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## The Cannabinoid Receptor 2 Is Critical for the Host Response to Sepsis<sup>1</sup>

Johannes Tschöp<sup>2,\*§</sup>, Kevin R. Kasten<sup>2,\*</sup>, Ruben Nogueiras<sup>‡</sup>, Holly S. Goetzman<sup>\*</sup>, Cynthia M. Cave<sup>\*</sup>, Lisa G. England<sup>\*†</sup>, Jonathan Dattilo<sup>\*</sup>, Alex B. Lentsch<sup>\*</sup>, Matthias H. Tschöp<sup>‡</sup>, and Charles C. Caldwell<sup>3,\*†</sup>

<sup>\*</sup>The Laboratory of Trauma, Sepsis and Inflammation Research, Department of Surgery, University of Cincinnati College of Medicine, Cincinnati, OH 45267

<sup>†</sup>Department of Research, Shriners's Hospital for Children, Cincinnati, OH 45229

<sup>‡</sup>Department of Psychiatry, Obesity Research Centre-Genome Research Institute, University of Cincinnati, Cincinnati, OH 45226

<sup>§</sup>Department of Anesthesiology, Klinikum Grosshadern, Ludwig-Maximilians University, Munich, Germany

### Abstract

Leukocyte function can be modulated through the cannabinoid receptor 2 (CB2R). Using a cecal ligation and puncture (CLP) model of sepsis, we examined the role of the CB2R during the immune response to an overwhelming infection. CB2R-knock out (KO) mice showed decreased survival as compared with wild-type mice. CB2R-KO mice also had increased serum IL-6 and bacteremia. Twenty-four hours after CLP, the CB2R-deficient mice had increased lung injury. Additionally, CB2R-deficiency led to increased neutrophil recruitment, decreased neutrophil activation, and decreased p38 activity at the site of infection. Consistent with a novel role for CB2R in sepsis, CB2R-agonist treatment in wild-type mice increased the mean survival time in response to CLP. Treatment with CB2R-agonist also decreased serum IL-6 levels, bacteremia, and damage to the lungs compared with vehicle-treated mice. Finally, the CB2R agonist decreased neutrophil recruitment, while increasing neutrophil activation and p38 activity at the site of infection compared with vehicle-treated mice. These data suggest that CB2R is a critical regulator of the immune response to sepsis and may be a novel therapeutic target.

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<sup>3</sup>Address correspondence and reprint requests to Dr. Charles C. Caldwell, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Medical Science Building, Surgical Research Unit G479 ML 0558, Cincinnati, OH 45267-0558. E-mail address: charles.caldwell@uc.edu.

<sup>2</sup>J.T. and K.R.K. contributed equally to the study.

### Disclosures

The authors have no financial conflict of interest.

A body of research has shown that leukocyte function can be modulated by endocannabinoids (reviewed in Ref. 1). These compounds mediate their actions through specific receptors, of which to date two have been identified, cannabinoid receptor-1 (CB1R)<sup>4</sup> (2) and -2 (CB2R) (3). CB1R is highly expressed in neural tissue (4) but has also been reported to be found in adipose and liver tissue (5). CB2R is found primarily on leukocytes (6). Activation of both receptors inhibits adenylate cyclase, resulting in the decrease of intracellular cAMP (7). It has also been shown that CB2R ligation leads to increased activation of p38 and NF- $\kappa$ B (8).

Two arachidonic acid-containing molecules have been identified as endocannabinoids: anandamide and 2-arachidonyl glyceride (2-AG). Although anandamide was originally discovered in the brain (9), 2-AG has been identified in the gut (10) as well as in brain (11). Anandamide has been shown to be increased in blood drawn from endotoxin-treated humans as compared with that drawn from healthy controls (12).

The role of endocannabinoids in the immune response is still unresolved. It has been found that topical application of 2-AG resulted in dermal inflammation, which was abolished by treatment with a selective CB2R antagonist (13). In contrast, several investigators reported that 2-AG and the CB2R are involved in attenuation of inflammatory reactions and immune responses. Particularly, 2-AG has been shown to inhibit cytokine production, including TNF- $\alpha$  released from both LPS-treated rat microglial cells (14) and murine macrophages (15) and IL-2 secretion in activated murine splenocytes (16). Furthermore, it has been shown that anandamide inhibits lymphocyte proliferation and induces cell death by apoptosis (17). Thus, it is unclear whether endocannabinoids mediate pro- or anti-inflammatory actions by leukocytes and through which receptor subtype they are acting.

The pharmacological agent, AM281 is a potent, selective CB1R antagonist/inverse agonist ( $K_i$  values are 12 and 4200 nM for CB1R and CB2R, respectively) (18). In vivo pharmacology studies with this compound have been shown to reduce mortality in rats following cecal ligation and puncture (CLP) (19). Additionally, AM281 treatment during the rat peritonitis model prevented neurological dysfunction as well as changes in systemic hemodynamics (19, 20). Finally, AM281 administration attenuated serum TNF- $\alpha$  and IL-1 $\beta$  levels up to 3 h following an LPS injection (21). However, there have been no studies reported using cannabinoid receptor-knock out (KO) mice to determine the role of these receptors during the course of sepsis.

Recently, it was reported that a new, highly specific, CB2R agonist, *N*-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-1,4-dihydro-6-methylindeno[1,2-*c*]pyrazole-3-carboxamide (GP1a) was synthesized (22). The  $K_i$  values for this compound are 0.037 and 363 nM for the CB2R and CB1R, respectively, representing a 4 log higher affinity of this compound for the CB2R. Although commercially available, to our knowledge, its use as a tool in a physiological model has not been reported.

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<sup>4</sup>Abbreviations used in this paper: CB1R, cannabinoid receptor-1; CB2R, cannabinoid receptor-2; 2-AG, 2-arachidonyl glyceride; CLP, cecal ligation and puncture; KO, knock out; WT, wild type; BAL, bronchoalveolar lavage; KC, keratinocytederived chemokine.

Sepsis is a complex immune response that involves both proinflammatory and immunosuppressive responses to an infection. The consequences of these responses include multiple organ failure and death. To date, there are very limited clinical therapies for the treatment of sepsis. In this study, we describe a novel role for the endocannabinoid system via its receptor subtype CB2R in sepsis using a well-established murine model. These studies use a genetic loss of function approach to suggest that endocannabinoids play an important role in the functional coordination of the systemic immune response to sepsis. Complementary studies using a pharmacological gain of function approach corroborate those discoveries and further suggest that the CB2R represents a viable therapeutic target for the treatment of sepsis.

## Materials and Methods

### Mice

Breeding pairs of CB2R-KO and C57BL/6J wild-type (WT) mice were purchased from The Jackson Laboratory and bred at the University of Cincinnati. The CB2R-KO mice have been back-crossed seven times to C57BL/6J mice. Home-bred WT and CB2R-KO mice used for the CB2R-KO studies were male. For the studies using GP1a, 6-wk-old, male mice were obtained from The Jackson Laboratory and allowed to acclimate for 1–2 wk. These purchased mice showed a modest, increased susceptibility to sepsis as compared with home-bred mice. All home-bred and purchased mice were housed in standard environmental conditions and were fed with a commercial pellet diet and water ad libitum.

