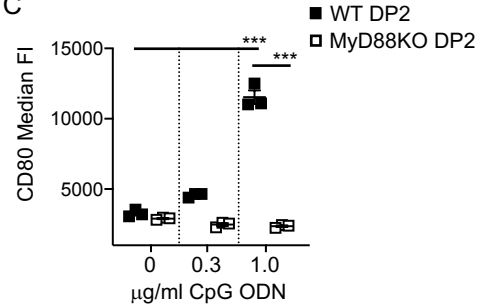
A



B



C



Supplemental Figure 9. Generation of MyD88KO DP2 Tg mice. (A) Schematic representation of the *Myd88* gene and Crispr-Cas9 targeting strategy. Two sets of RNA guides (red lines) for Cas9 were selected to target a ~160 bp region in exon one of the *Myd88* gene. Primers (black arrows) were used to amplify a 736 bp region in the WT gene. The founder selected for propagation had a 163 bp deletion in exon one. Sequencing of the targeted region showed that a frameshift occurred in the *Myd88* gene downstream of the Cas9 cleavage site that resulted in a stop codon and thus, disruption of MyD88 in the mutant strain. 5 of the predicted off-target sites were sequenced to confirm no alterations in the founder line. We intercrossed FVB/N DP2 *Myd88*^{+/-} mice to generate *Myd88*^{+/+} DP2 (WT DP2) and *Myd88*^{-/-} DP2 (MyD88KO DP2) littermates as bone marrow donors. (B) Analysis of BAL cells and LDLN cells from WT DP2 and MyD88KO DP2 bone marrow donors indicated that the mutant mice have a normal complement of mature immune cells in the airways and secondary lymphoid organs. (C) Analysis of responses to CpG/TLR9 activation in BMDC grown from WT DP2 and MyD88KO DP2 bone marrow. Upregulation of CD80 median fluorescence intensity (FI) on WT DP2 and MyD88KO DP2 BMDCs in response to CpG over 18hrs is shown and indicates functional disruption of MyD88 signaling.

Supplemental methods

Mice

C57BL6/J (B6, 000664), *Myd88*^{-/-} (MyD88 KO, 009088), B6(Cg)-*Zbtb46*^{tm1(HBEGF)Mnz/J} (zDC-DTR, 019506), *Nadph p47*^{-/-} (p47KO, 004742) and *Tnf*^{±/-} (TNFαKO, 005540) mice (all on the B6 background) were purchased from Jackson Laboratories. Mice that express GFP under the control of the ubiquitin promoter, C57BL/6-Tg(UBC-GFP)30Scha/J (B6 GFP) mice were kindly provided by Philippa Marrack (National Jewish Health, Denver, CO). HLA-DP2 transgenic (DP2) mice (1) on both the FVB/N and B6 background (backcrossed in the Fontenot laboratory using Jackson speed congenics until the mice were confirmed to be 98% identical to C57BL6/J) were obtained from Andrew Fontenot (CU Anschutz Medical Campus, Aurora CO). *Myd88*^{-/-} (MyD88KO DP2) were generated in FVB/N DP2 mice and validated at the Genomic Mouse Core Facility at National Jewish Health (Denver, CO). SgRNAs were designed to target a 163bp region in exon one of the *Myd88* gene (Supplemental Figure 9). *Myd88*^{-/-} (DP2) mice of this line exhibited a failure to thrive after weaning, as we observed previously after attempting to cross B6 DP2 mice with already established *Myd88*^{-/-} B6 mice. This FVB/N line was maintained with MyD88^{+/-} DP2 breeders and bone marrow was harvested after weaning to generate chimeras lacking MyD88 in the hematopoietic compartment. Male and female mice (8-12 weeks of age) were used in our experiments.

Primary tissue collection and processing

Bronchoalveolar lavage (BAL) was performed with three washes of 1ml DPBS and harvested into 15ml conical tubes containing 1ml cDMEM. The upper right mediastinal LNs (LDLNs) were dissected into 1ml cRPMI. Lungs were flushed by placing an incision in the left atrium of the heart followed by infusion of 10ml cold DPBS through the lung vasculature via insertion of a 25-gauge needle into the right ventricle of the heart. Flushed lungs were dissected into cRPMI, chopped into 3mm slices using razor blades, transferred into 15ml tubes containing 3ml cRPMI with 1 mg/ml collagenase and incubated 30 min at 37°C. Remaining tissue fragments were disrupted by serial passage through 16- and 18-gauge needles (5-6 times each). Lung digests were pelleted, and red cells were lysed for 2 min in ammonium chloride red blood cell lysis buffer (Invitrogen, 00-4333-57). Each digest was brought up to 15ml with DPBS, and the cells were strained through a 70 µm filters (Falcon). Cells were pelleted and resuspended in cRPMI media. LNs were teased apart and digested in 1 mg/ml collagenase in cRPMI for 30 min at 37°C, 5% CO₂. An equal volume 100 mM EDTA in DPBS (pH 7.4) was added to each digest for 5 min at RT, and cells were washed from the wells, strained through 70 µm filters and washed with DPBS.

