SUPPLEMENTAL METHODS

Binding and phagocytosis assay

Flow cytometric analysis of binding and phagocytosis were performed as previously described.[1] 500 μl mouse neutrophils (4×10^6/ml or 1×10^6/ml) in RPMI1640 (0.4 mM Ca^{2+}, Corning) without serum were added to Eppendorf tubes and FITC-zymosan (MOI=2) was added and incubated on ice for 20 min or at 37°C in 5% CO_2 for 30 min. Eppendorf tubes were placed on ice to stop phagocytosis, and the cell-zymosan suspension analyzed in the flow cytometer with and without the addition of Trypan Blue (Sigma-Aldrich, 0.1%) to quench the signal from uningested zymosan. Cells with trypan blue-quenched FITC-zymosan, representing cell surface-bound zymosan, or ingested FITC-zymosan were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Apoptosis assays

Cell apoptosis was determined with flow cytometry by the eBioscience Annexin V Apoptosis Detection Kit (Invitrogen) according to the manufacture's protocol. For in vitro experiment, 100 μl purified mouse neutrophils (4×10^6/ml or 1×10^6/ml) in RPMI1640 were mixed with zymosan (MOI=2) and added to Eppendorf tubes and incubated at 37°C in 5% CO_2 for various times. 1 μl APC-Annexin V (Invitrogen #17-8007) was added to 100 μl of the cell suspension and incubated 10 minutes on ice, then washed and stained with 5 μl 7-AAD Viability staining solution (Invitrogen # 00-6993) prior to flow cytometry. To assess neutrophil apoptosis during lung inflammation, mice were challenged with 20 μg sterile zymosan in 25 μl PBS by intranasal (IN) instillation and then euthanized at 14 hours. Single cell suspensions of lung tissue were incubated with anti-mouse CD16/32 (clone 2.4G2, BioXcell, West Lebanon, NH) to block Fc-receptors and stained with the following antibodies: BV510 Rat anti-mouse CD45 antibody (clone 30-F11, BD Horizon, San Jose, CA), V450 Rat anti-mouse Ly6G antibody (clone 1A8, BD Horizon, San Jose, CA), PE Rat anti-mouse CD11b antibody (clone M1/70, eBioscience, San Diego, CA). After wash, 5 μl APC-Annexin V was added to 100 μl of the cell suspension and incubated 10 minutes on ice, then washed and stained with 5 μl 7-AAD Viability staining solution prior to flow cytometry. Neutrophils were identified as $CD45^+Ly6G^+CD11b^+$.

Human neutrophil isolation

Human peripheral venous blood was obtained following informed consent from healthy volunteers and a CGD patient with a null mutation in *CYBB*, as approved by the WUSM Institutional Review Board. EDTA was the anticoagulant. PMN were isolated by centrifugation

with Polymorphprep (Axis-Shield PoC AS, Oslo, Norway) density gradient at 500g for 35 min at room temperature, according to manufacturer's protocol. After isolation, cells were washed in PBS and remaining red blood cells were lysed with hypotonic saline as for mouse PMN, then centrifuged at 400g at room temperature. PMN were resuspended in RPMI 1640 (0.4 mM Ca^{2+}, Corning) at density of $4x10^6$/ml. Purity was ≥ 95%.

Human neutrophils activated with zymosan in vitro

Normal or CGD PMN ($4x10^6$/ml) were incubated in 96 well tissue culture plate in RPMI 1640 with or without Diphenyleneiodonium chloride (DPI) (Invivogen) at the concentration of 10 μM for 10 min at 37°C and then stimulated with Zymosan (MOI=5) or serum opsonized zymosan (SOZ; MOI=5) with or without DPI. SOZ was prepared as previously described.[2] Plates were incubated at 37 °C for 1 hour. Final volume per well was 100 μl. After incubation, plates were centrifuged 400g for 10 min and supernatants were collected and stored in -80 °C for later use.

Impact of myeloperoxidase on LTB4 produced by WT and CGD mouse neutrophils

To study potential impact of reactive chlorine species produced by myeloperoxidase (MPO) on LTB4 generation or degradation, we used a specific MPO inhibitor 4-Aminobenzoic acid hydrazide (4-ABAH) or MPO-H_2O_2-Cl^- systems using H_2O_2 directly or Glucose and Glucose oxidase (GGO) as a sustained source of H_2O_2. For MPO inhibitor experiments, 100 μM 4-Aminobenzoic acid hydrazide (4-ABAH; Cayman) was preincubated with mouse PMN for 10 min at 37 °C, then mouse PMN ($4x10^6$/ml) were incubated in 96 well tissue culture plates in RPMI 1640 and stimulated with Zymosan (MOI=2) in the presence of 100 μM 4-ABAH for 1 hour at 37°C. Cell culture supernatants were collected for LTB4 ELISA. For MPO-H_2O_2-Cl^- experiments, 1 nM MPO (Sigma-Aldrich) and either 10 μM H_2O_2 (Sigma-Aldrich) or 0.01 μg/ml Glucose oxidase (GOD; Sigma-Aldrich) were added to RPMI 1640 (Corning Inc.), which 2 g/l D-glucose. We documented that GGO system (2 g/l Glucose in RPMI 1640 with 0.01 μg/ml Glucose oxidase) generated suitable amount of H_2O_2 compared to the ROS generated by WT PMN and zymosan using chemilumescence enhanced by luminol.[3] Mouse PMN ($4x10^6$/ml) were incubated in 96 well tissue culture plates and stimulated with Zymosan (MOI=2) in the presence of different MPO-H_2O_2-Cl^- system for 1 hour at 37°C, and cell culture supernatants collected for LTB4 ELISA. In other experiments, mouse PMN ($4x10^6$/ml) were stimulated with Zymosan (MOI=2) for 1 hour. Supernatants were collected and incubated with different MPO-H_2O_2-Cl^- system for 20 min at 37 °C as another approach to determine whether reactive chlorine species resulted in LTB4 degradation. LTB4 levels were quantified by LTB4 ELISA.

Activation of neutrophils in vitro with immobilized Immune complexes (IC)

Immune complexes were formed using human Lactoferrin (LFR, Sigma-Aldrich) and anti-Lactoferrin antibody (anti-LFR Ab, Sigma-Aldrich) as previously reported.[4] 20 μg/ml LFR in 0.05 M Carbonate-Bicarbonate buffer (PH=9.6, Sigma-Aldrich) was used to coat wells of 96-well tissue culture plates at room temperature for 1 hour. Wells were then blocked with 10% heat-inactivated FBS in PBS at room temperature for 1 hour, and anti-LFR Abs (1/500 dilution) in 10% FBS added and incubated at room temperature for 1 hour. Wells without the anti-LFR Ab incubation were regarded as controls.

