SUPPLEMENTAL INFORMATION

EXPERIMENTAL PROCEDURES

Reagents

Ultra-pure LPS, *E.coli* 0111:B4 was obtained from InvivoGen (CA, USA). Alexa594 conjugated LPS (LPS-Alexa594) from Invitrogen (Oregon, USA), IL-1 receptor antagonist (IL-1RA) (Kineret-Anakinra, Amgen, CA, USA), recombinant IL-1α (rIL-1α) was purchased from eBioscience, CA, USA.

Alveolar macrophage Isolation

BAL cells were obtained by bronchoalveolar lavage. Briefly, BAL cells were collected from individual mice by inserting a cannula into the trachea, and three 0.5 ml washes of PBS were infused in and out of the airways. The cells were centrifuged, and re-suspended in complete RPMI (10% FBS). 1 h later medium was removed and cultured with new complete RPMI for overnight.

Myeloperoxidase assay (MPO)

Briefly, lungs were homogenized in 1 ml of 50 mM potassium phosphate, pH 6.0, with 5% hexadecyltrimethyl ammonium bromide (HTAB) and 5mM EDTA, and then centrifuged at 12,000g for 10 minutes. The supernatant was diluted 1/50 HTAB buffer, mixed with 0.4 g/L tetramethylbenzidine (TMB) substrate solution (eBioscience) and measured by spectrophotometry at 450nm (SpectraMax).

Detection of cytokines and chemokines

The cytokine concentrations in the BALF, lung homogenates or culture supernatant were measured using ELISA kits for mouse IL-1β, IL-1α, and TNF-α ELISA kit (eBioscience). KC, MIP-2 and RANTES in BALF and lung homogenate were detected by ELISA kit (R&D systems, MN, USA). The assays were performed as described in manufacturers' protocols.

Flow Cytometry

BAL cells were collected from individual mice and probed with anti-F4/80 (clone BM8, eBioscience), annexin V and 7AAD (eBioscience) and the percentage of annexin V and 7AAD double-positive cells were determined for necrotic cells. For neutrophil staining, anti-Ly6G-FITC and CD11b-PerCp-Cy5.5 (eBioscience) was used. For lung single cell suspensions, the lungs were enzymatically digested with Liberase (0.2 mg/ml; Roche Diagnostics, IN, USA) and DNase I (0.1 mg/ml; Roche) in 2 ml of Hanks' balanced salt solution at 37°C for 30 min and filtering through a 70 mm cell strainer (BD Biosciences, CA, USA). Cells were stained with the following antibodies: CD16/32 (eBioscience), CD45.2-PECy5, CD31-APC, VE-Cadherin-PE, ICAM1-PE, and CD62E-PE (BioLegend, CA, USA). CD31 single positive cells were gated and VE-cadherin was analyzed by using a Cyan flow cytometer and analyzed by Summit V4.3 software package (both from BD Biosciences).

Vascular Permeability

Lung permeability was determined by assessing tissue accrual of Evans blue, as previously described (Green et al., 1988). Briefly, animals were administered 25 mg/kg Evans blue (Sigma) by tail vein (i.v.) injection 2 h before lung harvest. Lungs were perfused with 5ml PBS, and homogenized in 1ml PBS then washed twice. Evans blue was extracted by 1ml of Formamide at 60 °C for 18 h. The supernatant was separated by centrifugation at 5000 x g for 30 min. Evans blue concentration was quantitated by a dual wavelength spectrophotometric method at absorbencies of 620 nm and 740 nm, as determined by the following formula: E620 (Evans blue) = E620 - (1.426 X E740 + 0.030).

Western blotting

BAL fluid was pooled within experimental groups of mice and concentrated using by ultra-filter 10 kDa (Millipore, MA, USA) and whole cell lysate and supernatant from BMDMs stimulated with LPS and ATP. The samples were then subjected to SDS-PAGE and immunoblotting was performed with anti-IL-1α antibody (ALF-161, Santa Cruz Biotechnologies, CA, USA), followed by HRP-conjugated, anti-hamster secondary Ab. Detection was conducted using West Dura substrate (Thermo Scientific, MA, USA).

