Supplement Material

Materials and Methods

Materials:

Lipopolysaccharides (LPS) from Escherichia coli 0111:B4, C5a and IL-8 were from Sigma; PAR-1 peptide Ac-TFLLRNPNDK-NH2 was from Biosource; S3A mutant of cofilin [1] was a gift from Dr. Bernard; ketamine, xilazine, acepromazine were from Abbott Laboratories; anti-RhoA, anti-ZO-1, and anti-VE-cadherin antibodies were from Abcam; anti-Cdc42, anti-Rac1 antibodies were from BD Transduction Laboratories; anti-LIMK1 and anti-phospho-LIMK1/LIMK2 antibodies were from Cell Signaling Technology; anti-mouse Alexa Fluor 594 was from Molecular Probes.

siRNA against human LIMK1 was described previously [2, 3]. Double-stranded siRNA targeted against human *LIMK1*: CCU GGA GGG AAG AAC GUA UUU, and mismatch siRNA CCU GAA AGA AAA AAC GUA UUU (where 4 nucleotides were mutated G/A) were from Dharmacon (Chicago, IL). The specificity of the *LIMK1* siRNA was verified or the siRNA study was validated by using 1) mismatch controls, where mutation of only several nucleotides completely abolished the silencing effect; and 2) several siRNAs targeted against different regions on *LIMK1* mRNA, which showed similar silencing effects.

Animals

All animal procedures were carried out following the Public Health Service Policy on Humane Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of University of Illinois at Chicago. Mice were kept under standard conditions in the Biological Resources Laboratory facilities of the University of Illinois at

Chicago. The LIMK1 knockout mice generation and characteristics are described elsewhere [4]. *limk1+/-* pairs were used for breeding. The genotype of the mice was identified on DNA extracted from tail biopsy according to the manufacturer's protocol Wizard Genomic DNA purification kit Promega (Madison, WI) by polymerase chain reaction assay. Sex-matched *limk1+/+* littermates were used as controls for *limk1-/-* mice.

Lung preparation

According to an approved protocol of the University of Illinois at Chicago Animal Care Committee, *limk1-/-* mice or their wild-type littermates mice weighing between 25 and 35 grams were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg), xylazine (2.5 mg/kg) and acepromazine (2.5 mg/kg). The detailed protocol is described elsewhere [5]. Briefly, a tracheal cannula was inserted for positive-pressure ventilation and 100U heparin injected into the internal jugular vein for anticoagulation. The diaphragm was exposed through a midline abdominal incision, and excised from the rib cage. A sternotomy was performed and the thoracic cavity was exposed. A polyethylene cannula (PE-90, Becton Dickinsonm Sparks, MD) was inserted in the pulmonary artery through a small incision in the right ventricle. The cannula was secured using a suture around the pulmonary artery and aorta. The left atrium was cut free for drainage of the venous effluent. The lungs were perfused with RPMI 1640 solution at a constant flow rate (2mL/min), temperature (37° C) and pH (7.4), using a peristaltic pump (Gilson Minipuls 3). Positive-pressure ventilation (rate, 120 breaths/min; peak inspiratory pressure, 8-10 cm H_2O; peak end-expiratory pressure, 2 cm H_2O) was maintained by means of a dual mode ventilator (Kent Scientific, Litchfield, CT).

Capillary filtration coefficient determination was performed as described [5]

The lungs and heart were rapidly removed en bloc from the thoracic cavity and suspended from a lever arm connected to a force displacement transducer (FT03, Grass Telefactor, W Warwick, RI). Pulmonary arterial pressure was recorded during the experiment using a Gould pressure transducer (CP122 AC/DC strain gage Amplifier, Gould Instruments, Dayton, OH). Both transducers were connected to amplifiers (Grass Instrument Co, W. Warwick, RI) and the recordings were displayed on a computer monitor with the aid of an analog to digital converter (DAS 1800ST board; Keithly Metabyte, Solon, OH) and software for acquisition of data (Notebook Pro for Windows, Labtech, Andover, MA).

After mounting, the weight was zeroed and the lungs were given a 20-minute equilibration period. The lungs that were not isogravimetric at the end of this period were discarded. The pressure was increased abruptly by 8-10 cm H_2O for 5 minutes every 20 minutes, which gave a characteristic weight recording: an initial fast phase (almost vertical) followed by a slow phase that represented the net fluid extravasation. The venous pressure was assumed to be zero. For every mouse 4 capillary filtration coefficient determinations were made and the average was taken into consideration. At the end of experiment the lungs were cut free of any other tissue and placed in a oven for at least 48 hours until a constant weight was reached, representing the dry weight. Data analysis was carried out off-line, using a LOTUS 1-2-3 macro© developed in this laboratory.

