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CD47 Deficiency Protects Mice from Lipopolysaccharide-Induced Acute Lung Injury and *Escherichia coli* Pneumonia[1]

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CD47 modulates neutrophil transmigration toward the sites of infection or injury. Mice lacking CD47 are susceptible to *Escherichia coli* (*E. coli*) peritonitis. However, less is known concerning the role of CD47 in the development of acute lung inflammation and injury. In this study, we show that mice lacking CD47 are protected from LPS-induced acute lung injury and *E. coli* pneumonia with a significant reduction in pulmonary edema, lung vascular permeability, and bacteremia. Reconstitution of $CD47^{+/-}$ mice with $CD47^{-/-}$ neutrophils significantly reduced lung edema and neutrophil infiltration, thus demonstrating that $CD47^{+}$ neutrophils are required for the development of lung injury from *E. coli* pneumonia. Importantly, CD47-deficient mice with *E. coli* pneumonia had an improved survival rate. Taken together, deficiency of CD47 protects mice from LPS-induced acute lung injury and *E. coli* pneumonia. Targeting CD47 may be a novel pathway for treatment of acute lung injury. *The Journal of Immunology,* 2008, 180: 6947–6953.

Lipopolysaccharide binds CD14 on the surface of leukocytes resulting in cellular activation and production of proinflammatory cytokines and chemokines (1). Through CD18, neutrophils transmigrate across pulmonary endothelial barriers in the *E. coli* pneumonia and LPS-induced acute lung injury models (2, 3). Recent studies have shown that tissue-expressed CD47 (integrin associated protein) may also serve to modulate the acute inflammatory response by regulating the rate of polymorphonuclear leukocytes (PMN)[3] migration toward sites of injury or infection (4, 5). CD47 is an Ig superfamily transmembrane glycoprotein that is ubiquitously expressed in cells and tissues (6). The known ligands of CD47 are the membrane protein signal regulatory protein α and thrombospondin (6). CD47 interacting with signal regulatory protein α or thrombospondin has been implicated in multiple cellular processes, including PMN and monocyte transmigration (4, 5, 7, 8).

E. coli is a common cause of nosocomial pneumonia (9), which can lead to acute lung injury and acute respiratory distress syndrome (10). PMN mobilization and transmigration across pulmonary endothelial and epithelial barriers play a key role in development of acute lung injury and acute respiratory distress syndrome (11, 12). Because there are defects in PMN accumulation at the site of infection, phagocytosis, and oxidative burst, CD47-deficient mice succumb to *E. coli* peritonitis (13). Other observations have shown that CD47-deficient mice are less susceptible to *Staphylococcus aureus*-induced peritonitis and arthritis (14, 15). However, the contribution of CD47 to lung infection or inflammation has not been investigated.

CD47 is expressed on most cells, including endothelial and epithelial cells, and neutrophils. Prior studies have shown that endothelial or epithelial CD47, rather than neutrophil CD47, is important for neutrophil migration (4, 5). However, in vitro CD47 Ab studies suggest that anti-CD47 treatment of neutrophils blocks both transendothelial (16) and transepithelial (17) migration. Whether $CD47^{-/-}$ neutrophils have a delayed transmigration across cultured endothelial monolayers or pulmonary barriers (endothelial and epithelial cells) during inflammation and infection is still unclear.

We hypothesized that CD47 might regulate neutrophil transmigration, blood neutrophil count, cytokine production, and further influence the development of acute lung injury. Therefore, our first objective was to determine whether knockout of CD47 would alter neutrophil transmigration, blood neutrophil count, cytokine production, and pulmonary edema in a noninfectious LPS-induced acute lung injury mouse model. The second objective was to study whether lack of CD47 would affect neutrophil transmigration, neutrophilia, cytokine profile, pulmonary edema, and survival in an infectious mouse model (*E. coli* pneumonia). The final objective was to determine whether CD47 expression on neutrophils was critical to the development of acute lung injury following *E. coli* pneumonia.

Materials and Methods

Chemicals and reagents

LPS (*Escherichia coli* O55:B5) was purchased from Sigma-Aldrich.

Animals

Wild-type (WT) mice (C57BL/6J, 8 wk old) were purchased from The Jackson Laboratory. C57BL/6J mice deficient in CD47 were generated as previously described (13). Anesthesia was induced with an i.p. injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg). The committee

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[3] Abbreviations used in this paper: PMN, polymorphonuclear leukocyte; WT, wild type; BAL, bronchoalveolar lavage; MPO, myeloperoxidase.

on animal research of the University of California, San Francisco approved the protocols. In this study, baseline data were generated from normal $CD47^{+/-}$ and $CD47^{-/-}$ mice. $CD47^{+/-}$ instead of WT mice were used in the experiments because $CD47^{+/-}$ have never been shown different from WT mice and it allowed us to breed $CD47^{+/-}$ to $CD47^{-/-}$ to easily obtain 50% of each genotype in the offspring (13).

Direct visualized instillation

The anesthetized mice were suspended with their incisors attached to a ~60° wood support by 3/0 suture. A cold-light source (Dolan-Jenner Industries) with two 25-inch flexible fiber-optic arms allowed transillumination to visualize the glottis and vocal cords to deliver the LPS or *E. coli* into the air spaces (18).

LPS-induced acute lung injury mouse model

Mice were intratracheally instilled with LPS (5 mg/kg) by the direct visualized instillation method. Immediately before exposure to LPS, mice were given an injection of 0.05 μCi ^{125}I-albumin (Iso-Tex Diagnostics) via the right jugular vein. Mice were monitored for 4 and 24 h and sacrificed to carry out bronchoalveolar lavage (BAL) or measure excess lung water and lung vascular permeability.