Statistics

Data are reported as mean values ± SEM. Statistical significance was evaluated by Student's *t* test (two-tailed). In experiment where data was not normally distributed, the Mann-Whitney test was performed. For multiple comparison test, statistical significance

was evaluated by one or two-way ANOVA with Tukey's post-hoc test where appropriate. A p value of less than 0.05 was required to reject the null hypothesis

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Supplemental Figures

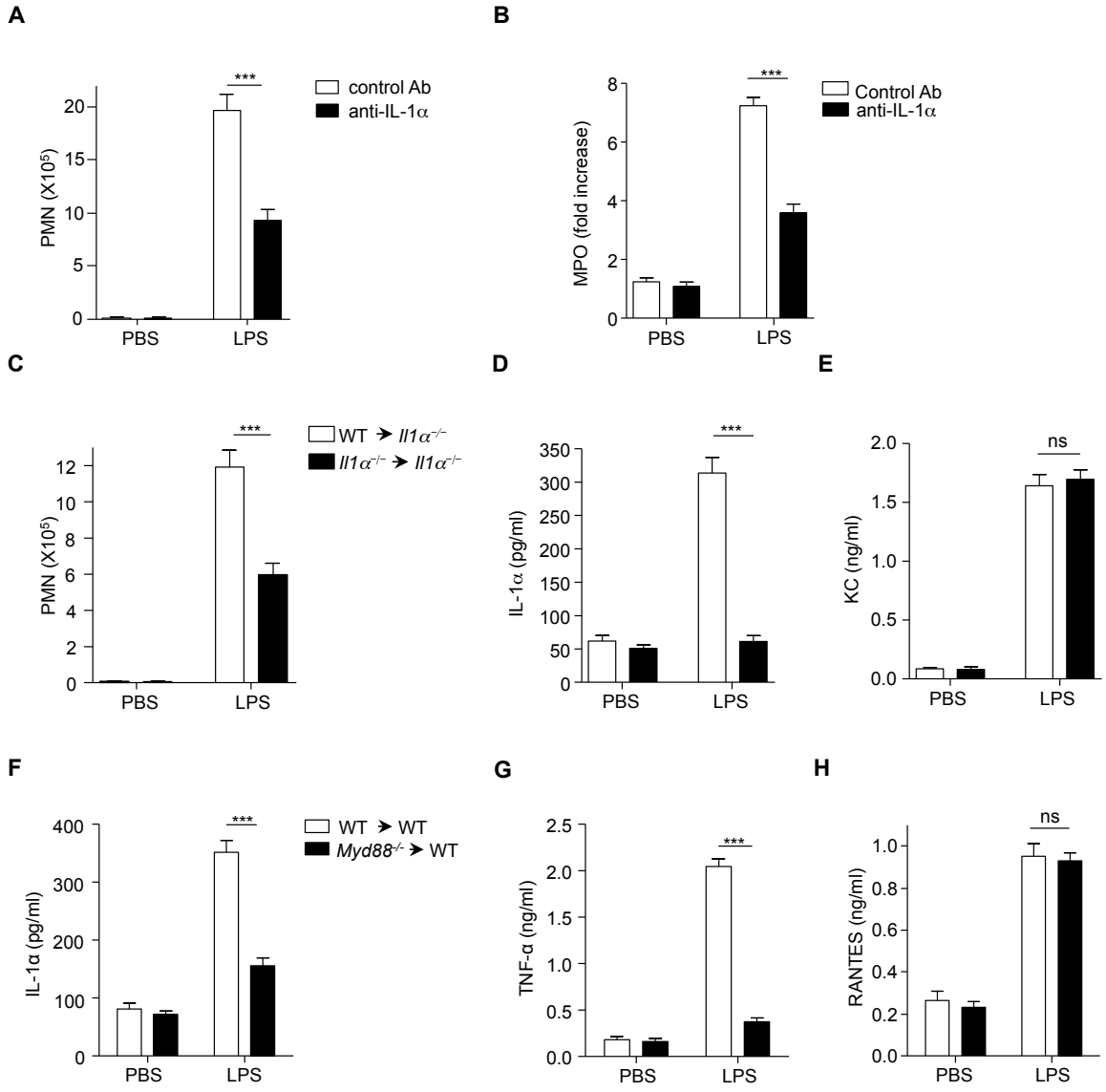

Figure S1. Related to Figure 2. IL-1a Is Crucial During LPS-Induced Acute Lung Injury

(A and B) WT mice were (i.t.) administered control or IL-1α neutralization antibody with PBS or LPS 6 h. (A) PMN numbers were counted in the BALF and (B) MPO activity was measured in the lung homogenate. (C-E) *Il1α*$^{-/-}$ mice received bone marrow cells from WT or *Il1α*$^{-/-}$ mice, and 12 weeks later mice were challenged (i.t.) with PBS or LPS 6 h. (C) PMN numbers were counted in the BALF, and (D) IL-1α and (E) KC concentrations in the BALF were determined by ELISA. (F-H) WT mice received bone marrow cells from WT or *Myd88*$^{-/-}$ mice, and 12 weeks later mice were challenged (i.t.) with PBS or LPS 6 h. (F) IL-1α, (G) TNF-α, and (H) RANTES concentrations in the BALF were determined by ELISA. Each group n=5. Results shown are representative of two experiments, mean ± SD. ***p < 0.001.