Drug infusion

The infusions were made at a constant rate of 0.2 ml/min using a side-port tube passing through a peristaltic pump and connected to the main line. The flow rate in the main line was decreased to 1.8 ml/min when infusion of the drugs was started. In experiments testing the effect of PAR-1 agonist peptide on the Kfc infusion was begun 5 minutes before the 1st Kfc measurement. In every case the perfusion of drug was stopped when the Kfc measurement was begun. PAR-1 agonist peptide was used at the same concentration (6 μM) in all the experiments.

Tissue preparation for biochemical assays

The blood was removed by a 10-minute perfusion with RPMI 1640 at RT and thereafter lungs were quickly cut free of any other tissue and snap-frozen in liquid nitrogen.

Gravimetric assessment of pulmonary water content

LPS at 23 mg/kg was injected into the peritoneal of conscious mice. Animals were anesthetized with ketamine/xylazine after 12 hours post-LPS and lungs were removed, immediately weighed to measure wet weight. Dry weight was determined after heating the lung at 80 ^{0}C for 36 hours. Pulmonary water content index was calculated by dividing the lung wet weight over the lung dry weight as described previously [6].

Survival studies

Wild type (n=21) and LIMK1 knockout mice (n=21) animals were injected with 23 mg/kg LPS intraperitoneally. Mice were returned to their cages and allowed access to food and water ad libitum. Animals were observed at regular intervals for occurrence of

mortality over the subsequent 7 days post-LPS. Moribund animals (defined as bradycardia to a heart rate less then 40; severe lethargy; and irresponsive to painful stimulations) were sacrificed with a lethal dose of ketamine/xylazine as defined by the University of Illinois at Chicago Animal Care Committee.

Electron microscopy studies

After removal of blood from the vascular space with a 10-minute HBSS perfusion, lungs were fixed in situ (20 min at RT) by injecting through a pulmonary artery cannula a mixture of 3% formaldehyde + 1.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. Excised specimens were further fixed in the same mixture (1h at RT) then post-fixed in 2% OsO_4 in acetate veronal buffer, pH 6.8 (1h on ice), stained in the dark (1h at RT) with Kellenberg uranyl acetate, dehydrated through graded ethanol and then embedded in Epon 812. Tissue blocks were cured (72 h at 90º C), cut on a Leica microtome, and 60 nm sections were counterstained with uranyl acetate and lead citrate and examined and photographed in a JEOL 1220 transmission electron microscope.

Immunocytochemistry

HUVECs grown to confluency on coverslips coated with gelatin were serum-starved for 6 hours before each experiment. Cells were washed with Hank's balanced salt solution (HBSS) and fixed with 3.3% paraformaldehyde for 30 min. Cells were permeabilized for 5 min with 0.1% Triton X-100/ PBS and washed extensively with HBSS. After blocking with 1%BSA/0.2% fish skin gelatin in HBSS for 1 h at room temperature (RT), cells were incubated with primary antibody in blocking solution for 1 h at RT followed by incubation with secondary antibodies. Slides were mounted using ProLong Antifade Kit (Molecular Probes). Microscopy was performed using Zeiss LSM

510 confocal microscope equipped with 63x water-immersion objective with appropriate filter sets.

Transfection and culturing of HUVECs:

Human umbilical vein endothelial cells (HUVECs) were obtained at first passage from Cambrex (Walkersville, MD, culture line CC-2519) and were utilized at passages 6-10. Cells were cultured in EBM-2 medium (Cambrex) supplemented with 10% (v/v) fetal bovine serum (Cellgro) and EGM-2 SingleQuots (Cambrex) and maintained at 37^0C in humidified atmosphere of 5% CO_2-95% air. Transient transfections of HUVECs with cDNAs were performed with SuperFect Transfection reagent (Qiagen) according to the manufacturer's protocol. Transfection of HUVECs with siRNAs was performed either with DharmaFect1 (Dharmacon) (for TER measurements and Western blotting) or using Amaxa Nucleofector (Lonza) (for Western blotting). TER experiments were performed from 12h to 48h after transfection (Figure 2C). Western blotting was performed 48h after transfection (Figure 2B).