Human or mouse neutrophils suspended at $4x10^6$/ml in RPMI1640 (0.4 mM Ca^{2+}, Corning) without serum were added to wells coated with immobilized IC and incubated at 37°C with 5% CO_2 for varying times. After centrifugation, supernatant was collected and stored in -80 °C prior to LTB4 ELISA. 10 μM DPI (Invivogen), 10 μM U75302 (Cayman), superoxide dismutase (SOD; #S7571, Sigma-Aldrich) and/or catalase (#C9322, Sigma-Aldrich) were also added in some experiments.

Measurement of intracellular calcium levels

Bone marrow neutrophil isolation and intracellular calcium measurements were performed as previously described.[5] BM was collected and red blood cells were lysed by hypotonic saline solution. Neutrophils were separated using a single step Percoll density gradient. Single cell suspensions in HBSS/HEPES were loaded with 3 μM Indo-1 AM and 2.5 mM probenecid (Molecular probes) at room temperature for 40 minutes in the dark. Then cells were washed and resuspended in cold HBSS/H (1 million/ml) with 0.5 mM Mg^{2+} and the indicated calcium concentration before use. Before analysis, cells were aliquoted into fluorescence-activated cell sorter (FACS) tubes containing HBSS/H/Mg/Ca, warmed to 37°C for 1.5 minutes, then run on the flow cytometer (LSR Fortessa, Becton Dickinson) for 30 seconds to establish a baseline reading. LTB4 (Cayman), thapsigargin (Sigma-Aldrich), ionomycin (Sigma-Aldrich) or cell stimulation cocktail (Invitrogen, ionomycin + phorbol myristate acetate (PMA)) was added and samples were analyzed in the presence of indicated calcium concentrations. For zymosan stimulation, cells were pre-incubated with zymosan (MOI = 10) on ice and then placed in a 37 °C water bath during flow cytometry to start synchronized stimulation. Data were analyzed by FlowJo Software (Tree Star Inc.). The data shown are representative kinetic tracings from WT/CGD pairs, or data compiled from independent experiments and quantified as AUC.

Neutrophil imaging in vitro and quantification

Mouse neutrophils (4×10^6/ml in RPMI1640) were primed with mouse TNF-α (50 ng/ml, R&D systems) at 37 ºC for 1 hour in 5% CO_2 incubator, then stained with Hoechst 33342 Trihydrochloride, Trihydrate (5 μg/ml, Invitrogen, Carlsbad, CA) at 37 ºC for 30 min in 5% CO_2 incubator, washed, and resuspended in RPMI 1640 medium (without L-glutamine and phenol red, Corning Inc.). Wells in a 4-well micro-insert 35 mm dish (Ibidi GmbH, Grafelfing, Germany) were coated with 10% heat-inactivated FBS at room temperature for 1 hour and washed twice with PBS. FITC-Zymosan (final concentration, 8×10^6/ml, Invitrogen, Calsbad, CA) and neutrophils (final concentration, 4×10^6/ml) were mixed well and 10 μl added into each well and incubated at 37 ºC for 1 hour in 5% CO_2 incubator. Hoechst 33342 stained cells (*Ex*405 nm/*Em*420-460 nm) and FITC zymosan (*Ex*488 nm/*Em*500-540 nm) were visualized using a Leica SP8X laser scanning confocal microscope, kept at 37ºC and 5% CO2 in an Okolab Cage incubator, using a 10x Apochromat air objective (N.A. 0.4). Images were acquired using LASX software (v 3.6.0; Leica Microsystems, Wetzlar, Germany). Aggregation of more than 10 neutrophils was treated as clusters. Cluster numbers were counted in a 950 μm × 950 μm image, and normalized to cluster number per 1 mm^2. The area of a neutrophil cluster was determined by the area of Hoechst 33342 nuclear staining. Analysis was performed using Volocity Quantification (v6.3.3; GeHealthcare, Chicago, IL) and Image J (NIH, Bethesda, MD).

Zymosan-induced lung inflammation

Mice were challenged with 20 μg sterile zymosan in 25 μl PBS by intranasal (IN) instillation and then euthanized at indicated time points. Bronchoalveolar lavage (BAL) samples were obtained by three sequential one ml lavages with ice cold PBS with 2mM EDTA and 2% FBS. The 1st ml of BAL supernatant was frozen at -80ºC for ELISA. After red cell lysis, pooled cells from all 3 ml were used to enumerate cell counts by hemocytometer and the leukocyte differential using Wright-Giemsa stained cytospins. For analysis of single cell lung suspensions, the right lung inferior lobe was digested as described[6] prior to cell counts and flow cytometry. For histology, lungs were inflated with 10% formalin prior to further processing. Homogenates for LTB4 ELISA were prepared from lungs snap-frozen in liquid nitrogen.

Neutrophil depletion during zymosan-induced lung inflammation

Neutrophil depletion followed a described protocol.[7] Mice were injected IP with 200 μg Anti-Ly6G antibody (clone: 1A8, BioXcell, West Lebanon, NH) on days 0 and 2 and 100 μg Anti-

Rat IgGk antibody (clone: MAR18.5, BioXcell) on day 1. Mice were challenged with 20 µg zymosan IN 4 hours after Anti-Ly6G antibody IP injection on day 2. Mice were euthanized 14 hours after zymosan challenge and evaluated for neutrophil depletion in peripheral blood obtained by cardiac puncture. White blood cell counts (WBC) were analyzed using a Hemavet and % of neutrophils was scored by Wright-Giemsa stained blood smears.

Lung tissue homogenates for LTB4 analysis

In some experiments, whole lung was collected after lavage and snap froze in liquid nitrogen. Steel beads and 1 ml PBS was added into the tubes. Samples were homogenized for 5 min and centrifuged at 12000 rpm for 20 min at 4°C. The homogenate supernatant was collected and frozen at -80 °C for later use. For subsequent analysis of LTB4 by ELISA, thawed homogenate supernatants were mixed with 4 times the volume of ethanol and mixed thoroughly. The mixture was incubated at 4°C for 5 minutes and centrifuged at 3000 g for 10 minutes, then dried using a rotary vacuum evaporator. Dried samples were resuspended in LTB4 ELISA buffer (Cayman) in a volume equal to the starting supernatant volume.