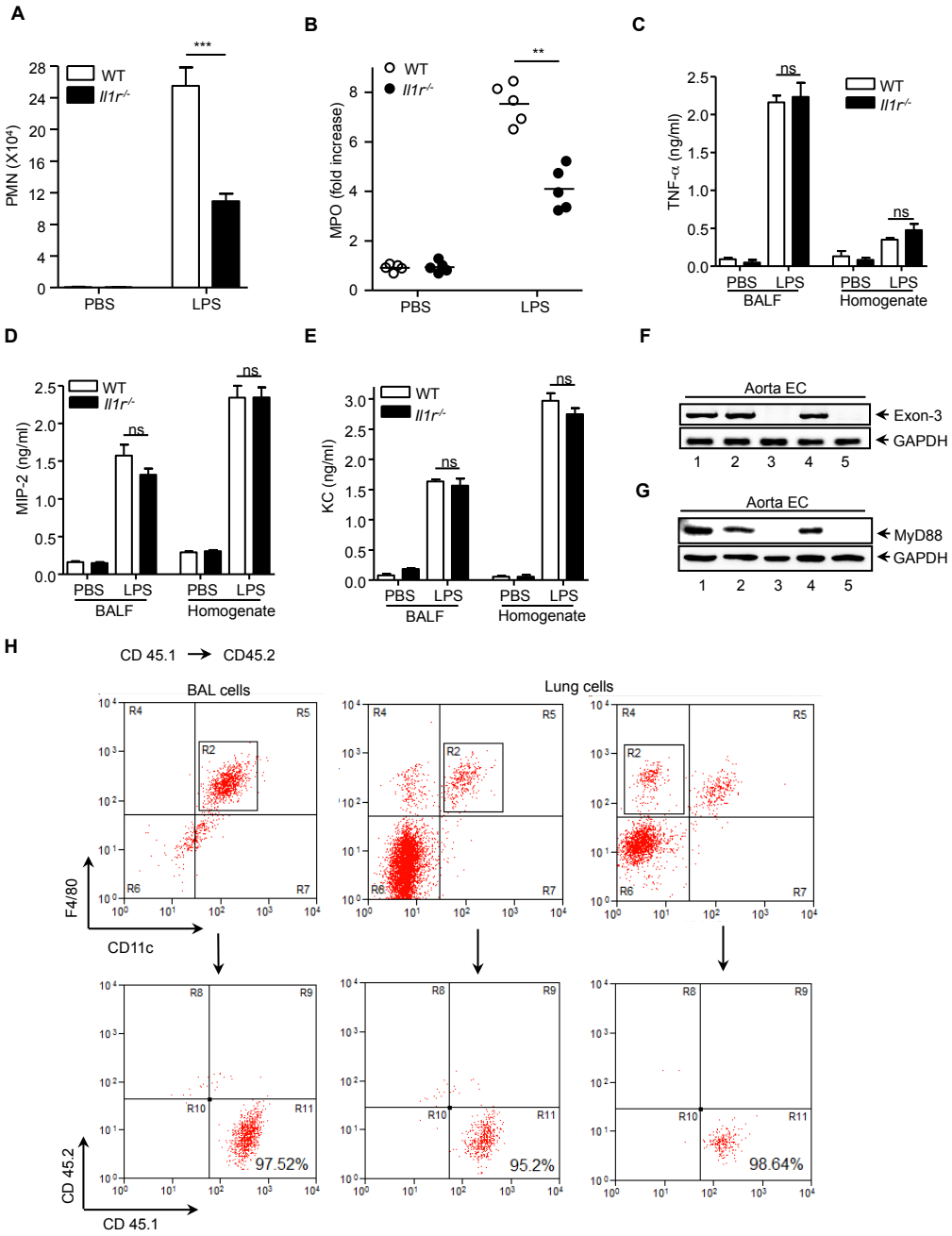

Figure S2. Related to Figure 3. IL-1R-MyD88 Is Important for Neutrophil Recruitment