Measurements of trans-endothelial electrical resistance

The total electrical resistance was measured dynamically across the HUVECs monolayer using an electrical cell-substrate impedance sensing system (ECIS) (Applied Biophysics, Troy) described previously1 [7]. Briefly, HUVECs were plated in a well containing a small gold electrode and transfected as described above. Culture medium was used as the electrolyte. The small electrode and the larger counter electrode were connected to a phase-sensitive lock-in amplifier. A 1-V, 4,000-Hz AC signal was supplied through a resistance of 1 megohm to approximate a constant current of 1 μA. The voltage change between the small electrode and the larger counter electrode was

continuously monitored, stored, and processed on a computer. The data are presented as the change in the resistive (in-phase) portion of the impedance normalized to its initial value at time zero.

Quantification of PMN infiltration into the lungs

After removal of blood from the vascular space with a 10-minute HBSS perfusion, lungs were fixed in situ (20 min at RT) by injecting through a pulmonary artery cannula 3% formaldehyde solution; and thereafter were kept in 3% formaldehyde solution overnight at 4^0C. Hemotaxilin/eosin and Leder (naphtol AS-D chloroacetate esterase) staining were performed according to the manufacturer's protocol (Sigma-Aldrich). Thereafter, lung sections were analyzed by light microscopy (magnification 40X). Infiltrated neutrophils were counted in a blinded manner to the mice genotype and experimental conditions. Data represent mean count from five different sections (four different fields were analyzed per each section) from each of 3 mice lung preparations per condition.

Chemotactic assay

Bone marrow derived neutrophils were used, chemotaxis was quantified using a Transwell system (polycarbonate filters with pore size 5μm). Cells were placed in the top well in a total volume of 100 μl, and 600 μl of chemoattractant or medium (RPMI 1640 with 1% FBS) was added to the lower chamber. After 40-min incubation at 37^0C, cells in the bottom chamber were collected and the number of migrated PMN was determined.

Superoxide production

Mouse bone marrow neutrophils were resuspended in BSA buffer (0.5% BSA in Hank's balanced salt solution with Ca^{2+} and Mg^{2+}, and 10 mM Hepes) at $5x10^6$ cells/ml. Superoxide anion released from neutrophils were measured as described elsewhere [8]. Briefly, isoluminol was added to the cell suspension to a final concentration of 50 μM and horseradish peroxidase to a final concentration of 40 U/ml. Cells (200 μl/well) were then seeded into 96-well flat bottom culture dish (E&K Scientific, Campbell, CA). Chemiluminescence was measured at least every minute using a Wallac Multilabel Counter plate reader (Perkin Elmer Life Sciences, Boston, MA) starting from 10 min before and continuing to 30 min after ligand stimulation. Unstimulated controls were recorded simultaneously.

Degranulation assay

Mouse bone marrow neutrophils were resuspended in BSA buffer (0.5% BSA in Hank's balanced salt solution with Ca^{2+} and Mg^{2+}, and 10 mM Hepes) at $6.25x10^6$ cells/ml and placed on ice. Cytocholasin B (10 μM, Sigma) was then added, cells were aliquotted ($5x10^5$ cells/tube), then returned to ice for 15 min. After 15 min, cells were transferred to 37°C and incubated for another 15 min. Agonist was prepared at 2x concentration and warmed to 37°C, then added to the cells to a final concentration of 1x. After 10 min, the reaction was terminated by transferring samples to ice. Cells were pelletted at 1500 rpm for 5 min at 4°C, and the supernatants were transferred to new tubes. Cell pellet was resuspended in 4x supernatant volume (640 μl) of 0.1% Triton X-100 in HBSS. Substrate (4-Methylumbelliferyl β-D-glucuronide hydrate, Sigma) was prepared fresh at a concentration of 2.5 mM in 0.1M Sodium acetate (pH 4.0) with 0.1% Triton X-100. Cell supernatant (40 μl) was then combined with 40μl substrate in a black

96 well plate (E and K Scientific), and then incubated for 15 min at 37°C. At the end of the incubation, 170 µl stop solution (50 mM glycine and 5 mM EDTA) was added to terminate the reaction. Fluorescence was measured on the Flex Station (Molecular Devices) with excitation wavelength at 365 nm and emission wavelength at 460 nm.

Actin polymerization

Bone marrow-derived neutrophils were stimulated for indicated amount of time with 20 nM C5a at room temperature and fixed in 3% formaldehyde in HBSS for 30 min. F-actin was stained by addition of 0.2 µM FITC-phalloidin/100µg/ml lysophosphatidilcholine in HBSS for 30 min. Fluorescence intensity was determined by flow cytometry.