ELISA

LTB4 was measured using a LTB4 ELISA kit (Cayman) according to the manufacture's protocol. Cytokines were analyzed using Mouse IL-1β ELISA kit (R&D systems), Mouse CXCL2 ELISA kit (R&D systems) and Mouse G-CSF ELISA kit (R&D systems).

Flow cytometry of BAL and lung samples

Single cell suspensions of lung tissue were incubated with anti-mouse CD16/32 (clone 2.4G2, BioXcell, West Lebanon, NH) to block Fc-receptors and stained with the flowing antibodies: BV510 Rat anti-mouse CD45 antibody (clone 30-F11, BD Horizon, San Jose, CA), V450 Rat anti-mouse Ly6G antibody (clone 1A8, BD Horizon, San Jose, CA), PE-Cy7 Hamster anti-mouse CD11c antibody (clone HL3, BD Pharmingen, San Jose, CA), APC Rat anti-mouse CD11b antibody (clone M1/70, eBioscience, San Diego, CA), PE Rat anti-mouse siglec-F antibody (clone E50-2440, BD Pharmingen, San Jose, CA), PerCP/Cy5.5 Rat anti-mouse Ly-6C antibody (clone HK1.4, Biolegend, San Diego, CA), with gating strategy shown in Supplemental Figure 2B . In PMN depletion experiments, an additional FITC Rat anti mouse Ly-6B.2 Alloantigen antibody (clone 7/4, Bio-rad, Hercules, CA) was used to detect PMN in mice treated with Anti-Ly6G, which blocks detection of Ly6G, with gating strategy shown in

Supplemental Figure 3. Data were collected on FACScan (BD, Franklin Lakes, NJ) and analyzed by FlowJo (Tree Star Inc.).

Histology and Immunohistochemistry

In some experiments, the left lung was fixed by inflation using 10% formalin, dehydrated by ethanol, embedded in paraffin, and cut into 5 μm sections. Tissue sections were stained with H&E. For immunohistochemistry, tissue sections underwent antigen retrieval with Antigen unmasking solution (Vector Laboratories, Burlingame, CA) with the manufacture's protocol , and then were blocked with 2% gelatin from cold water fish skin (Sigma-Aldrich) for 1 hour at room temperature, incubated with a 1/500 dilution of Rabbit anti-mouse myeloperoxidase antibody (ab139748, Abcam, Cambridge, CA) at 4^{o}C overnight, followed by incubation with biotinylated goat anti-rabbit antibody (1/500 dilution for 1 hour at room temperature, Vector Laboratories) and detected by VECTASTAIN ABC kit (Vector Laboratories) and DAB peroxidase substrate kit (Vector Laboratories). Sections were counterstained with hematoxylin (Gill's formula, Vector Laboratories). The images were captured with NanoZoomer digital slice scanner (Hamamatsu Photonics, Japan). Clusters of more than 10 MPO stained cells was treated as a foci. Foci number and diameter (longest distance in a foci) were analyzed by NDP.view2 software (Hamamatsu Photonics), and lung tissue section area was determined by Visiopharm software (Visiopharm, Medicon Valley, Denmark). Number of foci was normalized as total foci numbers per total lung tissue section area (10 mm^2).

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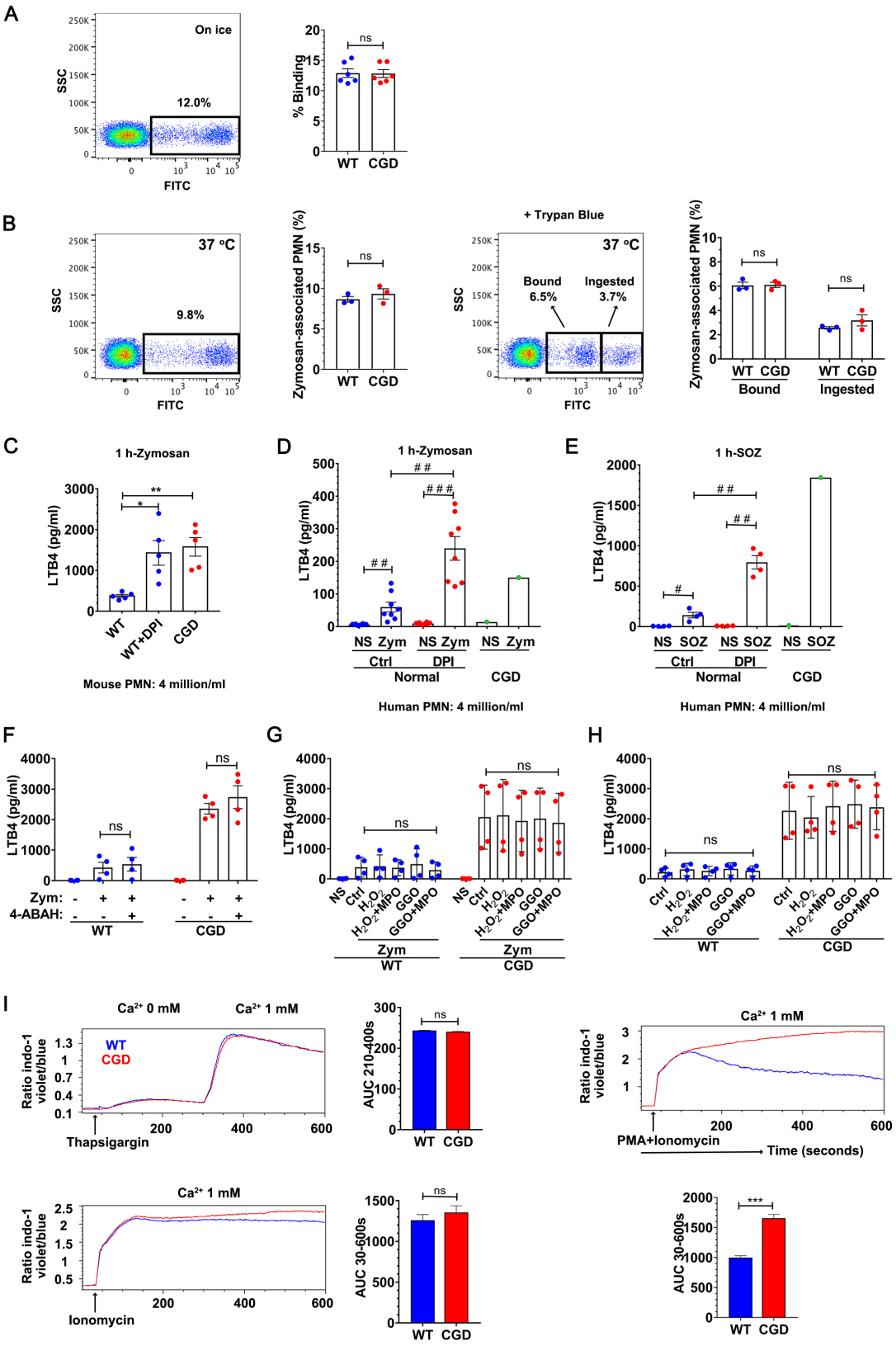
SUPPLEMENTAL FIGURE 1



