Supplemental Information

RIPK3 Activates MLKL-driven Necroptosis and NLRP3 Inflammasome in Macrophages to Protect Against *Streptococcus Pneumoniae*

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Supplemental Figure E1: (A) Densitometric analysis of RIPK3, p-MLKL, and MLKL protein expression of western blots (Figure 1C). (B) Ripk3 protein expression was assessed in lung tissue from wild-type (WT) and *Mlkl*$^{-/-}$ was collected at 72 hours post infection with S. pneumoniae (ATCC 6303). **P<0.01 and ****P<0.0001. Data are representative of 3 separate experiments, with N=10 per experiment. Data are presented as mean ± SEM.

Supplemental Figure E2: Wild-type, Ripk3-/-, and Mlkl-/- mice were instilled with PBS or infected with D39 (1x107 CFU). (A-D) At 72 hours post infection and (A) relative FITC fluorescence in plasma, (B) protein concentration in BAL, (C) wet to dry lung ratio, (D) CFU, and (E) survival were assessed. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001. Data are representative of 3 separate experiments, with N=10 per experiment. Data are presented as mean ± SEM.

Supplemental Figure E3: (A-C) Cell death was measured using the Real Time Glo Viability Assay. Changes in wild-type and *Ripk3*$^{-/-}$ macrophage cell death, as reflected by decreasing luminescence, in response to (A) ATCC 6303 and (B) D39 *S. pneumoniae* strains were quantified. (C) Macrophages were pre-treated with BAPTA AM (10μM, 20 minutes) prior to infection with ATCC 6303. Luminescence was assessed at 4 hours post infection. (D-E) Cell membrane integrity was assessed in wild-type and *Ripk3*$^{-/-}$ macrophages loaded with Calcein Red-Orange

AM (1μM, 1 hour) at select time points post (D) ATCC 6303 or (E) D39 infection (MOI=10). *$P<0.05$, ***$P<0.001$, and ****$P<0.0001$. Data are representative of 3 separate experiments, with N=3-5 per experiment. Data are presented as mean ± SEM.

Supplemental Figure E4: Increased proinflammatory cytokine production in *Mlkl*$^{-/-}$ lung during *S. pneumoniae* infection. At indicated times post-infection, lung tissues were harvested from WT or *Mlkl*$^{-/-}$ mice. Supernatants of lung tissue homogenates were collected by centrifuging. Cytokine levels were detected in the supernatants by ELISA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by one-way or two-way ANOVA test. Data are presented as mean ± SEM.

Supplemental Figure E5: Densitometric analysis of western blots included in Figure 7 was assessed using Image Lite Studio Software. (A) Reflective of calculations pertaining to blots associated with Figure 7B. (B) Reflective of calculations pertaining to blots associated with Figure 7D. Similar expression patterns were observed in ASC blots and are not included. (C) Reflective of calculations pertaining to blots associated with Figure 7E. (D) Reflective of calculations pertaining to blots associated with Figure 7F. (E) Reflective of calculations pertaining to blots associated with Figure 7G. *$P<0.05$, **$P<0.01$, ***$P<0.001$, and ****$P<0.0001$. Data are representative of 3 separate experiments, with N=3-10 per experiment. Data are presented as mean ± SEM.

Graphical abstract: Mechanistic interactions between RIPK3, mPTP, and NLRP3 activation in the macrophage in response to *Streptococcus pneumoniae*.

Supplemental Methods:

Mice: *Ripk3*$^{-/-}$ mice originated from Genentech (San Francisco, CA) and were backcrossed with C57Bl/6 mice. *Mlkl*$^{-/-}$ mice, were orignally obtained from Jiahuai Han (State Key Laboratory of Cellular Stress Biology and School of Life Sciences, Xiamen University, Xiamen, China).

Plasma processing: Plasma samples were collected after centrifugation of whole blood at 400*g* for 10 min at room temperature (RT) without brake. Immediately after centrifugation, the undiluted serum was then transferred to 15-ml polypropylene conical tubes, and aliquoted and stored at −80 °C for subsequent analysis.

Subcellular protein enrichment: The Mitochondria/Cytosol Fractionation Kit (BioVision #K256-100) was used to generate a highly enriched mitochondrial and cytosolic fractions. Plasma membrane enrichment was performed using the Plasma Membrane Protein Extraction kit (Abcam #ab65400).