Supplement figure legends:

Online Figure I. A, B, C) LPS at 23 mg/kg was given i.p., where indicated. Lungs were extracted 3 h upon LPS treatment. GTP-bound small GTPases pull-down assays were performed using whole lung homogenates from *limk1*$^{+/+}$ and *limk1*$^{-/-}$ mice as described elsewhere [9]. Precipitates and total lysates were analyzed by Western blotting using (A) anti-RhoA, (B) anti-Rac1, (C) anti-Cdc42 antibodies. Relative amount of GTP-bound GTPases over total amount of GTPases is presented on the bar graph (n=5 lung preparations per group).

Online Table I. Hemograms from *limk1*$^{+/+}$ and *limk1*$^{-/-}$ mice before and after LPS treatment. Animals were injected i.p. with LPS (23 mg/kg) and peripheral blood was collected from the retro-orbital sinus 6 h pos-LPS. Cell counts were performed using an automated cell counter with veterinary parameters and reagents. WBC, white blood cells; NE, neutrophils; LY, lymphocytes; MO, monocytes; EO, eosinophils; BA, basophils;

RBC, red blood cells; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelets. (n=3 mice per group).

Online Figure II. A) Superoxide production by PMNs of *limk1*$^{+/+}$ and *limk1*$^{-/-}$ mice. PMNs were stimulated with either fMLP or PMA for 15 minutes. Superoxide production was determined as described in Methods (n=3 per group). B) Degranulation of PMNs isolated from *limk1*$^{+/+}$ and *limk1*$^{-/-}$ mice. PMNs were stimulated with either fMLP or C5a for 30 minutes. Degranulation was determined as described in Methods (n=3 per group).

Online Figure III. Cytokine production in *limk1*$^{+/+}$ and *limk1*$^{-/-}$ mice before and after LPS treatment.

Animals were injected i.p. with LPS (23 mg/kg) and peripheral blood was collected 2 h later. Serum was isolated and cytokine production was measured according to the manufacturer's protocol (BioPlex, BioRad). (n=3 mice per group)