Supplemental Figure 1. Analysis of zymosan uptake, calcium responses to other agonists, and LTB4 production by DPI-treated mouse and human neutrophils

(A) Mouse BM neutrophils ($4x10^6$/ml) were mixed with FITC-zymosan (MOI=2) and were incubated on ice for 20 min. Samples were run by flow cytometry and % binding (FITC-zymosan positive neutrophils) was analyzed by FlowJo. Mean ± SEM, n=6. Student T test.

(B) Mouse BM neutrophils ($4x10^6$/ml) were mixed with FITC-zymosan (MOI=2) and were incubated at 37°C in 5% CO_2 for 30 min. Samples were run by flow cytometry with or without Trypan Blue (0.1%) and PMN with bound zymosan and PMN with ingested zymosan was analyzed by FlowJo. Mean ± SEM, n = 3. Student T test.

(C) Mouse BM neutrophils ($4×10^6$/ml) in RPMI1640 were pretreated with 10 μM DPI for 10 minutes and then stimulated with zymosan (MOI=2) for 1 hour in the presence of 10 μM DPI. LTB4 levels in culture supernatant was measured by ELISA. Mean ± SEM, n = 5 from 2 separate experiments. Student T test (*, P<0.05, **, P<0.01).

(D-E) Human normal or CGD neutrophils from peripheral blood ($4x10^6$/ml) were pretreated or not with 10 μM DPI for 10 minutes and then stimulated for 1 hour with zymosan (MOI=5) or SOZ (MOI=5) in RPMI1640 in the presence of 10 μM DPI or not. LTB4 levels in culture supernatant was measured by ELISA. Mean ± SEM. **(D)** n = 8 normal, n = 1 CGD. **(E)** n = 4 normal, n = 1 CGD. Paird T test (# #, P<0.01, # # #, P<0.001).

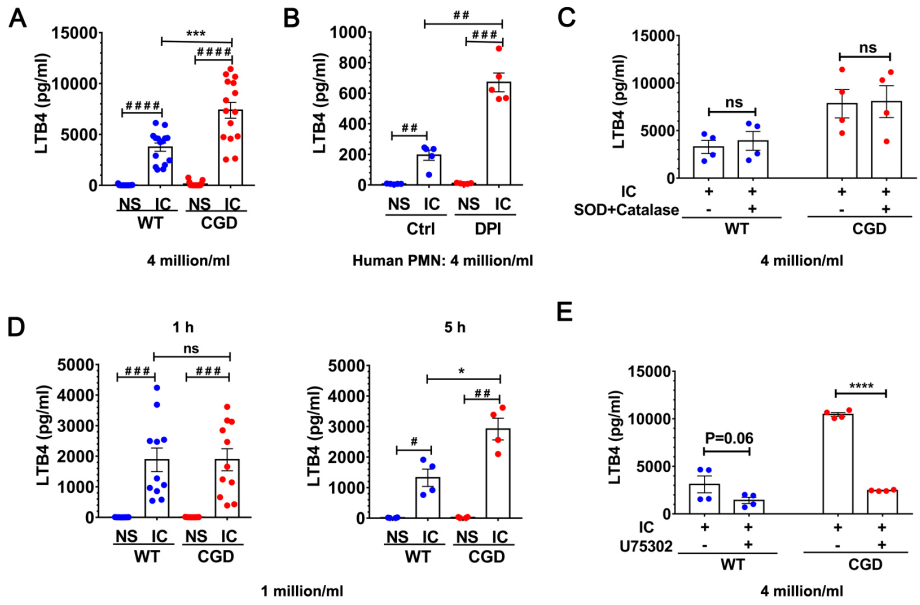
(F) Mouse BM neutrophils ($4×10^6$/ml) in RPMI1640 were pretreated with 100 μM 4-ABAH for 10 minutes and then stimulated with zymosan (MOI=2) for 1 hour in the presence of 100 μM 4-ABAH. LTB4 levels in culture supernatant was measured by ELISA. Mean ± SEM, n = 4 from 2 separate experiments. 1-way ANOVA.

(G) Mouse BM neutrophils ($4×10^6$/ml) in RPMI1640 were stimulated with zymosan (MOI=2) for 1 hour in the presence of 10 μM H_2O_2 or GGO (2 g/l Glucose in RPMI 1640 and 0.01 μg/ml Glucose oxidase) with or without 1 nM MPO. LTB4 levels in culture supernatant was measured by ELISA. Mean ± SEM, n = 4 from 2 separate experiments. 1-way ANOVA.

(H) Mouse BM neutrophils ($4×10^6$/ml) in RPMI1640 were stimulated with zymosan (MOI=2) for 1 hour. Then the Culture supernatant was incubated with 10 μM H_2O_2 or GGO (2 g/l Glucose in RPMI 1640 and 0.01 μg/ml Glucose oxidase) with or without 1 nM MPO at 37°C in 5% CO_2 for 20 min. LTB4 levels in culture supernatant was measured by ELISA. Mean ± SEM, n = 4 from 2 separate experiments. 1-way ANOVA.

(I) SOCE in neutrophils from WT and CGD mice was measured by flow cytometry. Mouse bone marrow neutrophils loaded with 3 μM Indo-1 were stimulated with 200 nM thapsigargin or 300 nM Ionomycin or 670 nM Ionomycin and 40.5 nM PMA with indicated extracellular calcium. Quantification of SOCE (area under the curve) is in the right panel. Mean ± SEM from 2 to 3 independent experiments. Student T test.

SUPPLEMENTAL FIGURE 2



Supplemental Figure 2. Increased LTB4 production in immune complex-activated mouse and human neutrophils in the absence of NADPH oxidase activity.

(A) Mouse BM neutrophils (1×10^6/ml) were stimulated for 1 hour with immobilized IC in RPMI1640. n = 15 form more than 3 separate experiments.