(A-E) WT and *Il1r*$^{-/-}$ mice were challenged (i.t.) with PBS or LPS 6 h later. (A) PMN numbers were counted in the BALF and (B) MPO activity was measured in the lung homogenate. (C) TNF-α, (D) MIP-2, and (E) KC concentrations in BALF and lung homogenates were determined by using ELISA. (F) MyD88 exon-3 deletion and (G) MyD88 protein expression in aorta EC was measured by PCR and WB respectively. WT (lane 1), *Tie2*-Cre (lane 2), *Myd88*$^{-/-}$ (lane 3), *Myd88*$^{fl/fl}$ (lane 4) and EC$^{MyD88-/-}$ (lane 5). GAPDH was used as loading control. (H) C57BL/6 (CD45.2+) mice received bone marrow from Ly5.1 (CD45.1+) mice and 12 weeks later, BAL cells and lung single cell suspension was performed. F4/80 and CD11c double positive or F4/80 single positive cells were gated and bone marrow cells reconstitution was measured by flow cytometry. Each group n=5. Results shown are representative of two experiments, as mean ± SD. **p < 0.01, ***p < 0.001.

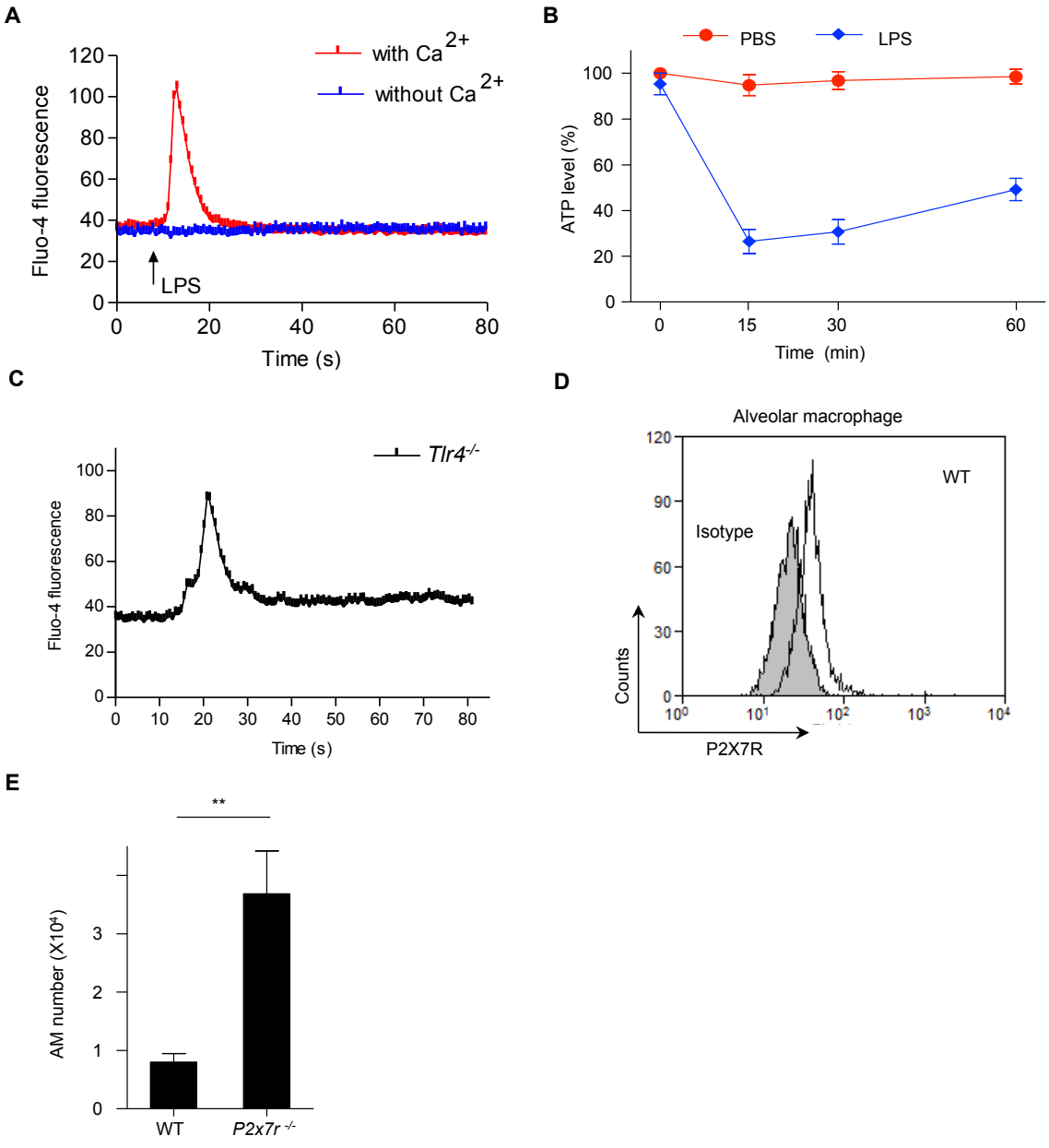

Figure S3. Related to Figure 4. LPS Mediates Calcium Influx in Alveolar Macrophages Expressing the P2X7 Receptor