Reagents

All stocks were prepared using buffers or solutions made with EndoGrade water (Hyglos, 607030) or DPBS (Hyclone, SH30028.02). Lack of endotoxin activity was confirmed to be <0.1EU/ml with the Endpoint Chromogenic Limulus ameocyte assay (Lonza, 50-647U). Beryllium hydroxide was precipitated from BeSO₄ solution using NaOH to pH 8.5, washed, and characterized as previously described (2). Be(OH)₂ was then adjusted to pH 7.4 (referred to in this study Be). BeO was purchased from Aldrich (202770), aluminum hydroxide was from InVivogen (vac-alu) and SiO₂

was a gift from US Silica Products (MIN-U-SIL, Frederick, MD). Prior to use, particles were placed in sterile DPBS in a sonicating water bath for 30 minutes to disperse crystal aggregates. Functional grade NA/LE rTNF α (410-MT) and Z-VAD-FMK (FMK001) were purchased from R&D Systems. Dexamethasone and etoposide were from the Apoptosis Inducer Kit from EMD Millipore (APT800). CaspGlow™ Fluorescein Active Caspase-3 (K183), -8 (K188), and -9 (K189) Kits, CaspGlow™ Red active caspase-8 Kit (K193), inhibitors for caspase 1 (Z-YVAD-FMK, 1012), caspase 3 (Q-DEVD-OPh, 1178), caspase 8 (Q-IETD-OPh, 1176), caspase 9 (Q-LEHD-OPh, 1177), the cathepsin B/L inhibitor Z-FF-FMK (1126), the Smac mimetic GDC-0917 (CUDC-427, B1918) and Enhanced Apoptotic DNA Detection Kits (K130) were all purchased from BioVision and used according to manufacturer instructions. Quick DNA Plus DNA (D4068) extraction kit was from Zymo Research. Cytochalasin D (1233), necrostatin-1 (2324) and GSK'872 (6492) were purchased from Tocris and reconstituted in DMSO (Sigma, D2438). Fixable Viability Dye (FVD) eFluor 506 (65-0866-14), the APC Annexin V Apoptosis Kit (88800774), IL-1 α ELISA kit (detects both the precursor/mature form) (88-5019-88), TNF α ELISA kits (88-7324-88), Sytox Green (S7020), Qubit dsDNA quantification kits (Q32851), ECL HRP substrate (32134), Halt™ Protease Inhibitor Cocktail (100X) (1860932), mouse genomic DNA (G309A), AccuCheck Count beads (PCB100), OneComp beads (01-1111-42), and IL-2 (3441-3-250, 3441-6-250) and IFN γ ELISPOT (3321-3-250, 3321-6-250) antibodies were from Invitrogen. Streptavidin-HRP (19534-050), AEC substrate (551951), and PHA (R30852801) were purchased from Invitrogen, BD Biosciences, and Remel. Collagenase from *Clostridium histolyticum* (C2139), CQ (C6628) and PMA (P8139) were purchased from Sigma. 1000X Zeocin selection reagent (ant-zn), RAW-Blue™ NF- κ B reporter cells (raw-sp), QuantiBlue SEAP detection reagent

(rep-qb1) and Vaccigrade LPS-EB (from *E. coli* o111:B4, vac-3pelps) were purchased from InvivoGen. MH-S cells (ATCC® CRL-2019™) were obtained from ATCC.