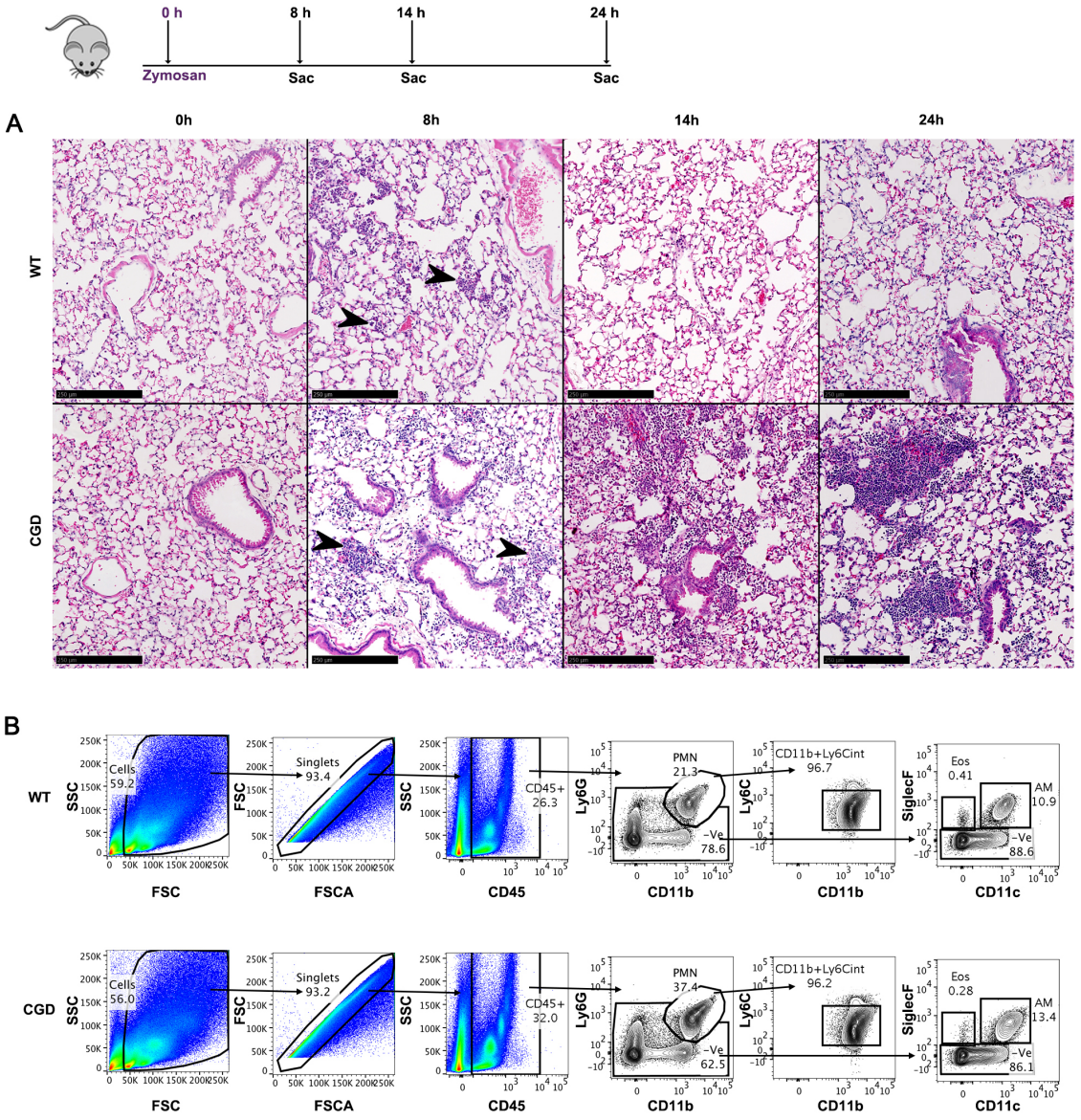
(B) Human neutrophils from peripheral blood ($4x10^6$/ml) were pretreated with 10 μM DPI for 10 minutes and then stimulated for 1 hour with immobilized IC in RPMI1640 in the presence of 10 μM DPI. n = 5 from 5 separate experiments. (A-B) Paired T test (# #, P<0.01, # # #, P<0.001, # # # #, P<0.0001) or Student T test (***, P<0.001).

(C) Mouse BM neutrophils (4×10^6/ml) were pretreated with 200 U/ml SOD and 200 U/ml Catalase for 10 minutes and then stimulated for 1 hour with immobilized IC in RPMI1640 in the presence of 200 U/ml SOD and 200 U/ml Catalase. n = 4 per group from 2 separate experiments. Paired T test.

(D) Mouse BM neutrophils ($1x10^6$/ml) were stimulated for 1 hour or 5 hours with immobilized IC in RPMI1640. n ≥ 4 form at least 2 separate experiments. Paired T test (#, P<0.01, # #, P<0.05, # # #, P<0.001) or Student T test (*, P<0.05).

(E) Mouse BM neutrophils ($4x10^6$/ml) were pretreated 10 μM U75302 for 10 minutes and then stimulated for 1 hour with immobilized IC in RPMI1640 in the presence of 10 μM U75302. Paired T test (****, P<0.0001). (A-E) LTB4 levels in culture supernatant was measured by ELISA. Data are shown as mean ± SEM.