### Cecal ligation and puncture

Male mice between 6–10 wk of age (20–26 g) were used. All experiments involving animals were performed under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Cincinnati. Polymicrobial sepsis was induced similarly as described (23). In brief, the CLP operations were always performed between 8 a.m. and 1 p.m. Normal fed mice were anesthetized to effect by 2.5% isoflurane in oxygen via facemask. The skin was shaved and disinfected. After a 1 cm laparotomy, the latter 80% of the cecum was ligated with a 3–0 silk tie (Ethicon) and punctured once on the anti-mesenterial side with a 23-gauge needle. A small amount of the bowel contents was extruded through the puncture hole to assure a full thickness perforation. Care was taken not to obstruct the bowel, and this was tested after the animals' death. The cecum was replaced in its original location and the midline incision closed by two-layer suture with 4–0 silk. The animals were resuscitated with 1 ml of sterile saline s.c. and kept on a heating blanket with additional oxygen supply for 1 h. Sham-treated controls underwent the same surgical procedures (i.e., laparotomy and resuscitation), but the cecum was neither ligated nor punctured.

### ELISA

Peritoneal fluid was harvested from mice by peritoneal lavage after aseptic preparation of the abdominal wall followed by injection of 9 ml of sterile saline into the peritoneal cavity and aspiration of peritoneal fluid. Serum was isolated from blood collected by cardiac puncture. IL-6, keratinocyte-derived chemokine (KC), MIP-2, MCP-1, and TNF- $\alpha$  levels

were analyzed by ELISA according to the manufacturer's protocol (PeproTech and BioSource).

### **Bacterial counts**

Bacterial counts were performed on aseptically harvested blood by cardiac puncture. Samples were serially diluted in sterile saline and cultured on tryptic soy agar pour plates. Plates were incubated at 37°C for 24 h and colony counts were performed.

### **Tissue histology**

Lung tissues were fixed in 10% neutral-buffered formalin, processed, and then embedded in paraffin for light microscopy. Sections were stained with H&E for histological examination. Control experiments have shown that maximum influx of leukocytes within the first 24 h after CLP occurs at 6 h. Interpretation of the histology was conducted by a pathologist blinded to the sample identity.

### **Bronchoalveolar lavage (BAL)**

BAL was performed 24 h after CLP. In brief, the trachea was exposed and cannulated with a polyethylene tube connected to a syringe. The lungs were washed by flushing with PBS solution through the tracheal cannula as one 1-ml aliquot and the recovered fluid saved as BAL. Protein concentration of the bronchoalveolar lavage fluid was determined by using a BCA Protein Assay Kit (Pierce).

### **Flow cytometry for surface and intracellular staining**

Single cell suspensions were prepared using standard procedures. Cell counts were determined using a Beckman-Coulter AcT 10 cell counter. Cells were resuspended in FACS buffer (PBS with 1% bovine albumin and 0.1% azide). Nonspecific binding to cells was controlled by adding 5% rat serum (Invitrogen) and 1 µg/tube of Fc Block (BD Pharmingen) to the FACS buffer. Myeloid cells were surface stained with the following Abs: Alexa Fluor 700-labeled CD11b (clone M1/70.15, BD Pharmingen); Alexa Fluor 488-labeled anti-neutrophil (clone: 7/4, Serotec); and allophycocyanin-labeled Gr-1 (clone: RB6-8C5, BD Pharmingen). Intracellular staining of peritoneal cells to evaluate phospho-p38 in situ was conducted in fixed, then permeabilized, cells using 90% methanol and stored at -20° C. After labeling with anti-phospho-p38 mAb (clone 28B10, Cell Signaling Technology), cells were analyzed by flow cytometry. LPS-activated cells were treated with 2 µM (final concentration) monensin (Calbiochem) for 4 h to evaluate cytokine production in situ. Cells were surface stained as above then fixed with 2% paraformaldehyde, and permeabilized using saponin buffer (PBS containing 0.1% (w/v) saponin, 0.1% BSA, 0.01 M HEPES, and 0.1% sodium azide). After staining with anti-TNF-α mAb, cells were analyzed by flow cytometry. Samples were run on a Becton Dickinson LSR II and analyzed by FACS Diva software (BD Biosciences). Nonviable cells and lymphocytes were excluded and the myeloid population included using the forward and side scatter parameters. Neutrophils were identified by bright 7/4 and Gr-1 expression, while macrophages were identified by dim 7/4 and Gr-1 expression. These neutrophil and macrophage gates were used to determine CD11b expression, phagocytosis, TNF-α, and phospho-p38 expression.

## Western blots

For Western blot analysis, cells at a concentration of  $15 \times 10^6$  per ml were solubilized in SDS-PAGE sample buffer with 2-ME, briefly sonicated, and heated at 60°C for 20 min. SDS-PAGE was conducted using 12% NuPAGE Novex Bis-Tris gels according to the manufacturer (Invitrogen). Separated proteins were transferred to PVDF membranes. After blocking with 5% nonfat milk in PBS, the membranes were incubated with primary Ab overnight at 4°C. After washing, the membranes were incubated for 2 h at room temperature with the appropriate fluorescein-conjugated secondary Ab (1/200 dilution). After washing, the membranes were incubated for 1 h at room temperature with an anti-FITC alkaline phosphatase-conjugated Ab (1/5,000 dilution). After washing, the membrane was developed using an ECF substrate (Thermo Scientific) and visualized on an Alpha Innotech FluorChem 8900 imager. The membranes were immunoblotted with the following primary Abs: p38, phospho-p38 (Cell Signaling Technology) and CB2R (Alexis Biochemicals).

## Pharmacologicals

Gp1a was purchased from Tocris Bioscience (Tocris Bioscience). Stock solution was freshly prepared in the corresponding buffer. Optimization experiments using bacteremia and serum IL-6 as endpoints were used to determine the 2.5 mg/kg per day dose of GP1a.

## Peripheral minipumps

For the survival experiments involving chronic peripheral treatment with GP1a, osmotic minipumps (Alzet Osmotic Pumps; Durect) were filled in the evening before surgery and primed in a water bath overnight at 37°C. Animals were randomized and were anesthetized to effect by 2.5% isoflurane in oxygen via facemask. Minipumps were implanted s.c. in the upper back directly after CLP pumping at a rate of 0.2  $\mu$ l/h for 14 days. The implantation of the pumps induces modestly increased tissue damage at the time of the CLP surgery as compared with the studies of CLP alone with CB2R-KO mice.

## Total lung single cell suspension preparation

Lungs were obtained from sham- or CLP-operated mice 6 or 24 h after surgeries. The single cell suspension was obtained using gentleMACS equipment (Miltenyi Biotec) using the manufacturer's "Preparation of single-cell suspensions from mouse lung with Collagenase D treatment" protocol. Cells were counted and prepared for flow cytometry as described above.

## Statistics

Statistical comparisons were performed using Kaplan Meier LogRank (survival), Student *t* test (two groups), or Tukey's test and ANOVA (more than two groups). StatView (SAS Institute) and GraphPad Prism 3.0 were used for statistical analyses. A value of  $p < 0.05$  was considered statistically significant.