Acute E. coli *pneumonia mouse model and survival study*

E. coli serotype K1 was originally isolated from the blood of a patient with biliary sepsis. The methods used to passage, store, amplify, and quantify the bacteria have been described as previously (19). *E. coli* 10^7 CFU were instilled into the air spaces of the lung. Immediately before exposure to *E. coli*, mice received 0.05 μCi ^{125}I-albumin via right jugular vein. Mice were monitored for 4 h and sacrificed to measure excess lung water and lung vascular permeability to protein or to carry out bronchoalveolar lavage. For a longer observation, mice received 2.5×10^6 CFU *E. coli* intratracheally and followed up for 48 h to measure excess lung water, blood neutrophil counts, and cytokines (MIP-2, IL-12) in the lung and plasma. In the survival experiments, mice were given 5×10^6 CFU *E. coli* intratracheally and followed up for 72 h. In preliminary studies, this dosage induced a mortality of ~50% 24 h after *E. coli* exposure in the WT mice.

Excess lung water and lung extravascular plasma equivalent

As previously described (20), the lungs were removed, counted in a gamma counter (Packard), weighed, and homogenized (after addition of 1 ml distilled water). The blood was collected through puncture of the right ventricle. The homogenate was weighed and a fraction was centrifuged (12,000 rpm, 8 min) for assay of hemoglobin concentration in the supernatant. Another fraction of homogenate, supernatant, and blood was weighed and then desiccated in an oven (60°C for 24 h). Excess lung water calculation was based on a standard formula. Lung extravascular plasma equivalents (index of lung vascular permeability to protein) were calculated as the counts of ^{125}I-albumin in the blood free lung tissue divided by the counts of ^{125}I-albumin in the plasma.

BAL and plasma cytokine and protein measurements

BAL and plasma samples were obtained from mice at 4 or 48 h in *E. coli* pneumonia and 4 or 24 h in endotoxin-induced acute lung injury. BAL was done after euthanizing the mice and then placing a 20-gauge catheter into the trachea through which 1 ml of cold PBS was flushed back and forth three times. TNF-α, MIP-2, and IL-12 were measured in BAL or plasma samples with ELISA kits (R&D Systems). Protein concentration was measured in the BAL fluid from all experimental groups, as an index of lung endothelial and epithelial permeability (Bio-Rad protein assay kit).

E. coli *colonies in the lung and blood*

Mice were euthanized at 4 h after intratracheal instillation of *E. coli*. The lungs were removed from the thorax, 1 ml PBS was added, and they were then homogenized under sterile conditions. The homogenate was serially diluted and cultured on a LB Plate (TEKnova) for 24 h. Blood was withdrawn by puncture of the right ventricle, and 0.1 ml blood was spread in the LB plate and cultured for 24 h for colony counts.

Measurement of leukocytes and neutrophils in blood and BAL

A sample of blood was placed in EDTA-coated vials (BD Microtainer) and a multispecies hematology instrument (Hemavet 950FS; Drew Scientific) was used to generate a complete blood count with cellular differential, hemoglobin, and hematocrit. BAL leukocytes and neutrophils were measured by a Coulter counter (Z1 series; Beckman Coulter) plus a differential white blood cell count by cytospin staining (Cytospin 3; Thermo Electron).

Determination of lung myeloperoxidase (MPO) activity

As previously described (21), aliquots of 10 μl supernatant of the lung homogenate were mixed with 200 μl of odianisidine HCl (Sigma-Aldrich, 1.65 mg/ml) KPO_4 solution buffer, and 10 μl of 0.1% H_2O_2 was added to initiate the reaction. Absorbance was recorded at 405 nm, every 6 s for six times using a Plate Reader (Dyx-Ex Technologies). The enzyme activity was calculated (units/min/ml) by dividing the rate of the change in the absorbance by the extinction coefficient, 1.13×10^{-2}.

Neutrophil depletion experiments

Neutrophil depletion was accomplished with a rat anti-mouse mAb against the neutrophil maturation Ag, Gr-1. The Gr-1 mAb was purified from the RB6–8C5 hybridoma (a gift from K. Ley, University of Virginia, Charlottesville, Virginia). The Gr-1 mAb (160 μg) was given i.p., yielding > 90% neutrophil depletion at 24 h.

Neutrophil isolation

CD47 heterozygous and deficient mice were euthanized and the bone marrow from the femurs and tibias was flushed with PBS using a 25-gauge needle. The whole bone marrow was centrifuged and washed in PBS, and the RBC were hypotonically lysed with 0.2% NaCl. This solution was restored to isotonicity with 1.2% NaCl and then filtered over a 70-μm nylon cell strainer (BD Biosciences-Discovery Labware). The solution was centrifuged and resuspended in PBS and then applied over a 62% Percoll gradient. The Percoll solution was centrifuged for 30 min at $1,500 \times g$. The neutrophil pellet was then isolated, washed, and centrifuged twice, and counted with a Coulter counter. Greater than 90% neutrophil purity was confirmed with a cytospin preparation and Diff-Quick staining.

Neutrophil reconstitution

Before neutrophil-depleted mice were intratracheally challenged with 10^7 CFU *E. coli*, 5×10^6 freshly isolated bone marrow neutrophils mixed with ^{125}I-albumin were i.v. injected to reconstitute blood neutrophils as previously described (22).

Statistical analysis

Statistics were done by SPSS software (SPSS). An unpaired *t* test (two-tailed) was used unless there were multiple comparisons, in which case we used ANOVA with post hoc Bonferoni test (significance level set at $p < 0.05$). The log-rank test was used for comparing survival data at 72 h by GraphPad Prism software (GraphPad Software). The results are shown as mean ± SD.