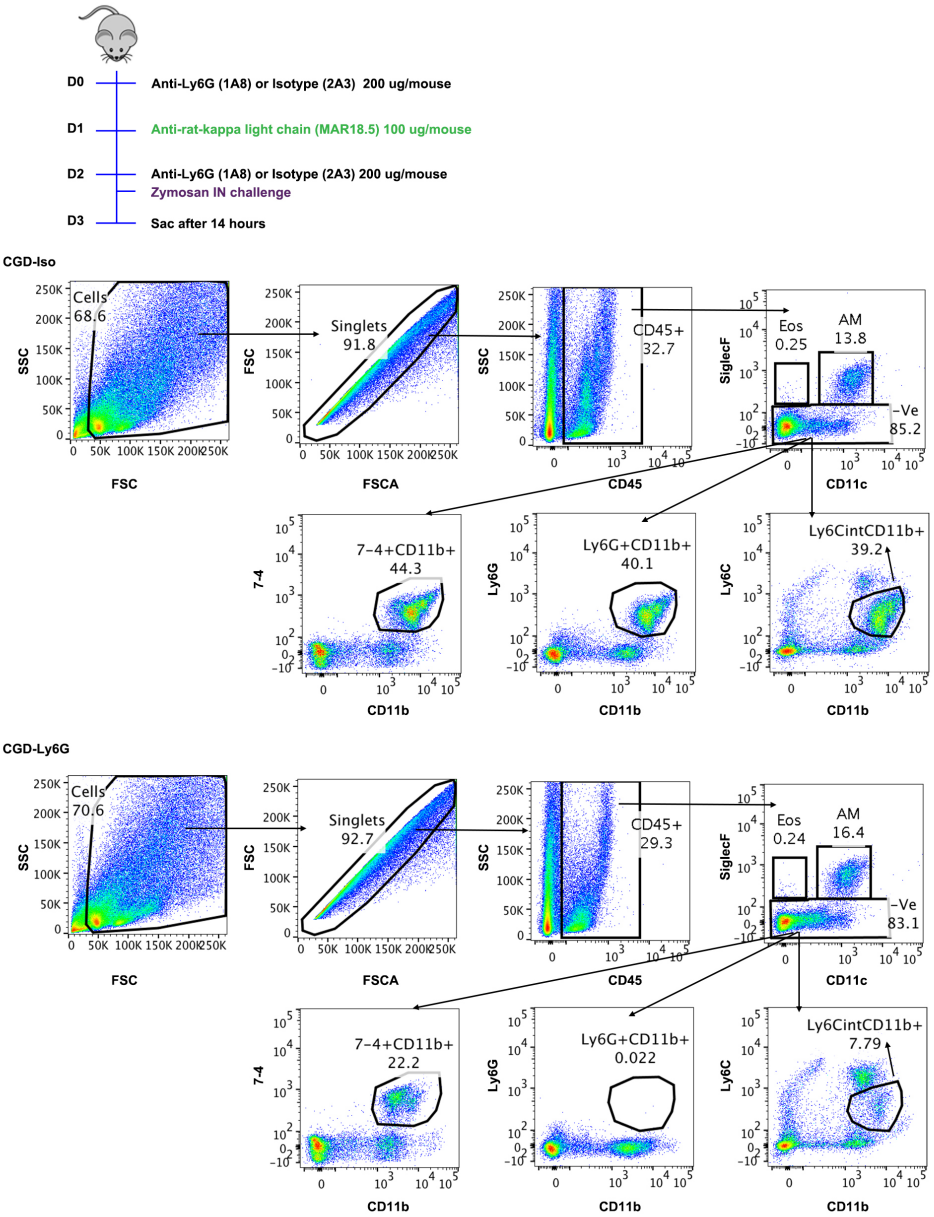
SUPPLEMENTAL FIGURE 3



Supplemental Figure 3. Histology and flow cytometry analysis of lung cell populations in zymosan-induced lung inflammation.

Mice were challenged with 20 µg sterile zymosan intranasally. **(A)** Formalin-fixed and paraffin embedded lung sections were stained with H&E. Bar = 250 µm. **(B)** Representative gating strategy for identifying lung neutrophil by flow cytometry. Lung inferior lobe was collected at 14 hours post challenge and digested. Cell population was determined by flow cytometry. Lung neutrophils were identified as $CD45^{+}Ly6G^{+}CD11b^{+}$, and also confirmed by $CD45^{+}Ly6C^{int}CD11b^{+}$. Eosinophils and alveolar macrophages were identified as $CD45+CD11c^{-}SiglecF^{+}$ and $CD45+CD11c^{+}SiglecF^{+}$ respectively. Similar markers and gating strategy were used for other lung samples of naïve mice and mice with sterile zymosan-induced lung inflammation.

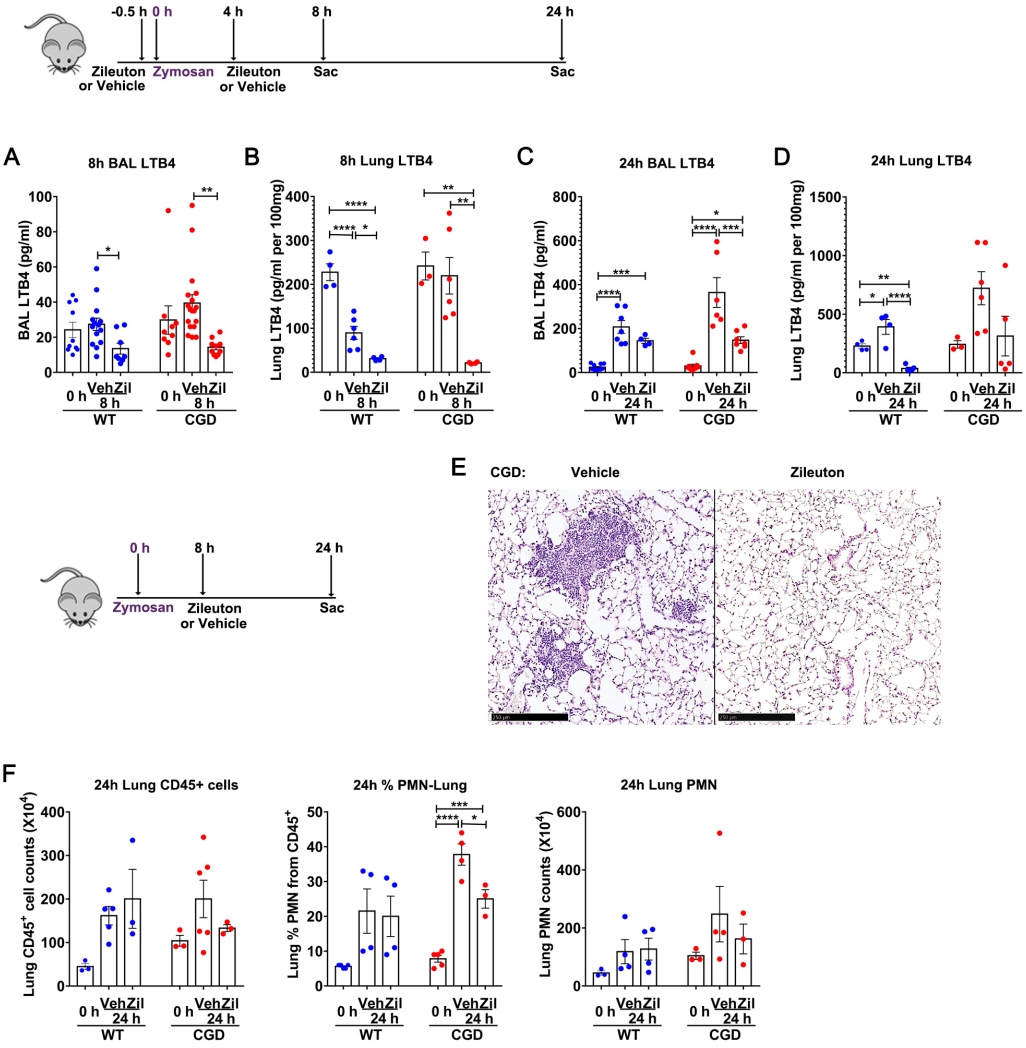
SUPPLEMENTAL FIGURE 4



Supplemental Figure 4. Flow cytometry analysis of cell populations in the lung without or with neutrophil depletion following zymosan challenge.