## Results

### Increased mortality in CB2R-KO after CLP-induced sepsis

To explore whether the function of the CB2R affects sepsis-induced mortality, the survival of CB2R-KO and WT mice was assessed over a 10-day period after CLP. WT mice had a significantly higher survival compared with mice lacking CB2Rs (54% vs 22%, respectively, Fig. 1A). As the level of IL-6 in the circulation 6 h after CLP is known to be predictive of outcome (24), serum from WT and CB2R-KO mice was measured for IL-6 6 h after CLP. As shown in Fig. 1B, levels of IL-6 were significantly higher in the CB2R-KO mice as compared with the WT control group. Twenty-four hours after CLP, we also found significantly increased serum IL-6 in CB2R-KO mice (Fig. 1B). The systemic bacterial load was greater by an order of magnitude in the CB2R-KO mice compared with WT mice (Fig. 1C). Sham-operated mice had no detectable serum IL-6 or bacteremia at 6 or 24 h (data not shown). Thus, these data show that the CB2R enhances survival and reduces bacterial spread during sepsis.

### CB2R deficiency increases lung injury during CLP-induced sepsis

We hypothesized that the increased CLP-induced mortality observed in the CB2R-KO mice would be associated with increased organ injury. To test this hypothesis, the lung and liver were examined 24 h following CLP. We observed no differences in liver injury as determined by ALT and histology (data not shown). As shown by histology, the lung had increased leukocyte infiltration and edema in the CB2R-KO mice as compared with WT mice (Fig. 2A). In a separate series of experiments, we collected BAL from CB2R-KO and WT mice 24 h following CLP. The BAL from CB2R-KO mice had increased bacterial load (Fig. 2B), numbers of neutrophils (Fig. 2, C and D), and permeability (Fig. 2E). Taken together, our data suggest that the CB2R mediates injury to the lung during sepsis induced by CLP.

### Neutrophil function and activation is decreased in septic CB2R-KO mice

It has been widely reported that dysregulated neutrophil function contributes to tissue damage observed during the course of sepsis and acute respiratory distress syndrome. Therefore, we next examined whether the CB2R mediates neutrophil recruitment and activation during the early stages of CLP-induced sepsis. First, we observed that the serum and peritoneal lavage levels of the murine neutrophil chemotactics, KC and MIP-2, were increased in CB2R-KO mice as compared with WT controls (Fig. 3, A and B). However, we did not observe significant differences in the MCP-1 (data not shown). We next enumerated peritoneal cells and found that the CB2R-KO mice had increased numbers of neutrophils but not macrophages (Fig. 3C). Additionally, we observed that neutrophils from WT mice had increased expression of the activation marker, CD11b (Fig. 3D). The MAPK p38 is known to play a key role in neutrophil function and priming. Additionally, the CB2R is known to mediate p38 activity. To determine whether the CB2R alters neutrophil p38 activity during sepsis, we isolated peritoneal cells from septic WT and CB2R-KO mice. We observed that these cells had no significant difference in total p38 expression from the two groups of mice. However, the cells from the WT mice had a 65% increase in p38 phosphorylation ( $p = 0.02$ )

as determined by densitometry (Fig. 3E). Thus, neutrophil numbers are increased in the absence of the CB2R, yet the cell's activation and p38 activity is decreased.

### Increased production of TNF- $\alpha$ by CB2R agonist

To determine the impact the CB2R agonist upon myeloid cells, we first isolated peritoneal cells from sham-operated and septic WT mice. By Western blot, we observed that these cells expressed the CB2R through the first 24 h of sepsis (Fig. 4A). We did not observe significant differences in CB2R expression during this time period. To further determine the impact of CB2R activation on myeloid cell activity, we added LPS to untreated or Gp1a-treated peritoneal cells isolated from sham-operated mice, and monitored for TNF- $\alpha$  production (Fig. 4, B and C) and accumulation (Fig. 4D). In this study, treatment with the CB2R-agonist, GP1a, resulted in increased production of TNF- $\alpha$  on a per cell basis and TNF- $\alpha$  accumulation over a 24-h period. Thus, addition of GP1a increased TNF- $\alpha$  production by cells at the site of infection in our sepsis model.

### Systemic CB2R agonist treatment improves neutrophil function, decreases tissue damage, and improves survival to CLP-induced sepsis

As our data show that CB2R-deficiency causes decreased neutrophil function, increased lung injury, and death after CLP, we sought to determine whether stimulation with a highly specific CB2R agonist might have beneficial effects. WT mice underwent CLP followed immediately by the implantation of a minipump that delivered a total of 50  $\mu$ g/day of the CB2R agonist, GP1a. Mice receiving the GP1a as compared with mice receiving the equivalent dose of ethanol vehicle (20  $\mu$ l/24 h) had a modest increase in survival (10 vs 0%, respectively) as well as mean survival time (91 vs 52 h, respectively) (Fig. 5A). Additionally, we found that GP1a treatment reduced CLP-induced serum IL-6 levels and bacteremia (Fig. 5, B and C). A comparison of Fig. 1A and Fig. 5A demonstrates there are mortality differences between the two control groups. To verify that these differences were due to the use of ethanol as a vehicle, untreated and ethanol-treated mice underwent CLP and were monitored for survival (supplemental Figure).<sup>5</sup> The results show that the increased mortality between the two control groups is due to ethanol treatment, consistent with previous reports (25, 26).

We next assessed the ability of GP1a treatment to reduce pulmonary damage. In contrast to CB2R-KO mice, we did not obtain significant numbers of cells in the BAL of GP1a-treated mice (data not shown). We observed that GP1a treatment reduced edema as compared with vehicle-treated mice after 24 h by histology (Fig. 6A). Next, we examined leukocytes from the entire lung at 6 and 24 h after CLP. We found no significant differences in CD4, CD8,  $\gamma\delta$ , or NK T cell as well as macrophage numbers between untreated and GP1a-treated mice (data not shown). However, 6 h after inflicting sepsis, we did observe a significant 4-fold increase in neutrophils in vehicle-treated lungs as compared with GP1a-treated lungs (Fig. 6B). We further observed a modest increase in CD11b expression on neutrophils isolated from the vehicle-treated lungs at this time point (Fig. 6C). Additionally, the BAL from the untreated mice had increased permeability (Fig. 6D). Finally, we isolated peritoneal

<sup>5</sup>The online version of this article contains supplemental material.

neutrophils at this time point and determined the oxidative burst with increasing doses of GP1a. In this study, we observed that GP1a treatment resulted in significant oxidative burst reduction (Fig. 6E). Thus, GP1a treatment resulted in decreased lung tissue damage and this was associated with decreased neutrophil numbers and oxidative burst.

To further determine whether GP1a treatment affected systemic and peritoneal chemokine expression, we screened plasma and peritoneal lavage fluid for KC and MIP-2 (Fig. 7, A and B). The serum expression of both KC and MIP-2 were both decreased by GP1a treatment, while serum MCP-1 concentrations were not altered (data not shown). A similar, but not significant, trend of chemokine concentrations was observed in the peritoneal lavage. The number of peritoneal neutrophils was decreased at the site of infection in the GP1a-treated mice (Fig. 7C). Additionally, neutrophils isolated from GP1a-treated mice were more activated as determined by elevated CD11b expression (Fig. 7D). To determine whether GP1a alters p38 activity in neutrophils during sepsis, we isolated peritoneal cells from septic WT and CB2R-KO mice and observed that neutrophils from the GP1a mice had increased phosphorylated p38 (Fig. 7E). Thus, pharmacological treatment with the highly specific CB2R agonist resulted in decreased numbers of neutrophils at the site of infection with increased activation.