Results

CD47-deficient mice are protected from LPS-induced acute lung injury

To test whether CD47 deficiency would reduce pulmonary edema and lung vascular permeability in LPS-induced acute lung injury, $CD47^{+/-}$ and $CD47^{-/-}$ mice were intratracheally instilled with LPS (5 mg/kg). At both 4 and 24 h later, blood was withdrawn and the lungs were removed to measure excess lung water and lung vascular permeability or to carry out BAL. At 24 h, excess lung water and extravascular plasma equivalents were reduced in the CD47-deficient mice (Fig. 1, *A* and *B*). The hemoglobin and hematocrit were lower in the $CD47^{-/-}$ mice (Fig. 1, *C* and *D*), suggesting less hemoconcentration in the $CD47^{-/-}$ mice. Plasma MIP-2 levels were reduced (Fig. 1*E*) and plasma IL-12 levels (Fig. 1*F*) were increased in the CD47-deficient mice.

To investigate whether the lack of CD47 would affect neutrophil transmigration in LPS-induced acute lung injury, $CD47^{+/-}$ and $CD47^{-/-}$ mice were intratracheally instilled with LPS and BAL was performed at 4 and 24 h. There was no difference in the leukocyte and neutrophil counts in the blood between $CD47^{+/-}$ and $CD47^{-/-}$ mice at baseline. However, leukocyte and neutrophil counts in the blood in the $CD47^{-/-}$ were significantly reduced

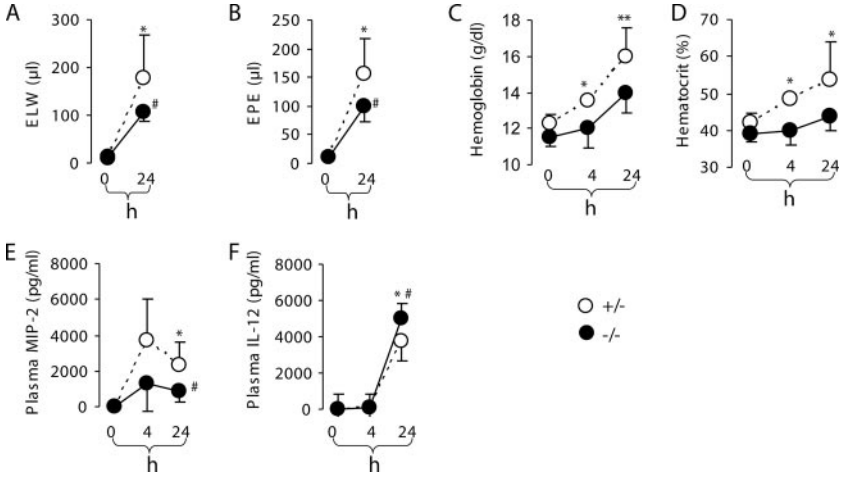

FIGURE 1. Lack of CD47 affected LPS-induced acute lung injury at 4 and 24 h. *A* and *B*, Excess lung water and lung vascular permeability were reduced in the CD47 knockout mice at 24 h. *, $p < 0.05$ vs $CD47^{-/-}$; #, $p < 0.05$ vs baseline. *C* and *D*, Less hemoconcentration in the CD47-deficient mice at 4 and 24 h. *, $p < 0.05$ or **, $p < 0.01$ vs $CD47^{-/-}$. *E* and *F*, Plasma MIP-2 level was reduced and plasma IL-12 was elevated at 24 h. *, $p < 0.05$; #, $p < 0.05$ vs baseline. $n = 3$ for baseline; $n = 6$–9 for the other group. Data were pooled from three experiments. Data are mean ± SD.

after LPS challenge (Fig. 2, *A* and *B*) at 24 h. Leukocyte and neutrophil counts in the BAL in the $CD47^{-/-}$ mice were also decreased compared with the $CD47^{+/-}$ mice (Fig. 2, *C* and *D*) at 24 h. The protein concentration in the BAL, an index of epithelial and endothelial permeability, was reduced in $CD47^{-/-}$ mice at 24 h (Fig. 2*E*). At 4 h, BAL MIP-2 and TNF-α were reduced in the $CD47^{-/-}$ mice. However, there were no differences in the BAL MIP-2, TNF-α, and IL-12 between $CD47^{+/-}$ and $CD47^{-/-}$ mice with LPS-induced acute lung injury at 24 h (Fig. 2, *F–H*).

CD47-deficient mice are protected from E. coli *pneumonia*

To study whether lack of CD47 would decrease pulmonary edema and lung vascular permeability in *E. coli* pneumonia, $CD47^{+/-}$ and $CD47^{-/-}$ mice were intratracheally instilled with 10^7 CFU *E. coli*. Four hours later, blood was withdrawn and the lungs were removed to measure excess lung water and lung vascular permeability. Excess lung water and extravascular plasma equivalents were significantly reduced in the CD47-deficient mice (Fig. 3, *A* and *B*). Hemoglobin and hematocrit in the blood were also lower in the $CD47^{-/-}$ mice (Fig. 3, *C* and *D*).

To determine whether lack of CD47 would affect BAL parameters in *E. coli* pneumonia, $CD47^{+/-}$ and $CD47^{-/-}$ mice were intratracheally instilled with 10^7 CFU *E. coli* and BAL was done at 4 h. Protein concentration, neutrophil counts, and MIP-2 levels in the BAL in the $CD47^{-/-}$ mice were significantly reduced compared with the $CD47^{+/-}$ mice (Fig. 3, *E–G*).