Representative figure of flow cytometry gating of the lung tissue cells with neutrophil depletion. Mice were sequentially injected IP with anti-Ly6G/Isotype and anti-Rat kappa light chain followed by intranasally instillation of 20 µg sterile zymosan. Lung inferior lobe was collected at 14 hours post challenge and digested. Cell population was determined by flow cytometry. Eosinophil and alveolar macrophage were identified as CD45+CD11c^{-}SiglecF^{+} and CD45+CD11c^{+}SiglecF^{+} respectively. Neutrophil were then gated from SiglecF^{-} population. Lung neutrophil were identified as CD45^{+}Ly6C^{int}CD11b^{+}, which was corresponding to the population of CD45^{+}Ly6G^{+}CD11b^{+} in Isotype treated mice. CD45^{+}7-4^{+}CD11b^{+} was also used to indicate neutrophil and monocyte population.

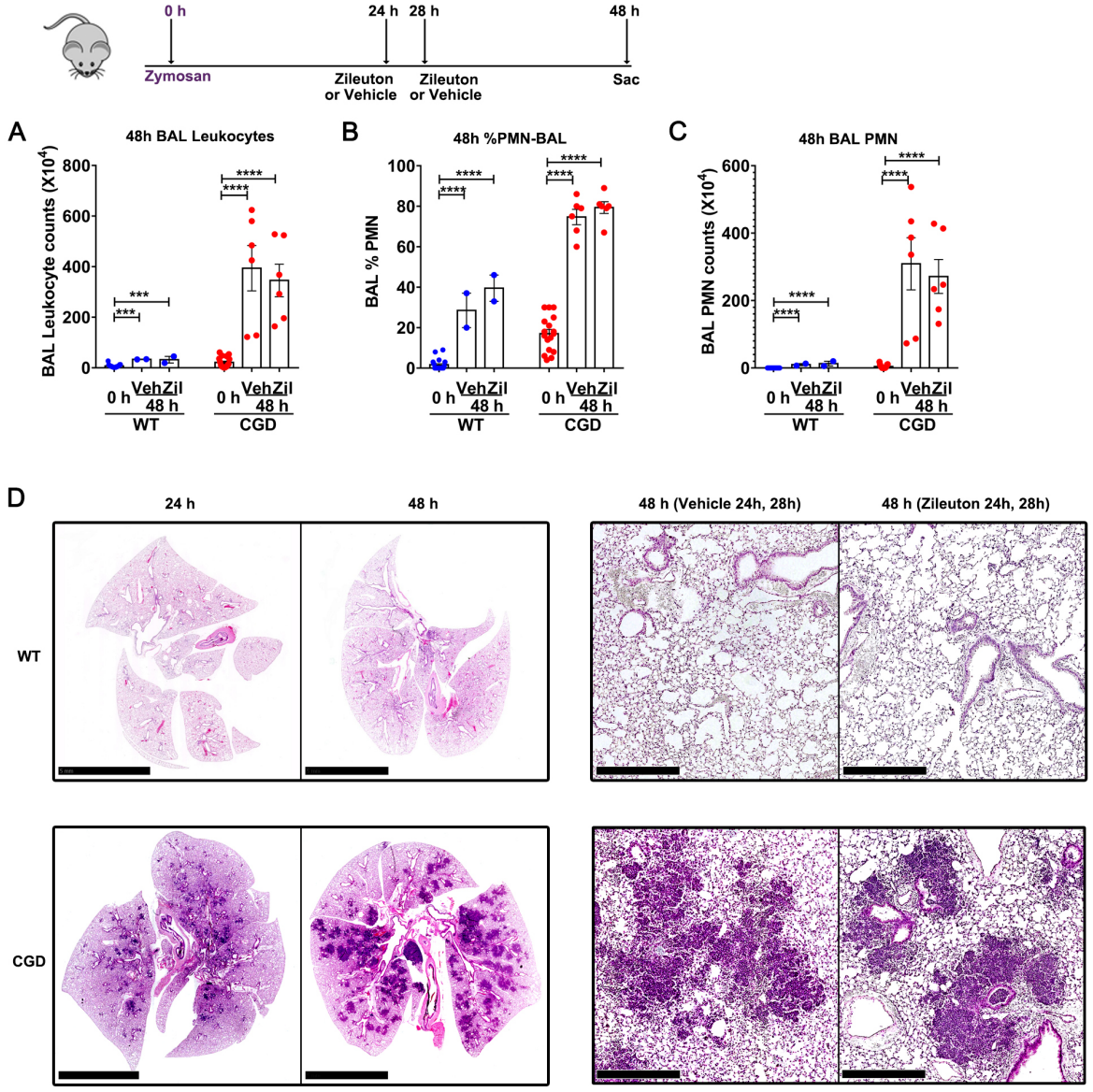
SUPPLEMENTAL FIGURE 5



Supplemental Figure 5. Zileuton reduced LTB4 levels and leukocyte numbers in BAL and lung following zymosan challenge and delayed administration of zileuton also reduced inflammation in CGD.

(A-D) Mice were gavaged with zileuton or vehicle 30 min before and 4 hours after 20 µg sterile zymosan challenge. BAL and Lung tissues were collected 8 hours or 24 hours after challenge. **(A-B)** 8 hours after zymosan challenge: (A) 1st ml BAL fluid supernatant LTB4 levels and (B) whole lung homogenate supernatant LTB4 levels were measured by ELISA. **(C-D)** 24 hours after zymosan challenge: **(C)** 1st ml BAL fluid supernatant LTB4 levels and **(D)** whole lung homogenate supernatant LTB4 levels were measured by ELISA. (E-F) Mice were gavaged with zileuton or vehicle 8 hours after intranasal 20 µg sterile zymosan. **(E)** Representative figures of lung section of CGD mouse stained with H&E. Bar = 250 µm. **(F)** Lung cell counts from right lung inferior lobe. % of $CD45^+$ cells were determined by flow cytometry. Lung $CD45^+$ cell counts were calculated by flow cytometry results. % of PMN ($CD45^+Ly6G^+CD11b^+$) was determined by flow cytometry. Lung PMN counts were calculated by flow cytometry results. n ≥ 3 per group from at least 2 separate experiments. Data are shown as mean ± SEM. 1-way ANOVA (*, P<0.05, **, P<0.01, ***, P<0.001, ****, P<0.0001).