(A) AM from WT mice were loaded with 4 mM of Fluo-4 for 30 min at room temperature and calcium mobility after LPS was measured by confocal microscopy. (B) WT mice were (i.t.) challenged with LPS at time 0 and ATP amounts were determined in AM by ATPlite detection assay. (C) AM from *Tlr4*$^{-/-}$ mice were loaded with 4 mM of Fluo-4 for 30 min and calcium mobility after LPS was measured by confocal microscopy. (D) BAL cells were isolated from WT mice (n=5). F4/80 and CD11c double positive cells (alveolar macrophage) were gated and P2X7R expression was measured by flow cytometry. (E) WT or *P2x7r*$^{-/-}$ mice were (i.t.) challenged with PBS or LPS 4 h and AM numbers counted in the BALF.

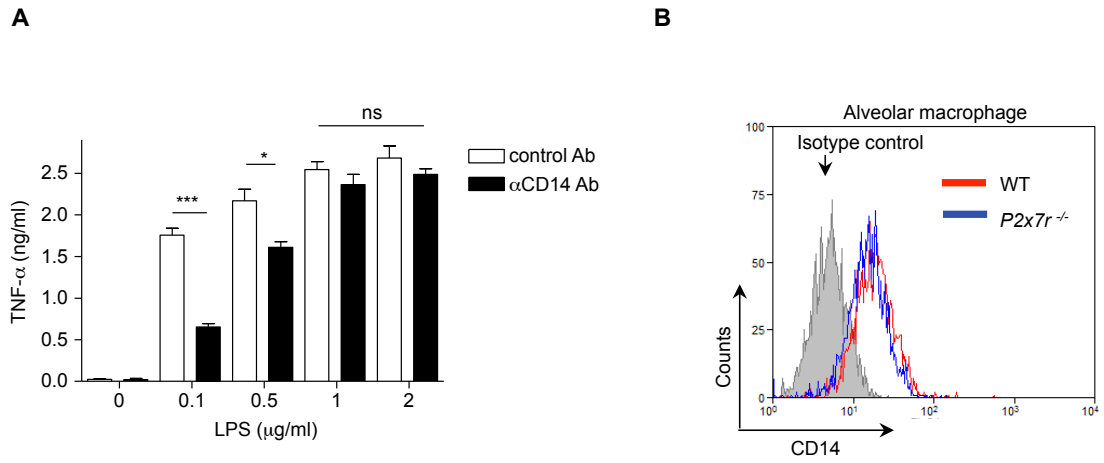

Figure S4. Related to Figure 5. CD14 levels Are Similar on AM in WT and *P2x7r* $^{-/-}$ Mice
(A) AM were isolated from WT mice and $3x10^4$ cells were plated in 96 wells. AM were pretreated with control or CD14 neutralization antibody (Ab) for 1 h, and stimulated with LPS at the indicated doses. TNF-α levels in supernatant were measured by ELISA after 4 h LPS stimulation. (B) BAL cells were isolated from WT or *P2x7r* $^{-/-}$ mice. F4/80 and CD11c double positive cells were gated and CD14 expression was measured by flow cytometry. Results shown are representative of two experiments, as mean ± SD. *p < 0.1, ***p < 0.001.

Supplemental Movie Legends

Movie S1. Related to Figure 5. P2X7R is not sufficient for Ca^{2+} Mobilization Induced by LPS

P2X7R were loaded with Fluo-4 (4 μM) for 30 min and calcium mobilization was measured by confocal microscopy upon LPS treatment.

Movie S2. Related to Figure 5. CD14 is not sufficient for Ca^{2+} Mobilization Induced by LPS

CD14 stably expressing HEK293 cells were loaded with Fluo-4 (4 μM) for 30 min and calcium mobilization was measured by confocal microscopy upon LPS treatment.

Movie S3. Related to Figure 5. CD14 is required for P2X7R Dependent Ca^{2+} Mobilization Induced by LPS

P2X7R and CD14 stably expressing HEK293 cells were loaded with Fluo-4 (4 μM) for 30 min and calcium mobilization was measured by confocal microscopy upon LPS treatment.

Movie S4. Related to Figure 5. ATP induces P2X7R Dependent Ca^{2+} Mobilization (positive control).

P2X7R and CD14 stably coexpressing HEK293 cells were loaded with Fluo-4 (4 μM) for 30 min and calcium mobilization was measured by confocal microscopy upon ATP treatment.