To test whether mice lacking CD47 affects blood neutrophil count and bacterial dissemination, $CD47^{+/-}$ and $CD47^{-/-}$ mice were intratracheally instilled with 10^7 CFU *E. coli*. Four hours later, lungs were removed and homogenized and blood was withdrawn for cell count and bacterial culture. As in the LPS model, blood leukocyte and neutrophil counts in the $CD47^{-/-}$ were significantly lower than in the $CD47^{+/-}$ mice (Fig. 4, *A* and *B*). Also, there were significantly higher numbers of bacteria present in the lungs in the $CD47^{-/-}$ mice (Fig. 4*C*). However, the number of bacteria in the blood in the $CD47^{-/-}$ mice was reduced (Fig. 4*D*).

To test whether the lack of CD47 prevents mice from *E. coli* penumonia at a later stage, $CD47^{+/-}$ and $CD47^{-/-}$ mice were intratracheally instilled with 2.5×10^6 CFU *E. coli*. Then, 48 h later, excess lung water was measured and found to be reduced in the $CD47^{-/-}$ mice (Fig. 5*A*). $CD47^{-/-}$ mice had a lower blood neutrophil count (Fig. 5*B*), hemoglobin, and hematocrit (Fig. 5, *C* and *D*), indicating less hemoconcentration. There was a reduction in MPO activity in the lung of $CD47^{-/-}$ mice, indicating fewer neutrophils were sequestered and had transmigrated into the lung

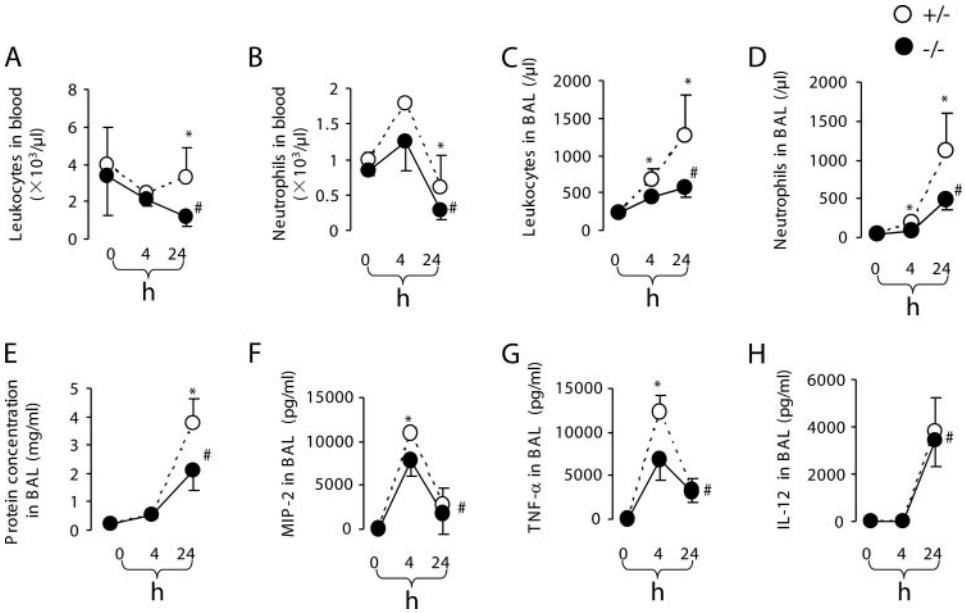

FIGURE 2. Deficiency of CD47 reduced neutrophil count in the blood and BAL in the LPS-induced acute lung injury at 4 and 24 h. *A*, Leukocyte in the blood; *B*, Neutrophils in the blood; *C*, Leukocyte in the BAL; *D*, Neutrophils in the BAL. *, $p < 0.05$ vs $CD47^{-/-}$; #, $p < 0.05$ vs baseline. Lack of CD47 decreased protein concentration in the BAL, decreased BAL MIP-2 and TNF-α at 4 h, but did not affect the cytokine production in the BAL at 24 h in LPS-induced acute lung injury. *E*, Protein concentration in the BAL. *F–H*, MIP-2, TNF-α, and IL-12 in the BAL. *, $p < 0.05$ vs $CD47^{-/-}$; #, $p < 0.05$ vs baseline. $n = 3$ for baseline; $n = 4$–5 for the other group. Data were pooled from four experiments. Data are mean ± SD.