***Streptococcus pneumonaie* strains:** Serotype 3 strain (ATCC 6303) was obtained from American Type Culture Collection. D39 strain was generously provided by Dr. Moon H. Nahm (University of Alabama). For instillation, either the ATCC 6303 or D39 strain of S. pneumoniae was cultured in Todd Hewitt Broth containing 2% yeast extract for 4 hours. Cultures were pelleted at 15,000 x g for 1 minutes and diluted for instillation with sterile PBS. All instillation titers were confirmed by plaque assay.

Membrane resealing assay: Prior to infection, macrophages were loaded with 1μM of Calcein Red-Orange AM (ThermoFisher Scientific #C34851) for 1 hour at room temperature. Macrophages were subsequently infected with either *S. pneumoniae* strain 6303 or D39 for the indicated times. Changes in fluorescence within the first 4 hours were quantified, with increased fluorescence intensity indicating cell membrane disruption and dye loss from the cell.

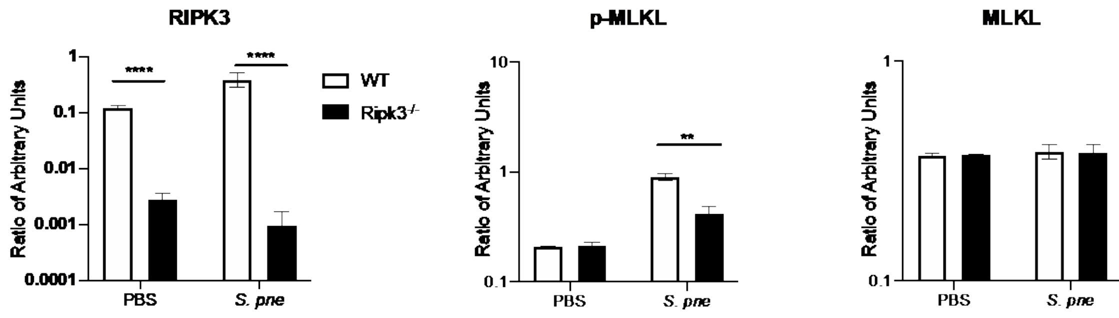
Measurements of lung injury: Bronchoalveolar lavage (BAL): Briefly, 0.8-ml of PBS was slowly injected and aspirated 4 times prior to saving the recovered lavage fluid on ice. Lavage was clarified at 7000 rpm for 10 minutes at 4°C. Protein levels were quantified by Bradford Assay (BioRad Laboratories #500-0201). Lung tissue collection: At select time points of infection lung tissue was collected from control and influenza infected young and aged adult mice. Tissue was snap frozen or placed into Allprotect (Qiagen) for future analysis. FITC-Dextran Lung Permeability Assay: Young and aged adult mice were intranasally instilled with 50-μL of FITC-Dextran (3mg/kg). After 1 hour, blood was collected from euthanized mice, and plasma was isolated after centrifugation (7000 rpm, 10 minutes). Fluorescence was assessed (excitation 485, emission 528). Lung Wet to Dry Ratio: Lung tissue was collected from control and influenza infected young and aged adult mice. Lung tissue weight was assessed at harvest (wet weight) and after being placed in a 60°C drying over for 48 hours (dry weight). Colony Forming Units: Serial dilutions of processed BAL or whole lung homogenates were plated on 10% sheep blood agar plates. Colony forming units were calculated based on colonies present on each plate per dilution. Histology: Tissue samples were processed, and H&E stained by the Translational Research Program at WCM Pathology and Laboratory of Medicine. Images were scanned using the EVOS FL Auto Imaging System (ThermoFisher Scientific). For all animal experiments, we used 10 mice per group and experiments were repeated at least three times.

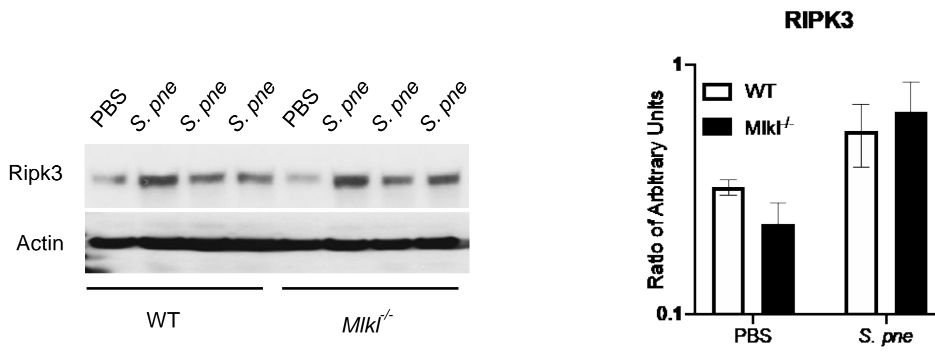
Western blot analysis: Equal amounts of protein (30–50 μg/lane) were loaded onto a 4–12% Bis-Tris Bolt gel (ThermoScientific) and run at 200V for 30 minutes. For western blot analyses, antibodies against β-actin (#8457), RIPK3 (#15828), MLKL (#37705), p-MLKL (Ser345, #37333), p-IP3R (Ser1756, #8548), PERK (#3192), GRP78 (#3177), CHOP (#2895), RIPK1 (#3493), MCU (#14997), UCP-2 (#89326), MICU1 (#12524), VDAC (#4661), NLRP3 (#15101), ASC (#13833),