## Discussion

Sepsis continues to be a prevalent clinical problem with few efficient pharmacological treatment options. Despite extensive efforts in both the clinical and laboratory settings, the molecular mechanisms of this disease state are only partially understood. The polymicrobial infection induced by cecal ligation in mice represents a sepsis model that mimics many features observed in patients in the intensive care unit (23). In this study, the CLP model was used to evaluate novel gain-of-function and loss-of-function experiments aimed at elucidating the function of CB2R during sepsis.

The genetic deletion of CB2R resulted in increased susceptibility to infection after CLP. Consistent with this observation, we found in mouse mutants deficient for CB2R increased serum IL-6 and increased bacterial load, all independent predictors of mortality during sepsis. Additionally, we observed that lung tissue damage was increased in the septic CB2R-KO mice. This is critical point as respiratory dysfunction proceeds heart, liver, and kidney failure in the development of multiorgan failure (27). Finally, we show that CB2R deficiency results in increased neutrophils with decreased activation and functionality. Significantly, CB2R gain of function during sepsis reversed the above phenotype and ultimately decreased susceptibility to sepsis.

It has been reported that the cannabinoid antagonist AM281 reduced the mortality rate and neurologic dysfunction after CLP in rats (19). This antagonist has a much higher affinity for CB1R as compared with CB2R. CB1Rs are predominantly located in the brain and nerves (2), while the majority of CB2Rs are found on immune cells (3). We speculate that selective antagonism of the CB1R in the brain may be beneficial during sepsis. This concept of the CNS involvement in the immune response to sepsis is consistent with a series of reports by Tracey and colleagues (28). They determined that stimulation of the vagus nerve inhibits



proinflammatory synthesis in WT mice through the nicotinic acetylcholine receptor  $\alpha 7$  subunit found on macrophages. In contrast, we found that the genetic deletion of the CB2R had the opposite effect upon survival as compared with the pharmacological inactivation of the CB1R.

Although the CB2R is found on multiple immune cells, we speculate that in this model, the CB2R on neutrophils is playing a key role. Properly regulated neutrophils clear bacteria while minimizing collateral tissue injury. Specifically, during sepsis, it has been demonstrated that granulocyte depletion decreases lung injury (29, 30) while decreased neutrophil recruitment was associated with increased survival (31). Further, the timing of neutrophil depletion is critical in that depletion at the onset of sepsis increases tissue damage, while depletion 12 h after sepsis decreases tissue damage (32). The data reported in this study complements these reports in that targeting of the CB2R can decrease the absolute number of pulmonary and peritoneal neutrophils, while decreasing mortality and tissue damage.

The intracellular concentration of cAMP is a key regulator of neutrophil function. In general, high levels of cAMP inhibit neutrophil activation and function, while decreased levels enhance activation. It has been established that ligation of the CB2R results in decreased intracellular cAMP and that endocannabinoids are elevated during sepsis and stress (33, 34). Therefore, it is likely that in CB2R-KO mice, cAMP will be increased, while in mice where the CB2R agonist was added, intracellular cAMP would be decreased. Studies have demonstrated that increased cAMP in neutrophils resulted in reduced oxidative burst and phagocytosis (35, 36). Consistent with these reports, we find that the bacterial load from mice lacking CB2R (increased cAMP) is increased, while the bacterial load from CB2R agonist-treated mice is decreased. Studies to determine the impact of CB2R gain- and loss-of-function upon phagocytosis is currently underway. Additionally, we observed a decrease of p38 MAP kinase activation in the CB2R-KO mice and an increase in the GP1a-treated mice. This may also represent an important mechanism responsible for proper neutrophil function or priming such that bacterial clearance is increased.

The immunopathogenesis of CLP-induced sepsis underlies a complex regulation regarding mediators and pathways involved in neutrophil mobilization and function. We show in this study, for the first time, that CB2R plays a novel role in neutrophil recruitment and bacterial clearance thereby acting as a major regulatory pathway of mortality in sepsis. Therefore, the use of CB2R agonists represents a novel therapeutic strategy.

## Supplementary Material

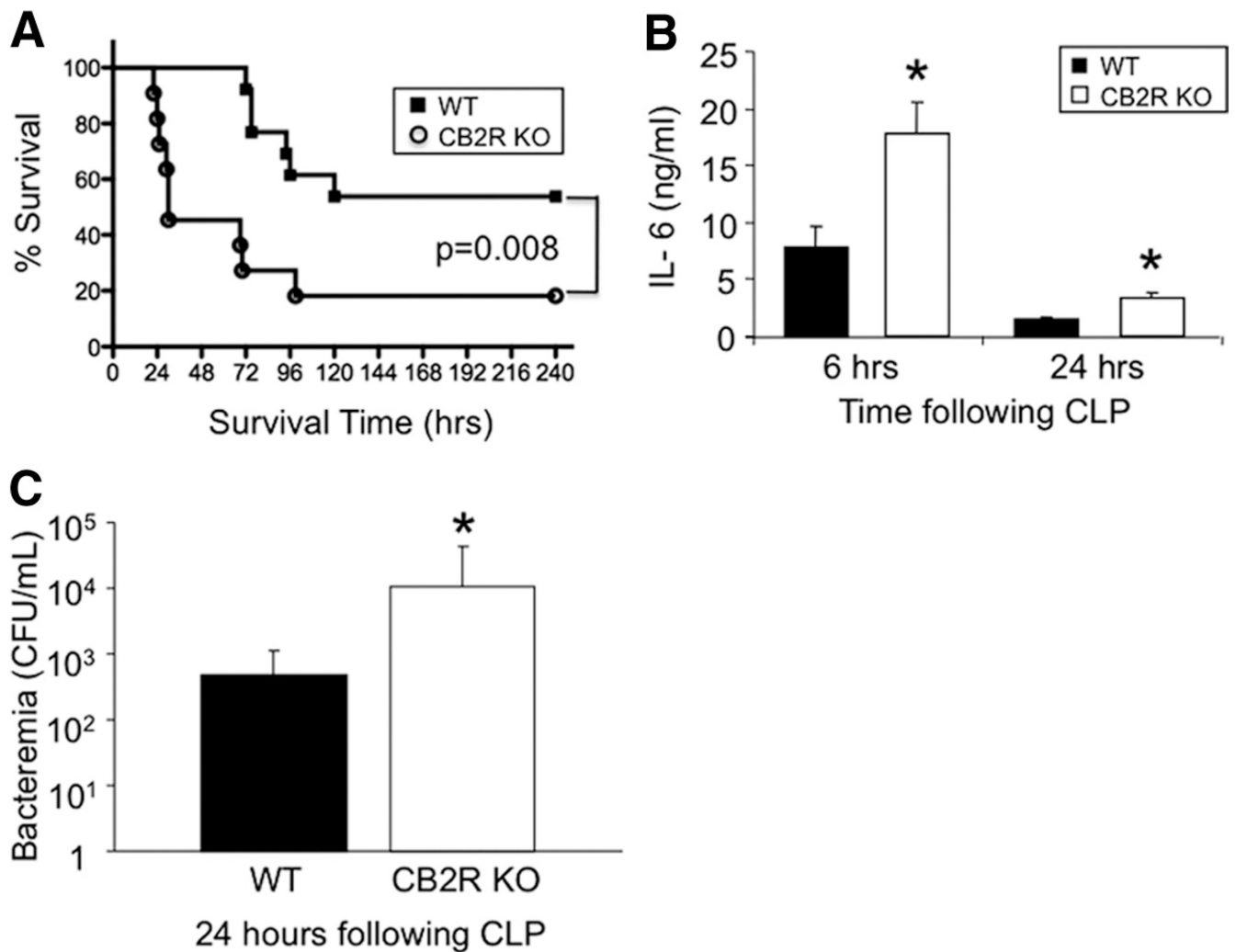
Refer to Web version on PubMed Central for supplementary material.