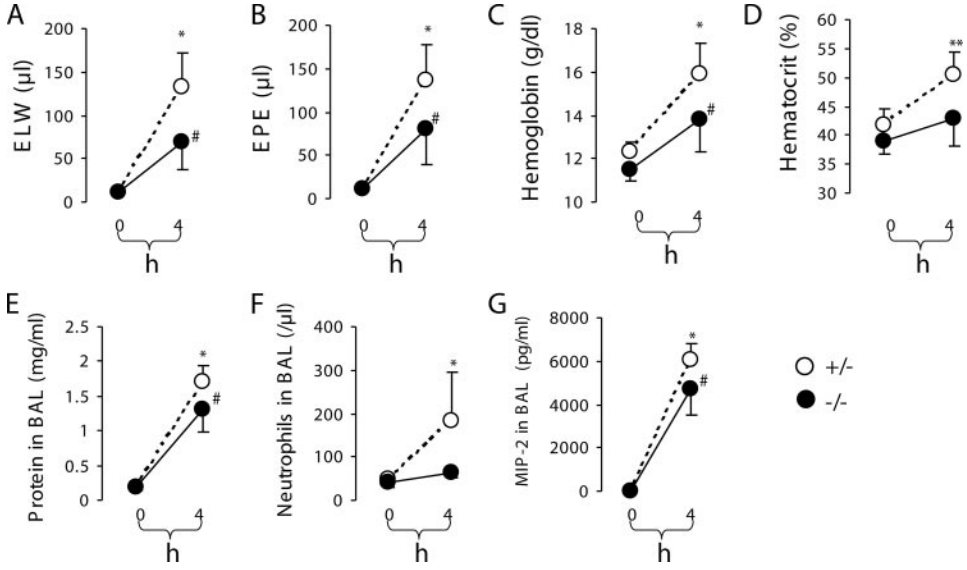

FIGURE 3. Deficiency of CD47 reduced lung injury in the *E. coli* pneumonia experiments at 4 h. *A* and *B*, Excess lung water and lung vascular permeability were reduced at 4 h. *C* and *D*, Hemoconcentration was attenuated in the CD47 deficient group. *E–G*, Lack of CD47 resulted in lower protein concentration, neutrophil counts, and MIP-2 levels in the BAL at 4 h. *, $p < 0.05$ or **, $p < 0.01$ vs $CD47^{-/-}$; #, $p < 0.05$ vs baseline. $n = 3$ for baseline; $n = 5–6$ for the other group. Data were pooled from three experiments. Data are mean ± SD.

(Fig. 5*E*). There were no differences in MIP-2 and IL-12 levels in the lung homogenate and IL-12 levels in the plasma between $CD47^{+/-}$ and $CD47^{-/-}$ mice (Fig. 5, *F–H*).

Neutrophils lacking CD47 contribute to protection of mice from E. coli *pneumonia*

To determine whether CD47 on the neutrophils exerts a more critical role in mediating lung inflammation and injury in *E. coli* pneumonia model than CD47 on lung cells, we selectively depleted endogenous neutrophils from the $CD47^{+/-}$ and $CD47^{-/-}$ mice by i.p. injecting Gr-1 mAb 24 h before the experiments, and then reconstituted mice with either $CD47^{+/-}$ or $CD47^{-/-}$ donor neutrophils via jugular vein injection followed by intratracheal instillation of 10^7 CFU *E. coli*. Mice were divided into four groups: 1) $CD47^{+/-}$ recipient/$CD47^{+/-}$ donor neutrophils; 2) $CD47^{-/-}$ recipient/$CD47^{+/-}$ donor neutrophils; 3) $CD47^{+/-}$ recipient/$CD47^{-/-}$ donor neutrophils; and 4) $CD47^{-/-}$ recipient/$CD47^{-/-}$ donor neutrophils.

Excess lung water and lung vascular permeability were reduced in the $CD47^{+/-}$ recipient mice that received $CD47^{-/-}$ donor neutrophils compared with $CD47^{+/-}$ recipients that received $CD47^{+/-}$ donor neutrophils. In contrast, excess lung water and lung vascular permeability were significantly increased in the $CD47^{-/-}$ recipient mice that received $CD47^{+/-}$ donor neutrophils compared with $CD47^{-/-}$ recipient mice that received $CD47^{-/-}$ donor neutrophils (Fig. 6, *A* and *B*).

Lung homogenate MPO (an index of neutrophil accumulation) was significantly decreased in the $CD47^{+/-}$ recipient mice that received $CD47^{-/-}$ donor neutrophils compared with $CD47^{+/-}$ recipient mice that received $CD47^{+/-}$ donor neutrophils. There was a trend for an increase in the lung homogenate MPO in the $CD47^{-/-}$ recipient mice that received $CD47^{+/-}$ donor neutrophils compared with $CD47^{-/-}$ recipient mice that received $CD47^{-/-}$ donor neutrophils (Fig. 6*C*).

There were significantly lower plasma MIP-2 levels in the $CD47^{+/-}$ recipient mice that received $CD47^{-/-}$ donor neutrophils compared with $CD47^{+/-}$ recipient mice that received $CD47^{+/-}$ donor neutrophils. There was a nonsignificant trend for an increase in plasma MIP-2 levels in the $CD47^{-/-}$ recipient mice receiving $CD47^{+/-}$ donor neutrophils compared with $CD47^{-/-}$ recipient mice that received $CD47^{-/-}$ donor neutrophils (Fig. 6*D*).

Because intravascular neutrophils also contribute to the lung MPO activity shown in Fig. 6*C*, endogenous neutrophil-depleted $CD47^{+/-}$ mice were administered donor $CD47^{+/-}$ or $CD47^{-/-}$ neutrophils i.v. and then intratracheally challenged with PBS or 10^7 CFU *E. coli*. Four hours later, mice were sacrificed and the lungs were perfused with 5 ml PBS through the