IκBα (#4814), p-IκBα (Ser32/36, #9246), Erk1/2 (#4695), p-Erk1/2 (Thr202/Tyr204, #4377), Akt (#4685), p-Akt (Ser473, #4060) were from Cell Signaling Technology. Antibodies to Calreticulin (ab22683), Tomm-20 (ab186735), and Anti-Sodium Potassium ATPase (ab58475) were purchased from Abcam. Antibody to IL-1β (AF-401-NA) was from R&D Systems. Densitometry was quantified using Image Studio Lite (version 5.0) software (LI-COR Biosciences) and averaged values of 3-5 gels were assessed.

Statistical Methods: The D'Agostino-Pearson normality test was used to examine Gaussian distribution and to compute a single P value from the sum of the discrepancies in our samples. Statistical analysis was assessed using the Mann-Whitney test for non-parametrically distributed and the Student's t-test for normally distributed populations. Statistical significance was considered by a *$P<0.05$, **$P<0.01$, ***$P<0.001$, and ****$P<0.0001$.

Supplemental Figure E1





Supplemental Figure E2

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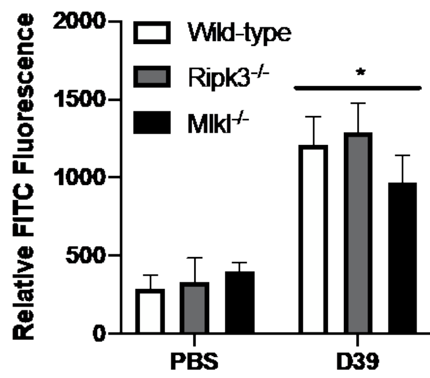

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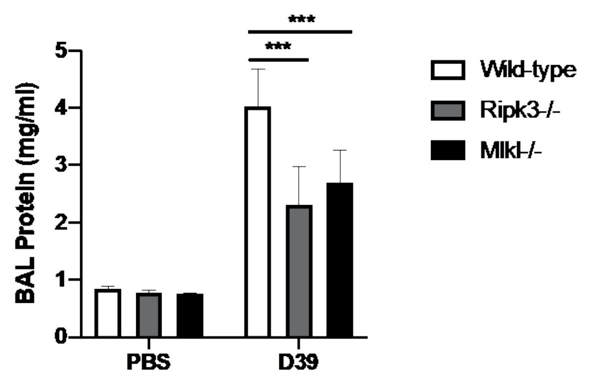

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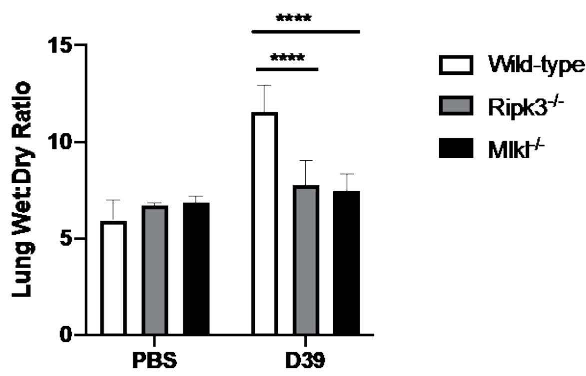

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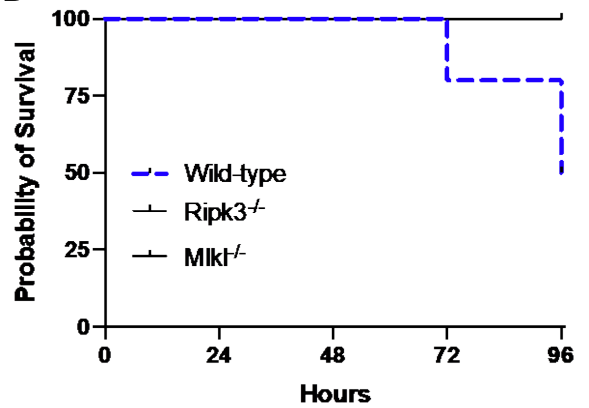