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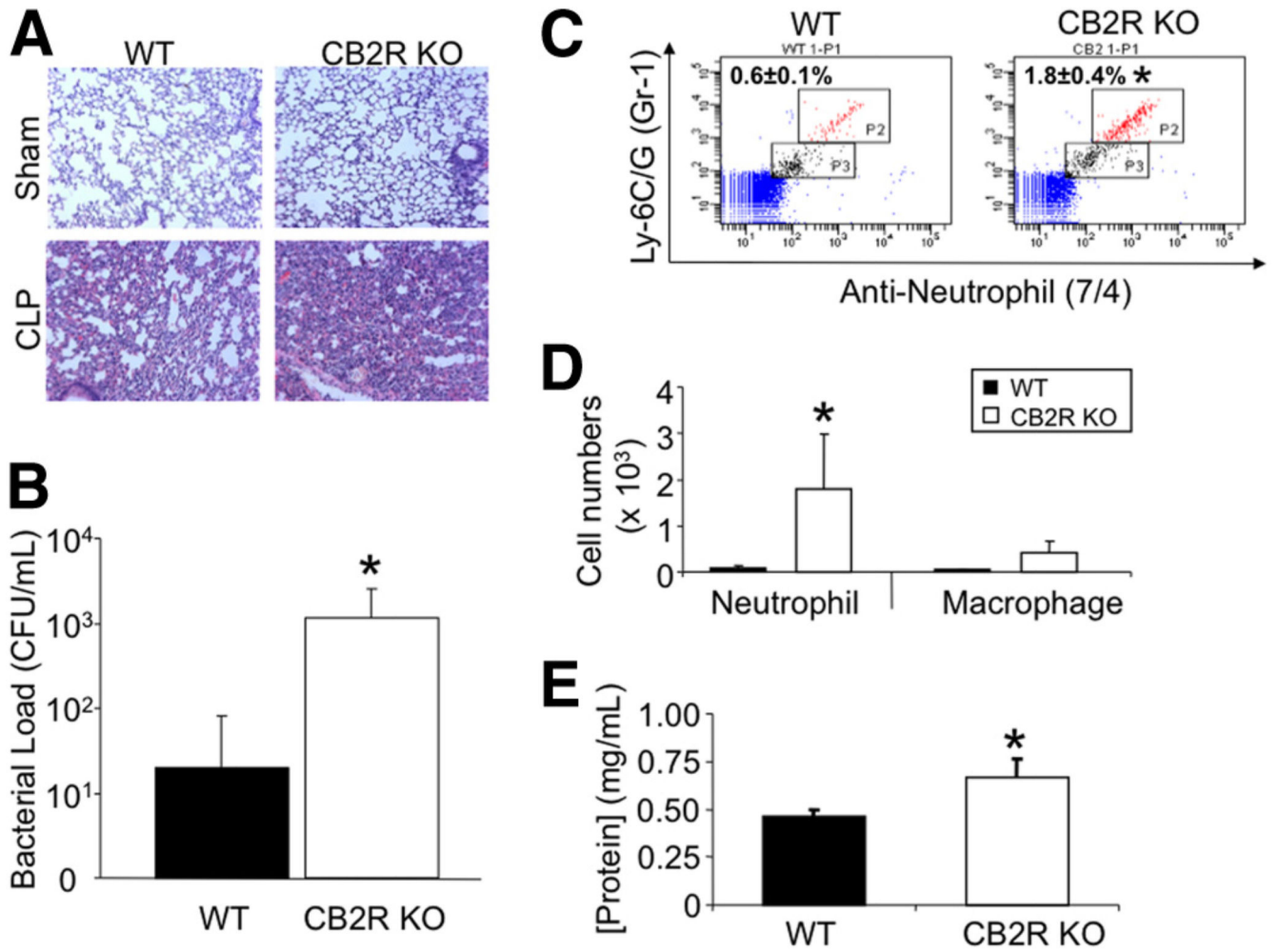
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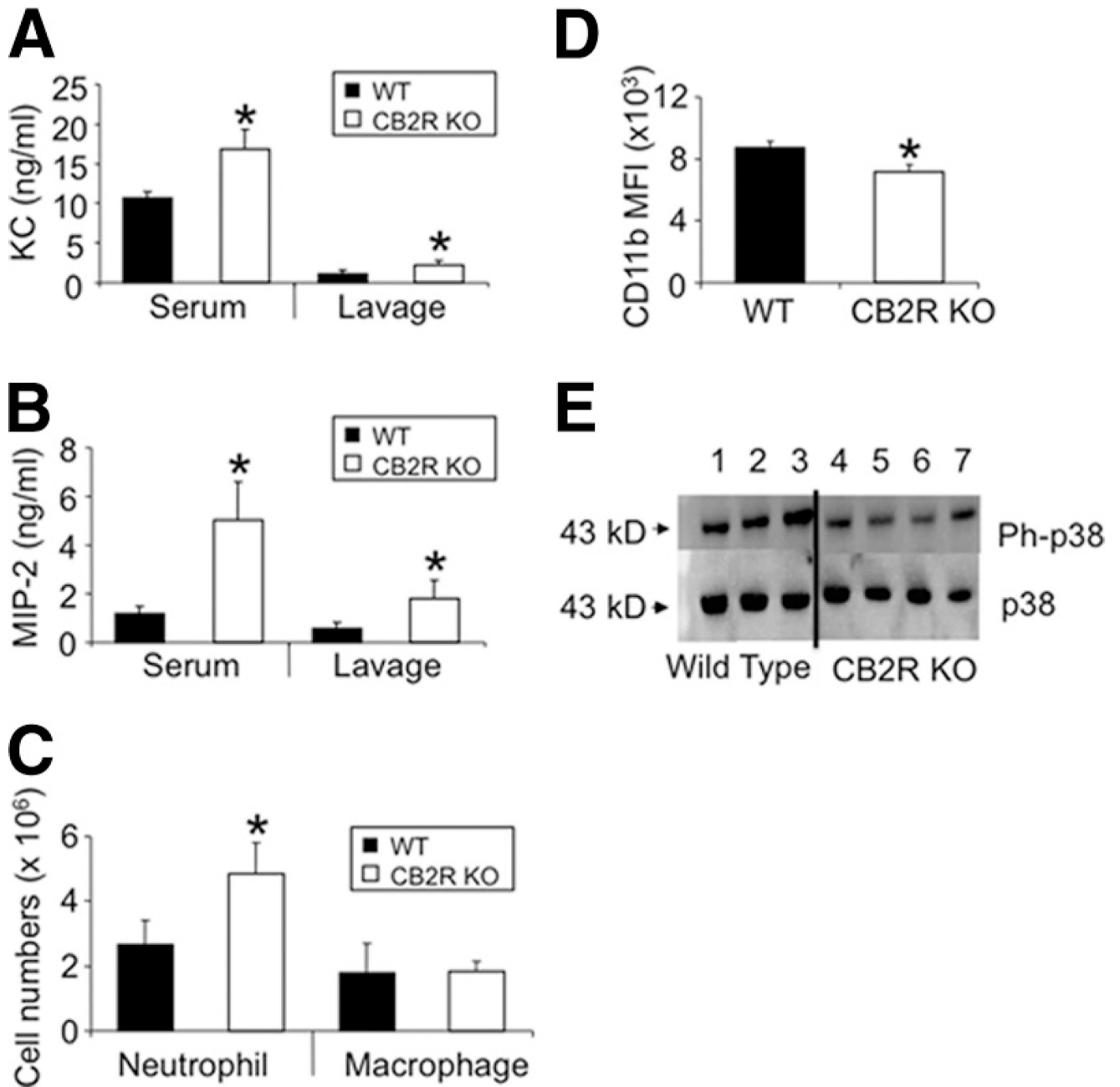
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**FIGURE 1.**