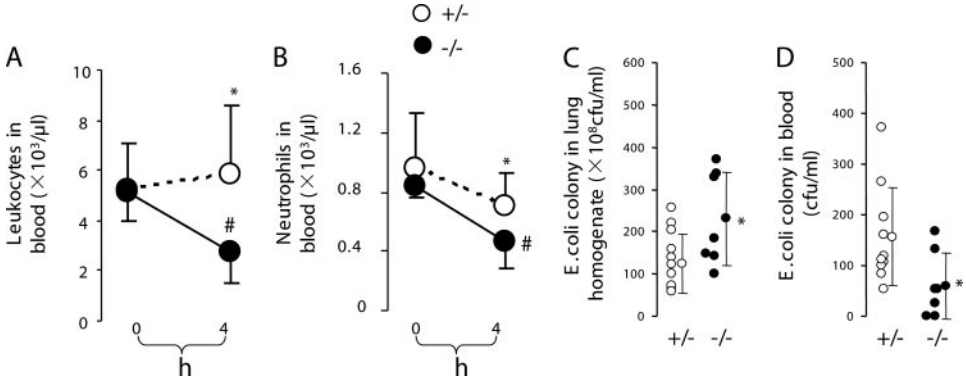

FIGURE 4. The number of neutrophils and *E. coli* colonies in the lung and blood stream in the *E. coli* pneumonia experiments at 4 h. *A* and *B*. Leukocyte and neutrophil counts in the blood with or without *E. coli* pneumonia. *, $p < 0.05$ vs $CD47^{-/-}$; #, $p < 0.05$ vs baseline. Data are mean ± SD. *C* and *D*, *E. coli* colonies in the lung homogenate (*C*) and blood (*D*) 4 h after initiation of infection. *, $p < 0.05$ for $CD47^{-/-}$ vs $CD47^{+/-}$ mice with pneumonia. $n = 3$ for baseline; $n = 7–9$ for the other group. Data were pooled from four experiments. Data are mean ± SD.

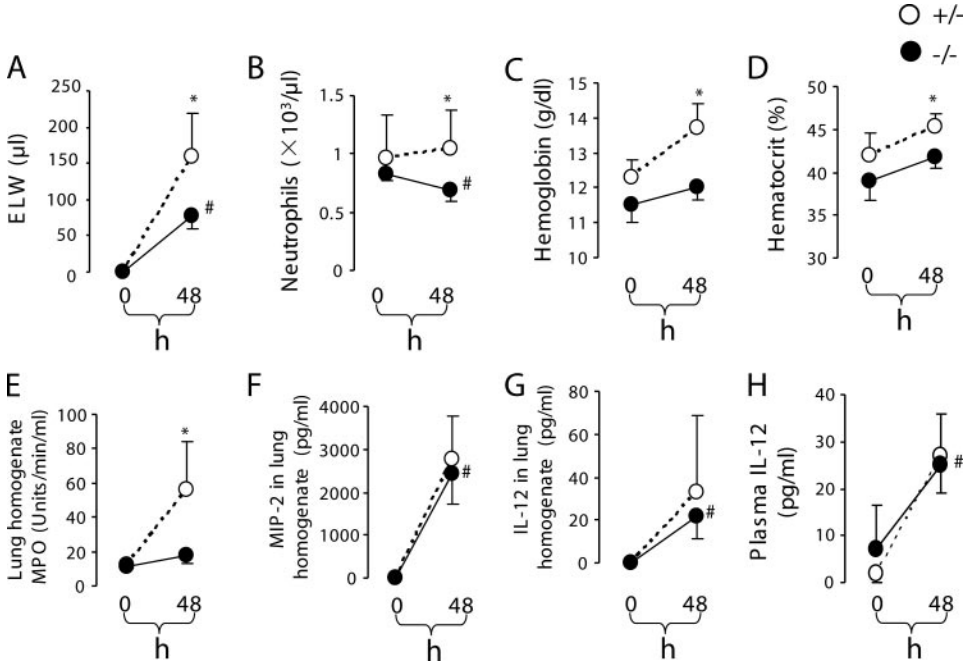

FIGURE 5. Deficiency of CD47-reduced lung injury in the *E. coli* pneumonia experiments at 48 h. *A* and *B*, Excess lung water and neutrophil counts were reduced at 48 h. *C* and *D*, Hemoconcentration was attenuated in the CD47-deficient group at 48 h. *E–H*, Lack of CD47 resulted in lower MPO activity in the lung homogenate, but did not affect MIP-2 and IL-12 levels in lung homogenate, and IL-12 in the plasma at 48 h. *, $p < 0.05$ for $CD47^{+/-}$ vs $CD47^{-/-}$; #, $p < 0.05$ vs baseline. $n = 3$ for baseline; $n = 4–6$ for the other group. Data were pooled from three experiments. Data are mean ± SD.

right ventricle to remove sequestered neutrophils from the pulmonary circulation. The flushed lungs were homogenized to measure MPO activity. There was no difference in lung MPO activity in the $CD47^{+/-}$ recipient mice that received $CD47^{+/-}$ and $CD47^{-/-}$ donor neutrophils challenged with PBS. However, the lung MPO activity was increased in the $CD47^{+/-}$ recipient mice that received $CD47^{+/-}$ donor neutrophils in *E. coli* pneumonia. There was a reduction in lung MPO activity in the $CD47^{+/-}$ recipient mice that received $CD47^{-/-}$ donor neutrophils (Fig. 6*E*).

Deficiency of CD47 improves survival with sublethal E. coli *pneumonia*

To test whether deficiency of CD47 affects survival in *E. coli* pneumonia, $CD47^{+/-}$ ($n = 17$) and $CD47^{-/-}$ ($n = 14$) mice were intratracheally challenged with 5×10^6 CFU *E. coli* and monitored