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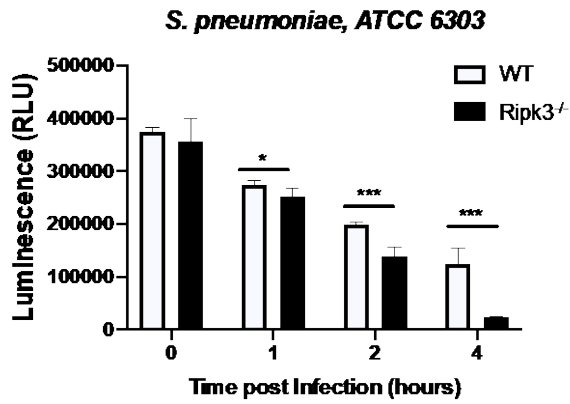

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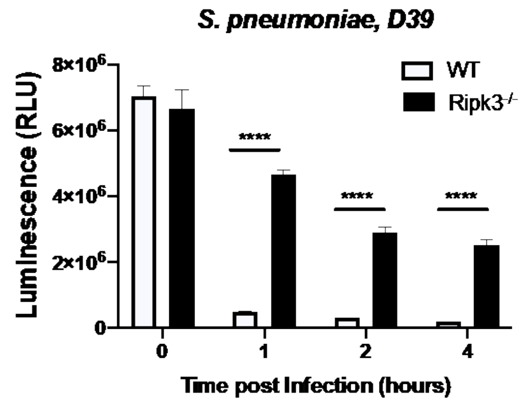

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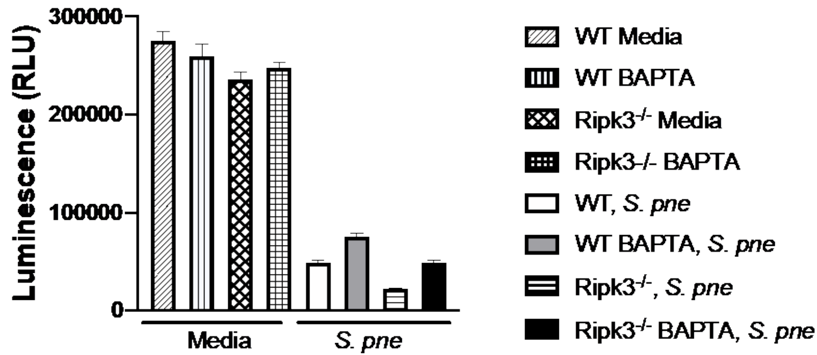

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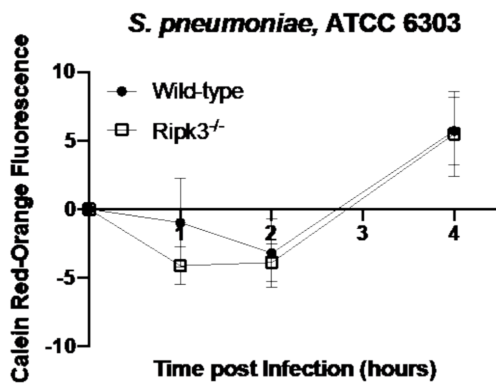