Antibodies

The following monoclonal antibodies (mAbs) against mouse targets were used for flow cytometry: PerCP-Cy5.5 anti-CD11b (M1/70/ 45-0112-82), eFluor 450 anti-F4/80 (BM8/ 48-4801-82), FITC anti-Ly6G (1A8-Ly6G/ 11-9668-80), FITC anti-CD103 (2E7/ 11-1031-82), eFluor 450 anti-MHCII (M5/114.15.2/ 48-5321-82), anti-CD19 (1D3/ 11-0193-82), PE-Cy7 anti-CD11c (N418/ 25-0114-82) and eFluor 450 anti-CD8a (53-6.7/ 48-0081-82) (all from ThermoFisher/Invitrogen); anti-CD16/CD32 (2.4G2/ 14-0161-85), PE anti-CD80 (16.10A1/ 553769), PE anti-CD64 (X54.5/7.1/ 558455), APC-Cy7 anti-CD45 (30F-11/ 561037) and PE anti-Siglec-F (E250-4440/ 552126) (BD Biosciences), Pacific Blue anti-Ly6G (1A8-Ly6G/ 127612), FITC anti-MerTK (2B10C42/ 151503), anti-CD4 APC (GK1.5/ 100412), APC/Fire™ 750 anti-CD11c (N418/ 117352) and Brilliant Violet 510 anti-CD45 (30F-11/ 103138) (all from BioLegend) and PE anti-CD3e (145-2C11/ 60-0031) from Tonbo. The functional grade NA/LE blocking mAbs against TNFR1 (Leinco Technologies Inc., 55R-593/ T245), TNFR2 (TR75-54.7/ BE0247), anti-mouse TNF α mAb (XT.3.11/ BE0058), HRPN isotype control (BE0088) and rat IgG1 (TNP6A7/ BE0290) (all from Bio X Cell) were used at a final concentration of 10 μ g/ml in cDMEM + 0.01% DMSO. Titration/optimization experiments determined this concentration/formulation with DMSO had maximal impacts of blocking the receptors and minimal inhibitory impacts of isotype controls.

Cell culture and media

Complete DMEM (cDMEM: high glucose DMEM (11995-065) containing 0.2 U/ml penicillin, 0.2 µg/ml streptomycin, 0.6 µg/ml L-glutamine (10378016) 10 mM HEPES (15630-080), 1 mM sodium pyruvate (11360-070) (all from Gibco) and 10% FCS (Hyclone, SH30.71.03) were used for in vitro experiments with primary AMΦs and RAW-Blue™ reporter cells. RAW-Blue™ reporter cells (containing chromosomal integration of a secreted embryonic alkaline phosphatase (SEAP) reporter construct inducible by NF-κB) were maintained according to manufacturer instructions, and reporter assays were detected with QuantiBlue™ reporter detection reagent. MH-S media (ATCC-formulated RPMI-1640 Medium (ATCC® 30-2001™) supplemented with 50 µM β-mercaptoethanol (Sigma, M6250) and 10% FCS) was used for the MH-S cell line. Experiments with MH-S and RAW-Blue cells were performed with cells having undergone < 5 passages and were maintained according to manufacturer instructions. Complete RPMI (cRPMI; RPMI 1840 (Corning, 10-040-CV) supplemented with 0.2 U/ml penicillin, 0.2 µg/ml streptomycin, 0.6 µg/ml L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 10% FCS) was used for ex vivo LN and lung cells. The following stimuli were used for in vitro experiments: 50 µg/ml sonicated Be, 10 µg/ml LPS + 40 µM Z-VAD-FMK (LZ) or 10 µM GDC-0917 Smac mimetic + 40 µM Z-VAD-FMK (SZ) to induce necroptosis, 100 µM dexamethasone or etoposide to induce apoptosis, 2.5 µM PMA to induce ROS or 10 µg/ml rTNFα to induce activation of NF-κB.

Assessment of extracellular cytokines and DNA

For cytokine analysis, BALF was concentrated 10-fold using 3K Amicon concentrators (UFC800396) per manufacturer instruction. Concentrated BALF and SNs were tested for IL-1α

and TNF α by ELISA. DNA in unconcentrated BALF and SNs was quantified using the HS dsDNA Qubit Assay or using 5 μ M Sytox Green as described below. All inhibitors and cell-free media were tested in order to control for their impacts upon the validity of the DNA quantification methods, of note MnTBAP interfered with DNA detection and was excluded from analysis. cDMEM (for in vitro experiments) and PBS/cDMEM mock BALF (for in vivo experiments) were used as blanks in determining DNA concentration to control for background levels of DNA in the FCS within the culture media or BAL. DNA was quantified using Qubit dsDNA detection kit and reader or for cytokines by ELISA and read on a PowerWave HT plate reader and analyzed with Gen5 software (both from BioTek). In some experiments extracellular DNA was quantified using 5 μ M Sytox Green, ran with a genomic mouse DNA standard curve in 96 well plates (Greiner, 655801) and read on a GlowMax microfluorimeter (Promega). Nucleosomes were detected using the Cell Death Plus ELISA kit (Roche, 11 774 425 001). A qPCR method (3) was used to determine the copy number of mtDNA or nuDNA present in the BALF of Be-exposed mice. BALF DNA was extracted using the *Quick-DNA*[™] Miniprep Plus kit from Zymo Research (D4068) per manufacturer instructions. Total copy number/ml of the original sample was calculated and multiplied by the total ng/copy number (as determined using molar equivalents) to calculate ng/ml of each type of DNA present in the BALF.