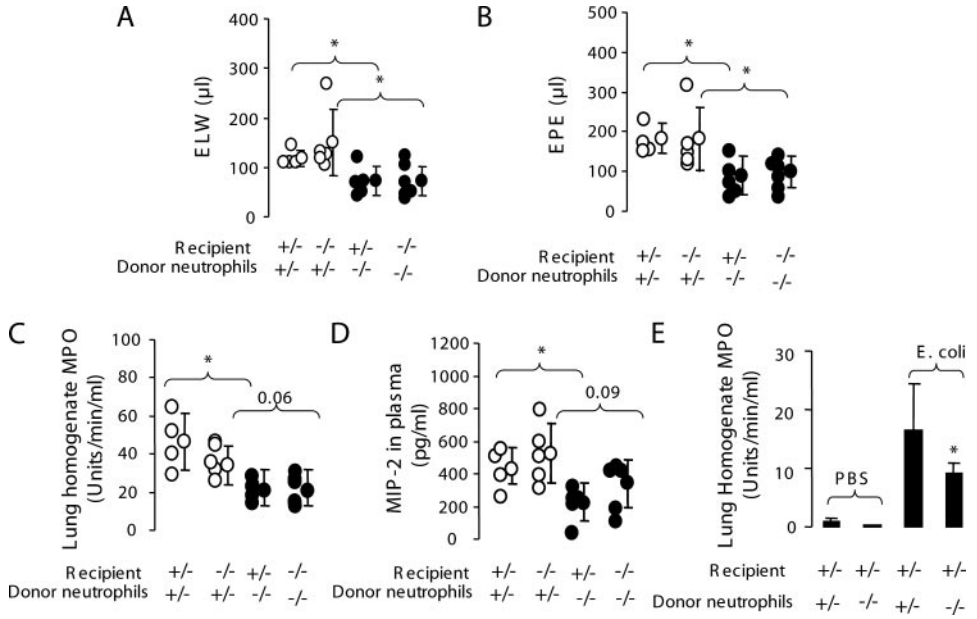

FIGURE 6. Neutrophil reconstitution experiments to demonstrate that CD47 expression on the neutrophils contributes to greater lung inflammation and injury in the *E. coli* pneumonia. *A* and *B*, Reconstitution of $CD47^{+/-}$ mice with $CD47^{-/-}$ neutrophils reduced excess lung water and lung vascular permeability compared with $CD47^{+/-}$ mice with $CD47^{+/-}$ neutrophils. Reconstitution of $CD47^{-/-}$ mice with $CD47^{+/-}$ neutrophils increased excess lung water and lung vascular permeability compared with $CD47^{-/-}$ mice with $CD47^{-/-}$ neutrophils. *C*, Reconstitution of $CD47^{+/-}$ mice with $CD47^{-/-}$ neutrophils significantly decreased lung homogenate MPO, an index of neutrophil infiltration. *D*, Reduced plasma MIP-2 production in the $CD47^{+/-}$ mice reconstituted with $CD47^{-/-}$ neutrophils. *E*, Reconstitution of $CD47^{+/-}$ mice with $CD47^{-/-}$ neutrophils significantly decreased lung homogenate MPO, an index of neutrophil infiltration after flushing away sequestered neutrophils in the pulmonary circulation. $n = 3$ for baseline; $n = 4–6$ for the other group. Data were pooled from six experiments. Data are mean ± SD.

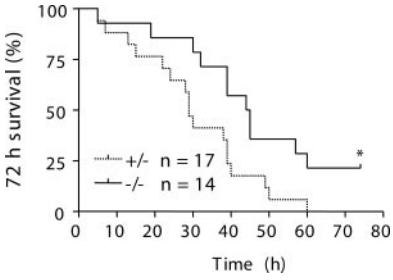

FIGURE 7. Lack of CD47 improved survival at 72 h after mice were intratracheally challenged with 5×10^6 CFU live *E. coli*. *, $p < 0.05$ using a log-rank test; $n = 17$ in the $CD47^{+/-}$ group; $n = 14$ in the $CD47^{-/-}$ group. Data were pooled from two experiments.

for 72 h (data were pooled from two batches of experiments). The survival during 72 h in the $CD47^{-/-}$ mice was significantly enhanced compared with the $CD47^{+/-}$ mice (Fig. 7).

Discussion

The present findings indicate that mice lacking CD47 were protected from LPS-induced acute lung injury as well as *E. coli* pneumonia as reflected by a significant reduction in excess lung water and lung vascular permeability to protein. The neutrophil transmigration into the air spaces of the lung was reduced in the CD47 deficient mice. In the reconstitution experiments with *E. coli* pneumonia, transmigration of $CD47^{-/-}$ neutrophils across the $CD47^{+/-}$ pulmonary endothelial barrier was reduced. CD47-deficient mice with *E. coli* pneumonia had less bacteremia and better survival.

Although previous studies had shown a role for CD47 in PMN influx to sites of infection, they had not clearly distinguished whether PMN or stromal CD47 was required for this effect. Now, we have shown clearly that PMN CD47 regulates PMN influx. These data suggest that, in vivo, PMN CD47 is required for neutrophil migration to a site of infection. In our model, it is likely that neutrophils contribute significantly to the lung damage that results in increased edema and increased bacteremia. Thus, the decreased PMN ingress in response to LPS or *E. coli* likely accounts for the improved clinical condition and survival of mice lacking PMN CD47.

Several studies have implicated CD47 is involved in the regulation of cytokine production. In monocyte-derived dendritic cells, CD47 engagement potently suppressed IL-12, TNF-α, IL-6, and GM-CSF, but did not affect IL-8 production (23, 24). In the early stage (4 h) of the LPS-induced acute lung injury or the *E. coli* pneumonia mouse models, MIP-2 or TNF-α in the BAL were reduced in the CD47-deficient mice. At 24 h, in the LPS-induced acute lung injury model, CD47-deficient mice had less MIP-2 and higher IL-12 in the plasma although there was no difference in cytokine levels in the BAL. The mechanisms that determine how CD47 regulates cytokine production remain unclear. However, in our in vivo models, reduced proinflammatory cytokines might contribute to attenuated pulmonary and systemic proinflammatory responses (lower lung water and lung vascular permeability, less hemoconcentration) in the $CD47^{-/-}$ mice in the early stage. At 48 h, there was no difference in IL-12 levels in the lung homogenate and the plasma between $CD47^{+/-}$ and $CD47^{-/-}$ mice in the *E. coli* pneumonia model, indicating IL-12 did not facilitate a protective role for $CD47^{-/-}$ mice with *E. coli* infection in the late stage (25).