CB2Rs decrease mortality, IL-6, and bacteremia after CLP. *A*, WT ( $n = 13$ ) and CB2R-KO mice ( $n = 11$ ) underwent CLP and were monitored for survival for 10 days. *B*, Serum IL-6 levels 6 and 24 h after CLP. IL-6 cytokine levels (ng/ml) were determined using ELISA. The sample size equals four to six per group. *C*, Blood bacterial load was determined 24 h following CLP. The sample size equals 15–16 per group. Data expressed as mean  $\pm$  SEM. \*,  $p < 0.05$  as compared with WT.

**FIGURE 2.**

The CB2R decreases lung injury during sepsis. WT ( $n = 6$ ) and CB2R-KO mice ( $n = 6$ ) underwent CLP. *A*, H&E-stained lung sections of CB2R-KO or WT mice 24 h after a sham- or CLP-operation. *B*, Lung BAL bacterial load was determined 24 h following CLP. The sample size equals seven per group. *C*, Representative FACS plot and *D*, Enumeration of neutrophils (P2 gate) and macrophages (P3 gate) isolated from the BAL of septic mice. *E*, Lung permeability as determined by protein recovered in the BAL. The sample size equals 10–11 per group. Data expressed as mean  $\pm$  SEM. \*,  $p < 0.05$  as compared with WT.

**FIGURE 3.**

Neutrophil recruitment, activation, and p38 activity are mediated by the CB2R during sepsis. Serum and peritoneal lavage were collected 24 h following CLP. The serum and lavage levels of *A*, KC, and *B*, macrophage inflammatory protein-2 (MIP-2), were determined by ELISA. The sample size equals 10–11 per group. Cells from peritoneal lavages were collected 24 h following CLP. *C*, Peritoneal neutrophils and macrophages were enumerated by flow cytometric analysis. The sample size equals 10–11 per group. *D*, The CD11b expression on neutrophils as analyzed by flow cytometry. *E*, Western blots showing p38 and phospho-p38 (Ph-p38) expression on peritoneal cells. Lanes 1–3 and 4–7 represent samples

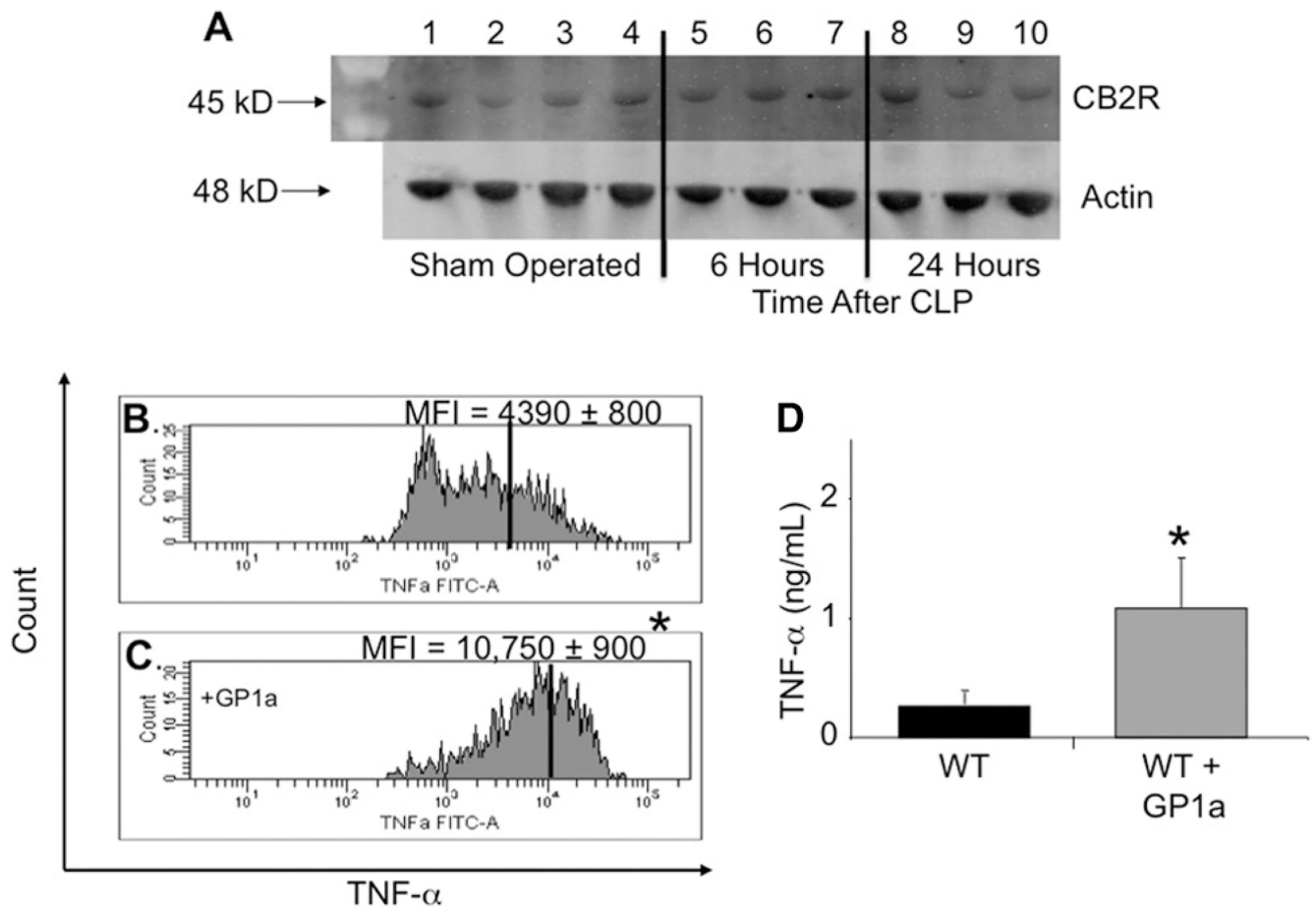
from septic WT and CB2R KO mice, respectively. Data expressed as mean  $\pm$  SEM. \*,  $p < 0.05$  as compared with WT.