Quantification of intracellular IL-1 α

AM Φ s in BAL from naïve mice were counted, pelleted and lysed with five freeze/thaw cycles in liquid N₂. 0.5ml lysate buffer (DPBS containing 0.05% Tween 20 (VWR, 0777-1L) in DPBS and 1X Halt[™] Protease Inhibitor) was added to the cells. Cells were subjected to three to five 20-

second pulses with a mechanical tissue disruptor on ice. Cell lysates were centrifuged for 10 min at 10,000 xg to remove non-soluble particulates/debris, filtered through a 0.5 µm filter and precise volumes were recorded. IL-1α was quantified in duplicate by ELISA that detects both the precursor and mature form and used to determine pg cytokine/1x10⁵ AMΦs in the original samples.

Flow cytometry

To analyze cell death, AMΦs and BAL cells stained with eFluor 506 Fixable Viability Dye 1:500 in DPBS for 30 min on ice, followed by washing the cells with Annexin V Binding Buffer and incubating the cells for 15 min RT with Annexin V APC. Gates were based on unstained and stained fresh BAL cells or HK BAL cells as previously described (2, 4). For ex vivo analysis of innate cells in the airways of PBS or Be treated mice or for counting AMΦs, BAL cell populations were analyzed with the following mAbs: Fc Block, PE anti-Siglec F or CD64, FITC anti-MerTK or Ly6G, eFluor 450 anti-Ly6G or anti-MHCII (IA/IE), PerCP-Cy5.5 anti-CD11b, APC-Cy7 anti-CD45, and PE-Cy7 anti-CD11c. To assess cDCs, LDLN cells were stained with the following mAbs: Fc Block, FITC anti-CD103, PE anti-CD80, PerCP-Cy5.5 anti-CD11b, eFluor 450 anti-IA/IE, APC-Cy7 CD45 and PE-Cy7 anti-CD11c. For analysis of blood mononuclear cells in reconstituted bone marrow chimeras, cells were stained with Fc Block, BV510 anti-CD45, FITC anti-CD19, APC anti-CD4, e405 anti-CD8, PerCP Cy5.5 CD11b, APC-AF780 CD11c and PE-Cy7 CD3.

NF-κB reporter assay

RAW-Blue™ cells were maintained per manufacturer instructions in cDMEM under Zeocin selection to 80% confluence. The cells were harvested by incubating on ice with DPBS for 30 min, detached from culture flasks with a cell scraper and plated (50,000 cells/well) in cDMEM overnight at 37° C, 10% CO₂. The cells were treated +/- 50 µg/ml Be for 2hrs, washed three times with warm cDMEM to removed free Be, and cultured for 24hrs in cDMEM +/- 10 µg/ml rTNF α . Plates were spun at 400 xg for 5 min, and supernatants were harvested and tested for the presence of the NF- κ B reporter using the QuantiBlue™ Substrate per manufacturer instructions. Assay plates were read for development of the substrate at 605nm, and NF- κ B activity is expressed as relative units (OD absorption).

Microscopy

MH-S cells were added to an 8-chamber plate (Ibidi) 1 x 10⁵/well in MH-S media and allowed to adhere for 30 min in 10% CO₂ at 37°C. The cells were taken out of incubator and washed twice with PBS to remove non-adherent cells. The endosomes were loaded with 4 kDa FITC-Dextran (Sigma, 46944-100MG-F) and washed three times with PBS. Nuclei were stained with Hoechst (Sigma, 62249) for 10 min at RT. The cells were then washed twice in (DPBS containing 1% BSA (Sigma, A2153) and resuspended in 200 µl of phenol red-free cRPMI (Invitrogen, 11835-030). The cells were imaged on a Zeiss LSM780 2 photon microscope at 60X magnification every 160 sec before and after treatment with nothing (media), 50 µg/ml Be or 50 µg/ml SiO₂ for 1hr. FITC-dextran fluorescence intensity was measured in the nuclear regions of interest (ROIs) in individual cells before and after particle exposure using Imaris image analysis software. Data were expressed using a three-point rolling mean (using the time point before and after each value) of the FITC

fluorescence density in nuclei ROIs at each time point. Time 0 values were normalized to equal 1 for each cell track. Ten cells that remained in focus for the duration of the time lapse were imaged per treatment group were analyzed. Immunofluorescence images were analyzed using Fuji/ImageJ Software. H&E sections were scanned using a Leica Aperio AT2 digital scanning microscope and analyzed with ImageScope Software (Leica). Bronchioles on each section were enumerated (on average ~40 bronchioles were captured/lung scan) and the number of bronchioles with an inflammatory infiltrate were enumerated to determine the percent of bronchioles with infiltrates per section. The software was then used to measure the diameter of each infiltrate from the edge of the bronchiole epithelium to the radial edge of the infiltrate.

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