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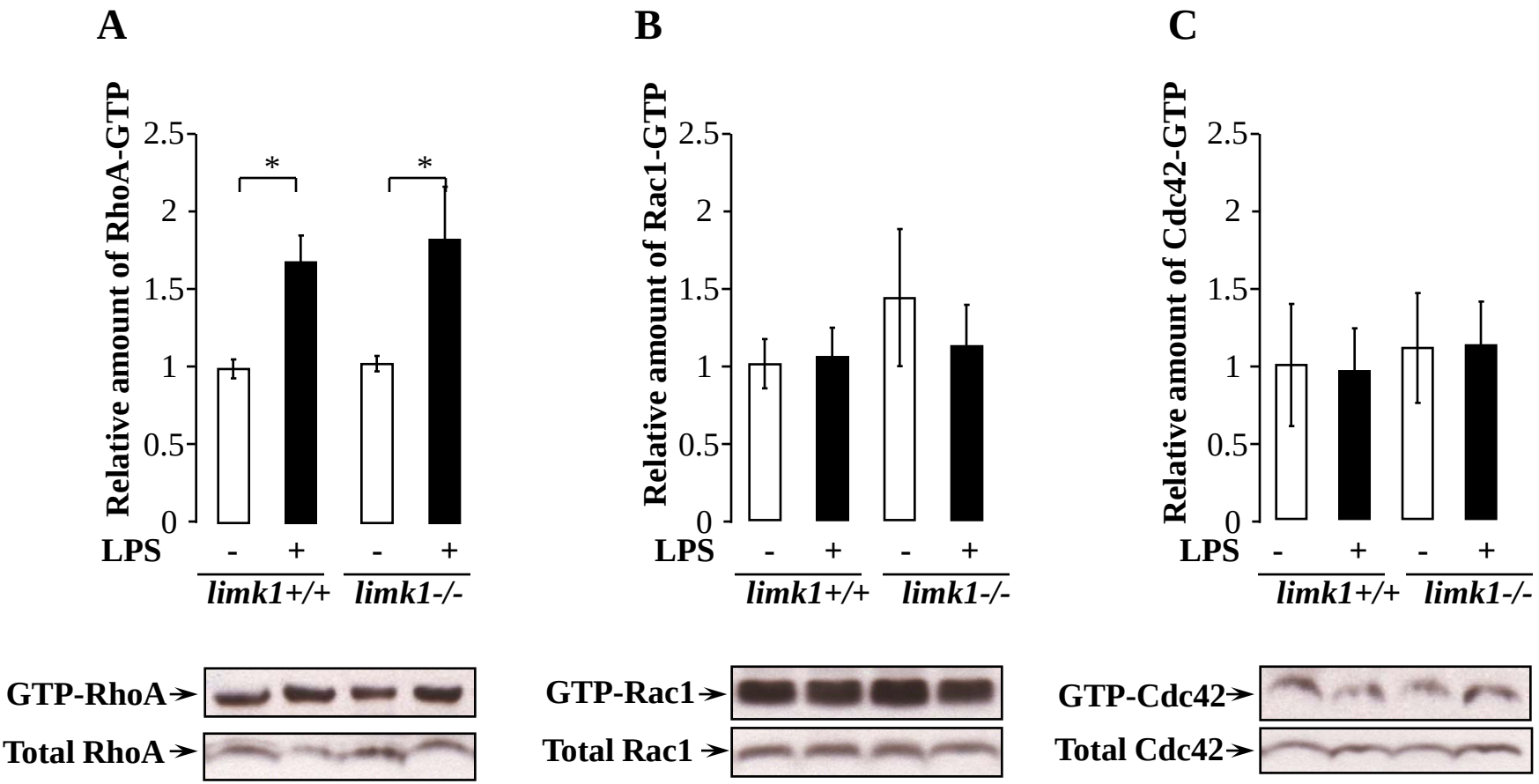
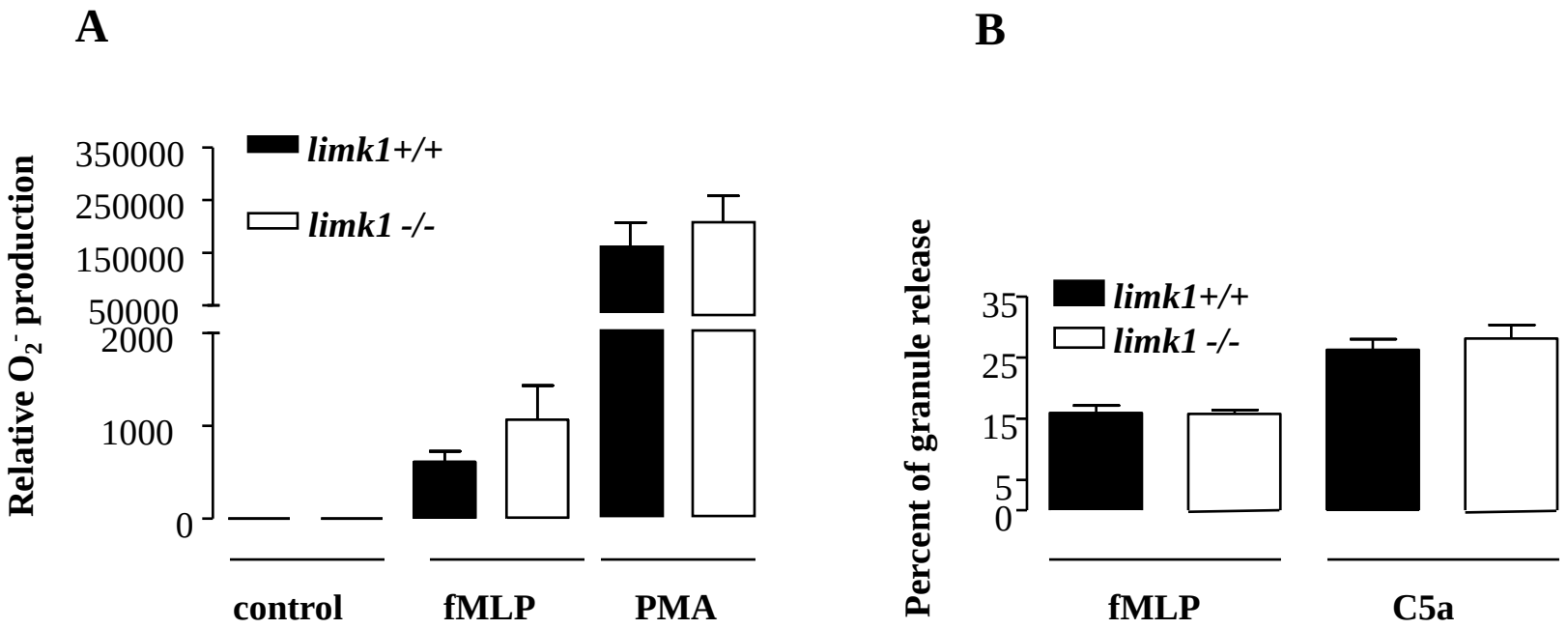