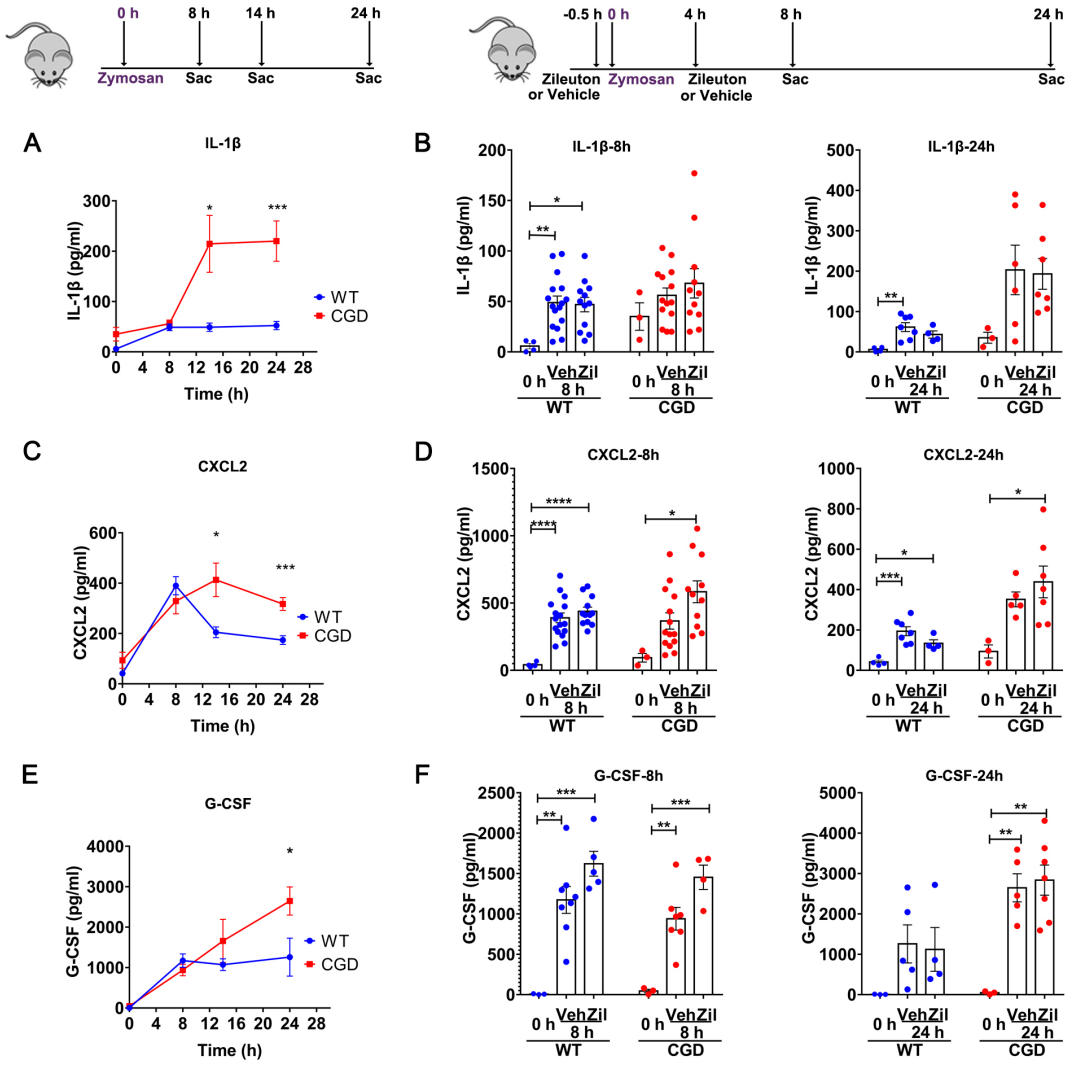
SUPPLEMENTAL FIGURE 6



Supplemental Figure 6. Administration of zileuton after 24 hours following zymosan challenge did not reduce CGD lung inflammation at 48 hours.

Mice were gavaged with zileuton or vehicle 24 hours and 28 hours following intranasal challenge with 20 μg sterile zymosan. BAL and Lung tissues were collected 48 hours after challenge. **(A)** Total leukocytes counts from 3 ml BAL fluid. **(B)** % of PMN were determined by cytospin with Wright-Giemsa stain. **(C)** BAL PMN counts was calculated by cytospin results. **(D)** Representative figures of lung section stained with H&E. Left Panels, Bar = 5 mm; Right Panels, Bar = 500 μm. n =2 in WT 48 hours group from 1 experiment. n ≥ 4 in other groups from at least 2 separate experiments. Data are shown as mean ± SEM. 1-way ANOVA (*, P<0.05, **, P<0.01, ***, P<0.001, ****, P<0.0001).

SUPPLEMENTAL FIGURE 7



Supplemental Figure 7. BAL levels of IL-1β, CXCL2 and G-CSF following zymosan challenge of WT and CGD mice, which were not reduced by zileuton.

Mice were intranasally challenged with 20 μg sterile zymosan. BAL was collected at different time points after zymosan challenge. **(A)** IL-1β level, **(C)** CXCL2, and **(E)** G-CSF level in 1st ml BAL of naive mice or zymosan challenged mice were determined by ELISA. **(B, D, F)** Zileuton or vehicle was given 30 min before and 4 hours post the infection **(B)** IL-1β level, **(D)** CXCL2, and **(F)** G-CSF level in 1st ml BAL of zileuton or vehicle administrated and zymosan challenged mice were determined by ELISA. Mean ± SEM, n ≥ 3 in each group from at least 2 separate experiments. (A, C, E) Students' T test (*, P<0.05, ***, P<0.001). (B, D, F) 1-way ANOVA (*, P<0.05, **, P<0.01, ***, P<0.001, ****, P<0.0001).