The pulmonary neutrophilic response in LPS-induced acute lung injury or *E. coli* pneumonia may occur in two phases: an initial recruitment of neutrophils and persistent influx of bone morrow-released neutrophils (26). In the $CD47^{+/-}$ mice with LPS-induced acute lung injury or *E. coli* pneumonia, blood leukocyte and neutrophil counts were significantly higher than in the $CD47^{-/-}$ mice and more neutrophils transmigrated into the air spaces. The mechanism that explains how the lack of CD47 contributes to less neutrophilia in our mouse studies is not clear. However, one study has shown that neutrophil mobilization depends on IL-8-induced stimulation and the number of circulating neutrophils (27). In the CD47-deficient mice, less MIP-2 and lower neutrophil counts in the blood might indicate reduced capacity of neutrophil mobilization from the bone marrow compared with the CD47 expressing mice. Consequently, fewer circulating neutrophils in the CD47-deficient mice might contribute to fewer neutrophils migrating into the air spaces in addition to a defect in neutrophil transmigration. Another possibility is that the relative neutropenia in $CD47^{-/-}$ mice results from enhanced margination of the circulating PMN. Clinically, neutropenia is a sign of severe acute infection, generally thought to result from intravascular activation of PMN with consequent adhesion in various vascular beds, not necessarily related to the site of infection. Perhaps, in the absence of CD47, activation of this aberrant adhesion is exaggerated.

In the PMN migration across the epithelial monolayer experiments, inhibition of CD47 did not halt migration, but delayed neutrophil migration, and the total number of neutrophils that eventually migrated was the same as that observed with controls (5). In this study, the number of neutrophils in the BAL in the LPS-induced acute lung injury and *E. coli* pneumonia models in the $CD47^{-/-}$ mice was significantly reduced compared with $CD47^{+/-}$ mice. These findings are consistent with the prior report that neutrophil influx was diminished in $CD47^{-/-}$ mice compared with $CD47^{+/-}$ littermates in the peritoneum of an *E. coli* peritonitis model at 4 h (13) and a *S. aureus* peritonitis model at 48 h (14). Two factors may contribute to fewer neutrophils in the BAL: 1) neutrophil transmigration is delayed without CD47 expression; 2) the number of neutrophils available to the air spaces from the blood in the $CD47^{-/-}$ mice is less. Less MIP-2 in the air spaces of the lung may contribute to the delayed influx of neutrophils into the air spaces in the CD47 null mice at the early stage (4 h), but not at the later stages (24 and 48 h) in our mouse studies because there was no difference in the lung MIP-2 levels.

In the reconstitution experiments with *E. coli* pneumonia, the lung homogenate MPO levels were reduced in the $CD47^{+/-}$ mice receiving $CD47^{-/-}$ neutrophils after flushing the neutrophils from the pulmonary circulation. This finding indicates that transmigration of neutrophils across lung endothelia is reduced. This result is consistent with the previous data that anti-CD47 treatment of neutrophils blocks both transendothelial (16) and transepithelial (17) migration. The explanation for why $CD47^{-/-}$ neutrophils had less migration across $CD47^{+/-}$ pulmonary endothelial barrier remains unclear. In our in vitro study, $CD47^{-/-}$ neutrophils are not innately defective in their ability to migrate through an uncoated filter in response to chemoattractant (data not shown). However, reduction of transmigration of $CD47^{-/-}$ neutrophils in our in vivo models is more complicated than the in vitro setting. In the in vivo setting, there are several other factors (cytokines, cell-cell interactions, blood flow, and ventilation) that are not modeled by the in vitro studies. For example, impairment of engagement with ligands in the endothelial and epithelial cells in the $CD47^{-/-}$ neutrophils may reduce neutrophil sequestration, adhesion, and transmigration (28). However, the exact mechanism requires further investigation.

Although more bacteria accumulated in the lung in the $CD47^{-/-}$ mice at 4 h, the severity of lung injury was less and the survival from *E. coli* pneumonia was enhanced in the $CD47^{-/-}$

mice. This finding contrasts with the *E. coli* peritonitis model (13), but is consistent with the report that CD47-deficient mice are resistant to *S. aureus*-induced peritonitis (14) and arthritis (15). One explanation for this difference is that the peritoneal barrier (mesothelial cells) (29) is different from the pulmonary barriers (endothelial and epithelial cells). For example, mice lacking NKCC1 (Na^+-K^+-Cl^- transporter 1) are protected from *Klebsiella Pneumoniae* pneumonia but not protected from *K. Pneumoniae*–induced peritonitis (30). In our Gram-negative pneumonia model, the delayed influx of neutrophils into the air spaces might downregulate the intensity of the pulmonary proinflammatory responses, and eventually the reduced number of neutrophils are still sufficient to confine the *E. coli* in the lung in the $CD47^{-/-}$ mice, particularly because there is less lung endothelial and epithelial injury. In addition, CD47 deficient mice had less bacteremia, perhaps in part because of less injury to the endothelial and epithelial barriers of the lung. Most importantly, $CD47^{-/-}$ neutrophils, not lung cells, explain the protective effects in *E. coli* pneumonia. $CD47^{-/-}$ neutrophils given to the recipient with $CD47^{+/-}$ lung cells produced a reduced neutrophil transmigration and plasma MIP-2 levels.

Taken together, deficiency of CD47 protects mice from LPS-induced acute lung injury and *E. coli* pneumonia by reducing neutrophil transmigration, neutrophilia, proinflammatory cytokine production, and bacteremia. CD47 might be a novel therapeutic target to treat acute lung injury.

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Disclosures

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