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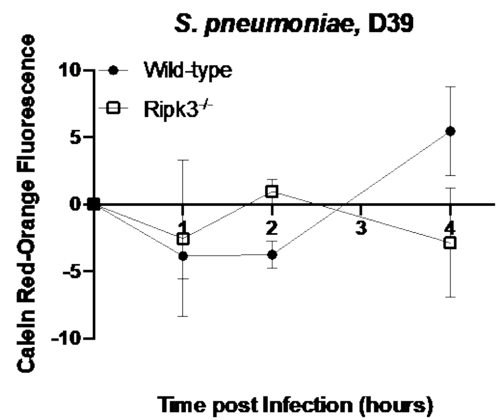
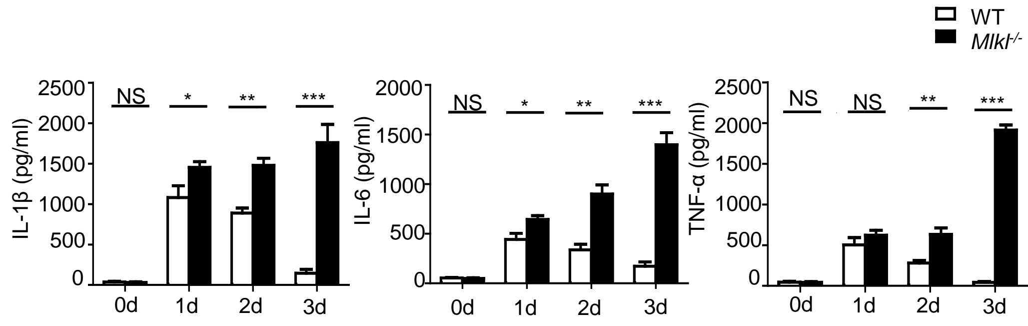
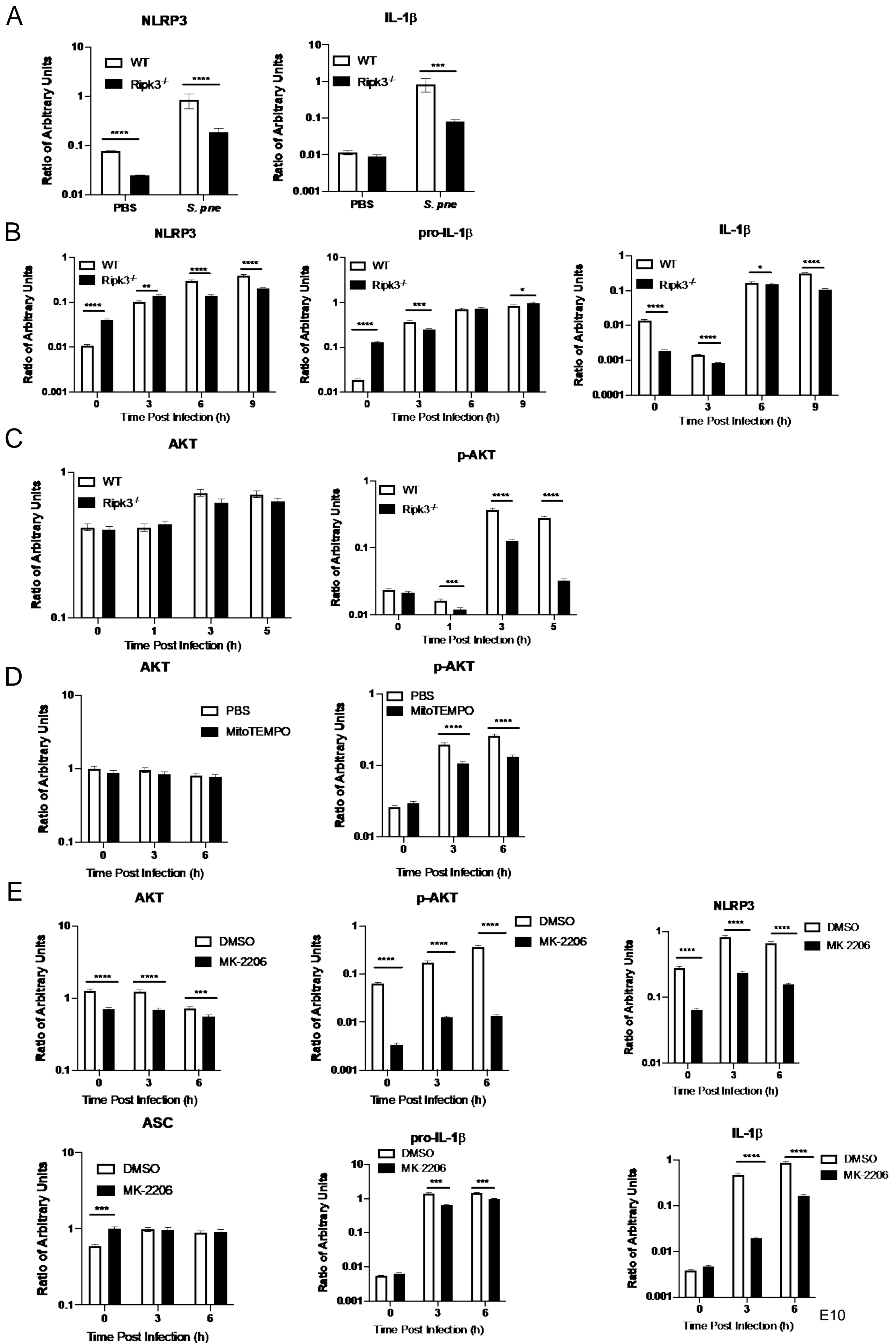
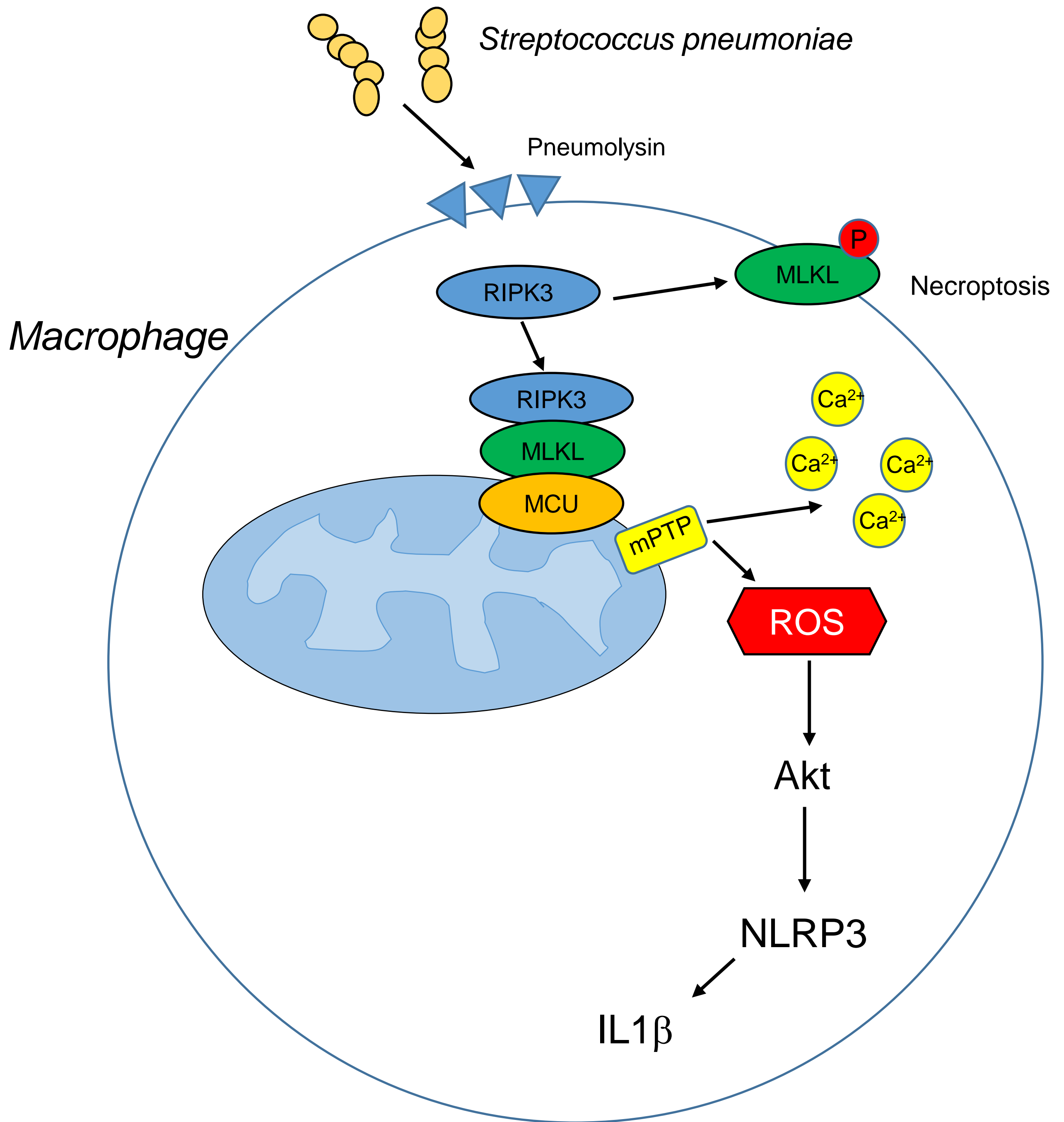


Supplemental Figure E4



Supplemental Figure E5




Streptococcus pneumoniae
Pneumolysin
Macrophage
RIPK3
MLKL
P
Necroptosis
RIPK3
MLKL
MCU
mPTP
Ca2+
Ca2+
Ca2+
Ca2+
ROS
Akt
NLRP3
IL1β