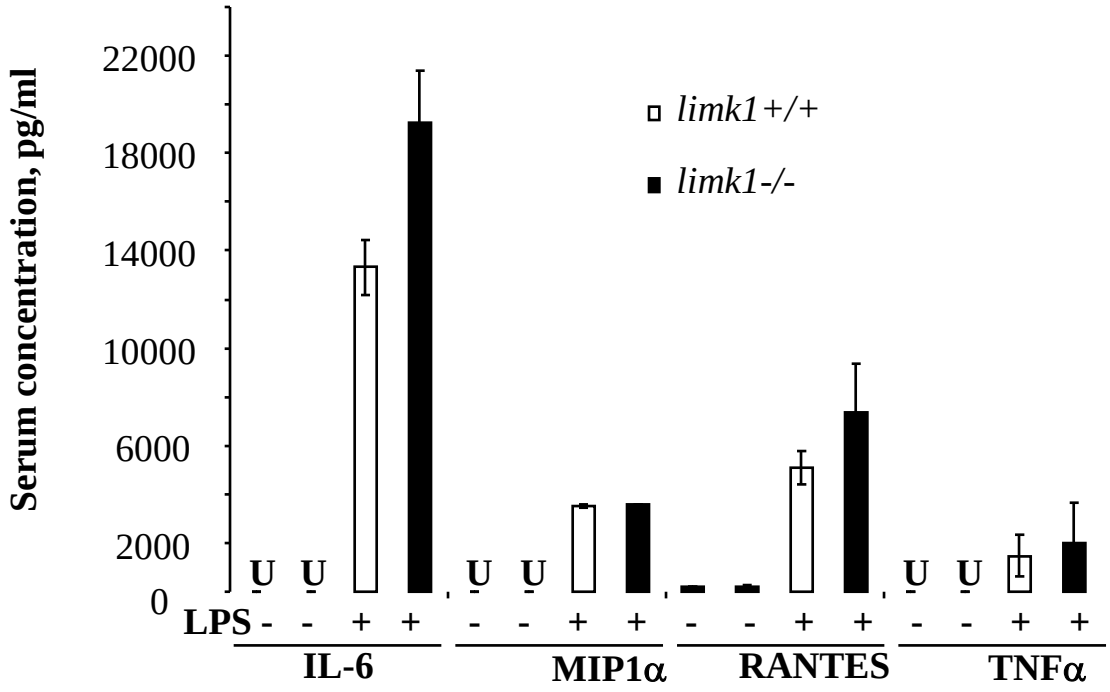
Online Figure I



Online Figure II

	limk1+/+		*limk1*-/-		*limk1*+/+, LPS		*limk1*-/-, LPS	
Variable	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
WBC (x10^9/L)	7.226667	1.120238	6.406667	1.540692	1.6	0.480833	2.05	0.381838
NE (x10^9/L)	1.543333	0.347035	1.42	0.38223	0.61	0.169706	0.56	0.325269
LY (x10^9/L)	5.323333	0.680759	4.65	1.162282	0.905	0.233345	1.35	0.014142
MO (x10^9/L)	0.263333	0.037859	0.236667	0.110151	0.055	0.06364	0.06	0
EO (x10^9/L)	0.073333	0.092916	0.073333	0.049329	0.025	0.021213	0.06	0.056569
BA (x10^9/L)	0.023333	0.025166	0.023333	0.023094	0.005	0.007071	0.02	0.028284
RBC (M/µL)	8.92	0.600083	8.946667	0.855005	8.425	0.516188	9.9	2.390021
Hb (g/dL)	12.66667	0.929157	13.63333	1.582193	12.55	0.636396	14.2	2.404163
HCT (%)	40	2.751363	42.93333	3.126233	43.8	3.252691	48.1	5.515433
MCV (fL)	44.83333	0.305505	48.06667	1.209683	52	0.707107	49.35	6.29325
MCH (pg)	14.23333	0.378594	15.2	0.458258	14.9	0.141421	14.45	1.06066
MCHC (g/dL)	31.66667	0.665833	31.73333	1.436431	28.7	0.707107	29.45	1.626346
PLT (x10^9/L)	622.6667	231.7096	387	115.113	416	70.71068	339	226.2742

Online Table I



Online Figure III