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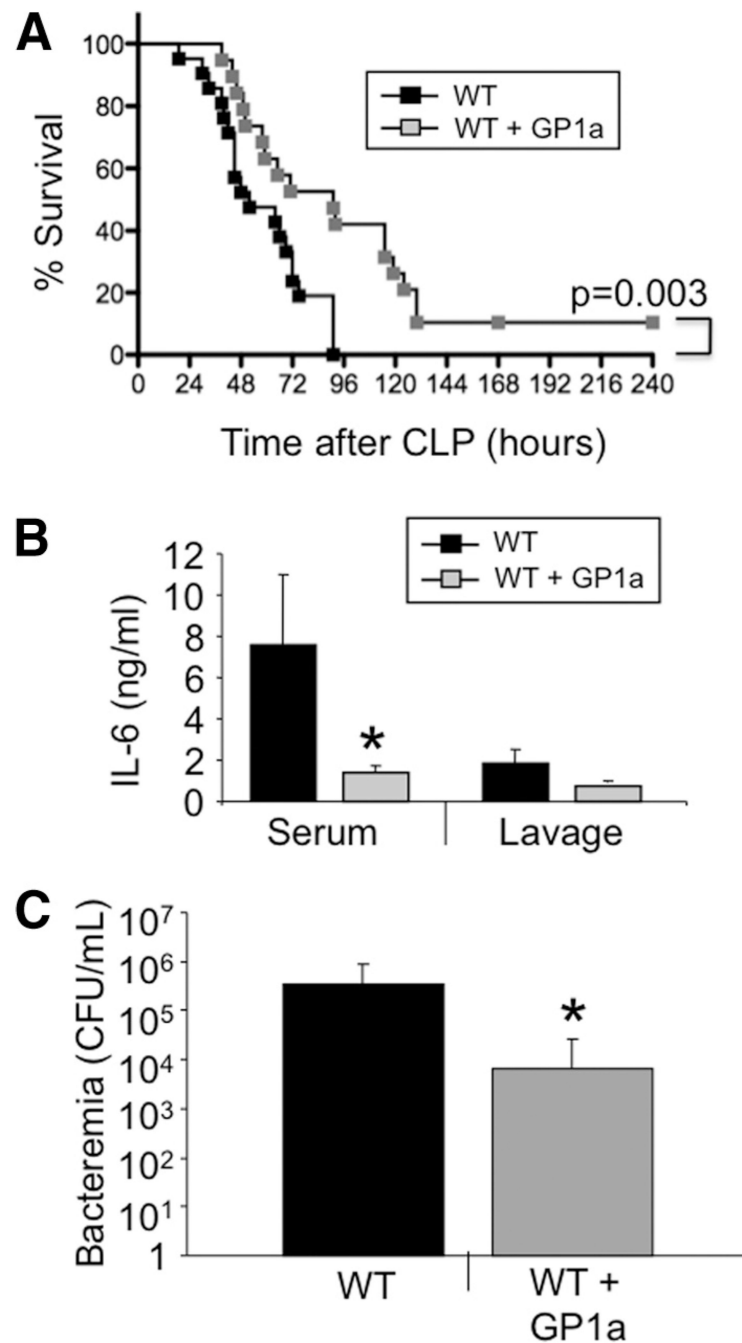
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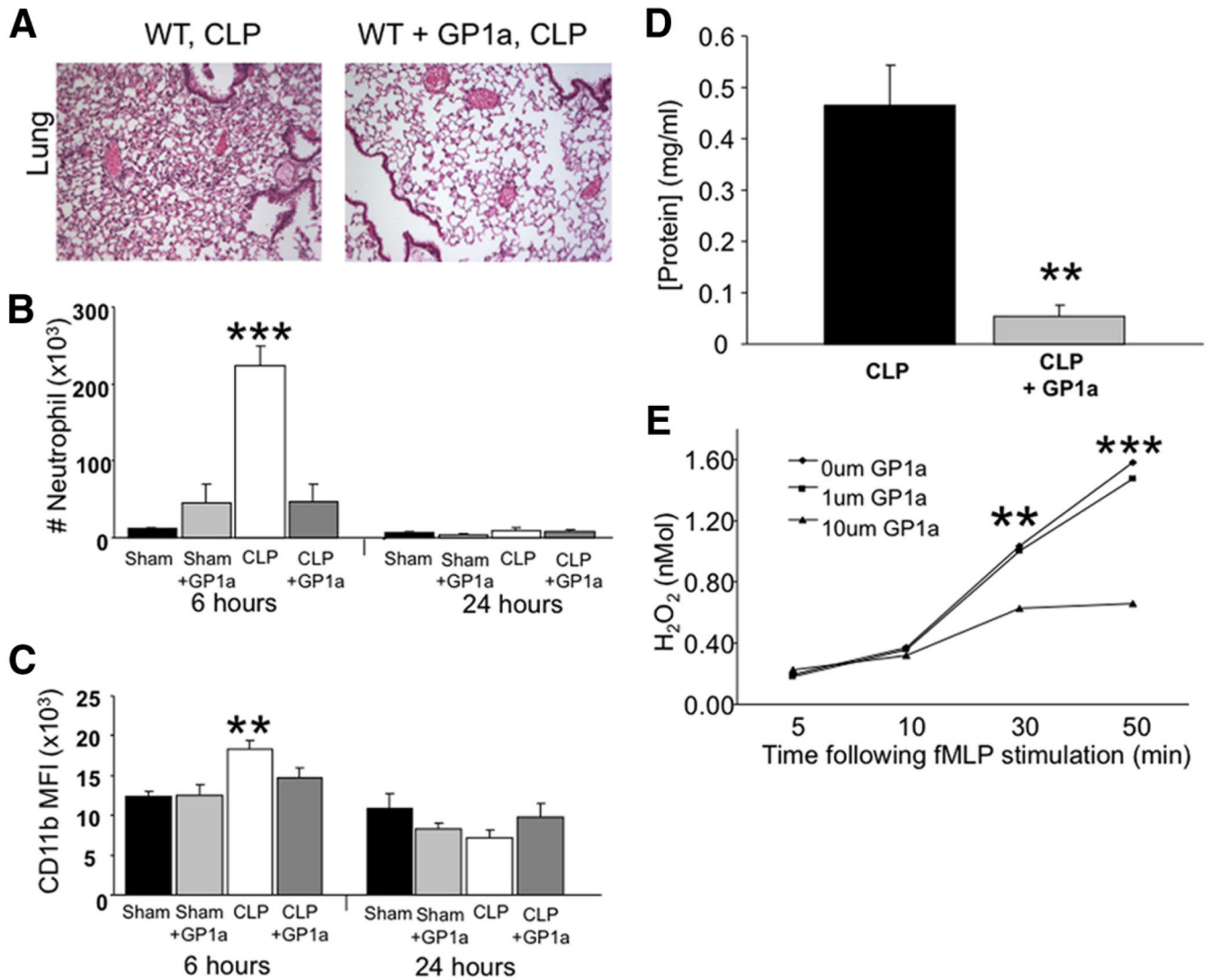
**FIGURE 4.**

Activation of the CB2R increases neutrophil TNF- $\alpha$  production. *A*, Blots showing CB2R expression on peritoneal cells obtained from nonseptic and septic mice. *Lanes 1–4* are peritoneal samples from sham-operated mice, while *lanes 5–7* and *8–10* represent peritoneal samples from CLP-operated mice 6 and 24 h following surgery, respectively. Peritoneal cells were isolated from sham-operated mice. The cells were activated with LPS in vitro and additionally treated with vehicle (*B*) or Gp1a (*C*). TNF- $\alpha$  production was determined by intracellular cytokine flow cytometric analysis. The sample size for the mean fluorescent intensity (MFI) equals four per group  $\pm$  SEM. *D*, TNF- $\alpha$  accumulation from in vitro LPS activated vehicle-treated and Gp1a-treated peritoneal cells from sham-operated mice was determined by ELISA after 24 h. The sample size equals 10–11 per group. Data expressed as mean  $\pm$  SEM. \*,  $p < 0.05$  as compared with WT.

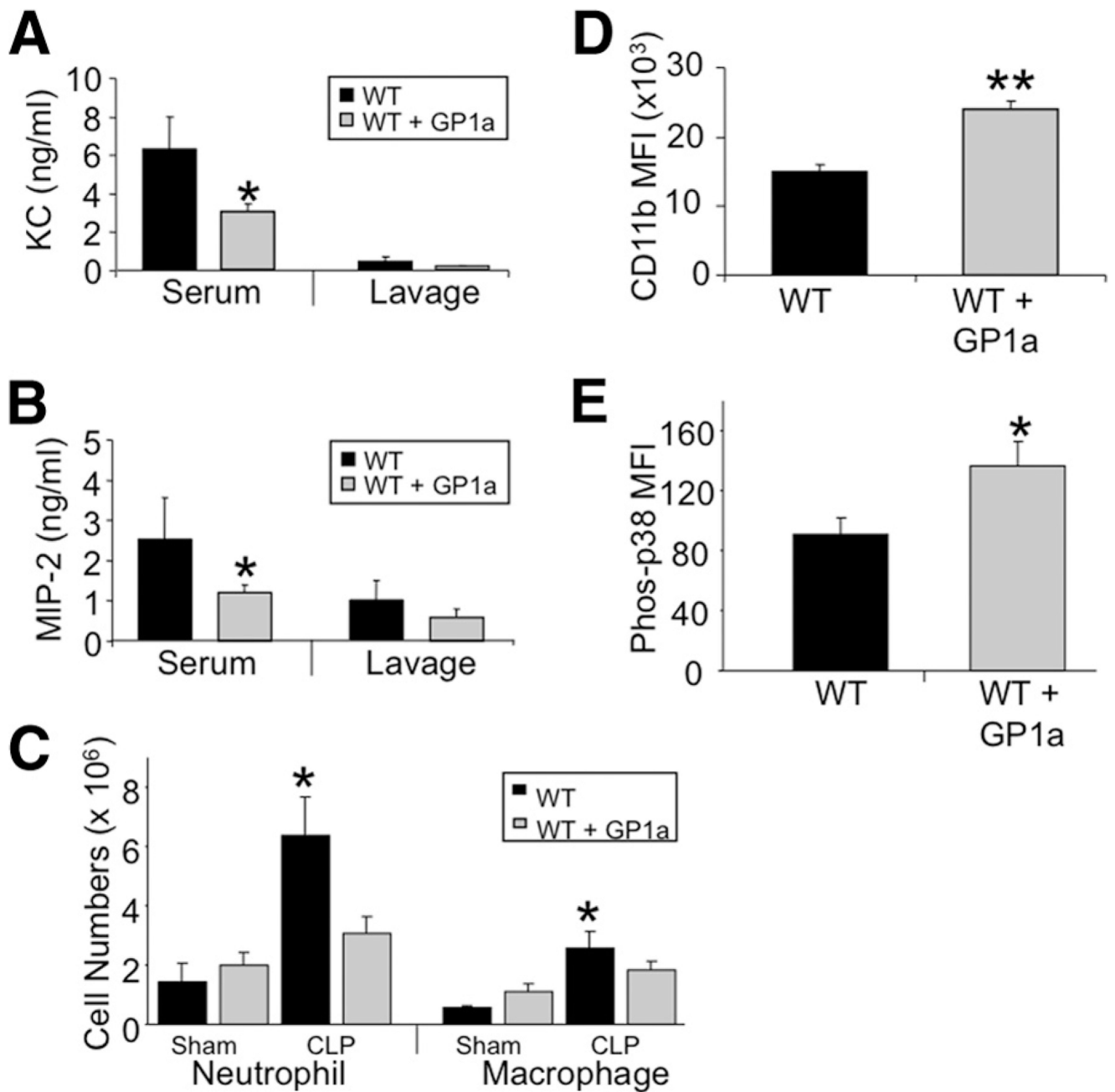


**FIGURE 5.**

Systemic treatment with the CB2R agonist decreases mortality, IL-6 and bacteremia following CLP. **A**, WT mice treated with the CB2R agonist ( $n = 20$ ), GP1a, or vehicle ( $n = 21$ ) underwent CLP and were monitored for survival. The data presented are three experiments combined. **B**, Serum and peritoneal IL-6 (ng/ml) levels 24 h after CLP were determined using ELISA. **C**, Blood bacterial load was determined 24 h following CLP. **B** and **C** sample size equals 11 per group. Data expressed as mean  $\pm$  SEM. \*,  $p < 0.05$  as compared with WT.

**FIGURE 6.**

Systemic treatment with the CB2R agonist decreases neutrophil recruitment to the lung and pulmonary injury. WT ( $n = 6$ ) and GP1a-treated mice ( $n = 6$ ) underwent CLP. *A*, Representative H&E-stained lung sections of WT mice treated with GP1a or vehicle 24 h after CLP. *B*, Total lung tissue neutrophils were acquired via GentleMACS of lungs from sham treated with vehicle, sham treated with GP1a, and WT mice treated with GP1a or vehicle 24 h after CLP. GentleMACS homogenate was layered on Lympholyte M and the cellular layer obtained was FACS stained for enumeration and *C*, CD11b expression by flow cytometric analysis. The sample size equals four to six per group. *D*, Lung permeability as determined by protein recovered in the BAL of WT mice treated with GP1a or vehicle 48 h after CLP. The sample size equals six per group. *E*, Peritoneal lavage cell oxidative burst from WT mice 6 h after CLP when treated with 0, 1, and 10  $\mu$ m GP1a in vitro. The sample size equals five mice per group. Data expressed as mean  $\pm$  SEM. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  as compared with WT.

**FIGURE 7.**

Neutrophil recruitment, activation and p38 activity are mediated by the CB2R agonist during sepsis. Serum and peritoneal lavage were collected 24 h following CLP. The serum and lavage levels of KC (A), and macrophage inflammatory protein-2 (MIP-2) (B), were determined by ELISA. The sample size equals eight to nine per group. Cells from peritoneal lavages were collected 24 h following CLP. C, Peritoneal neutrophils and macrophages were enumerated by flow cytometric analysis. The sample size equals eight per nine per group. D, The CD11b expression on neutrophils as analyzed by flow cytometry. E, The expression of

p38 and phospho-p38 (Ph-p38) on peritoneal neutrophils as determined by flow cytometry. Data expressed as mean  $\pm$  SEM. \*,  $p < 0.05$ ; \*\*,  $p < 0.05$  as compared with WT.

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