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*J Immunol* published online 12 June 2009
http://www.jimmunol.org/content/early/2009/06/12/jimmunol.0900203

**Supplementary Material**  http://www.jimmunol.org/content/suppl/2009/06/18/jimmunol.0900203.DC1

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The Cannabinoid Receptor 2 Is Critical for the Host Response to Sepsis

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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. The Journal of Immunology, 2009, 183: 499–505.

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)4 (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-κB (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 derived chemokine.

These compounds mediate their actions through specific receptors, of which to date two have been identified, cannabinoid receptor-1 (CB1R) and -2 (CB2R). CB1R is highly expressed in neural tissue but has also been reported to be found in adipose and liver tissue. CB2R is found primarily on leukocytes. Activation of both receptors inhibits adenylate cyclase, resulting in the decrease of intracellular cAMP. It has also been shown that CB2R ligation leads to increased activation of p38 and NF-κB.

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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 neuroendocrine dysfunction as well as changes in systemic hemodynamics (19, 20). Finally, AM281 administration attenuated serum TNF-α and IL-1β 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-methylindenol[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

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0900203

The Journal of Immunology

Received for publication January 22, 2009. Accepted for publication May 4, 2009.

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1 This work was supported by the Shriners of North America Project No. 8904. The project described was supported by Award Number R01GM072760 from the National Institute of General Medical Sciences. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health.

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4 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, keratinocyte-derived chemokine.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0900203

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commercially available, to our knowledge, its use as a tool in a physiological model has not been reported.

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 pelleted 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 30% 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 isolated from blood collected by cardiac puncture. IL-6, keratinocyte-derived chemokine (KC), MIP-2, MCP-1, and TNF-α were isolated from blood collected by cardiac puncture. IL-6, keratinocyte-derived chemokine (KC), MIP-2, MCP-1, and TNF-α levels were measured by ELISA according to the manufacturer’s protocol (PeproTech and BioSource).

Peritonitis

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-α 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 the 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 alkaline phosphatase-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 × 10⁶ 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% Nu-PAGE 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; Duract) 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% isoflu- rane in oxygen via facemask. Minipumps were implanted s.c. in the upper back directly after CLP pumping at a rate of 0.2 μl/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 a predictor 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. 1C). 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. 2C 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

![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 ± 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 ± SEM. *, *p* < 0.05 as compared with WT.]
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. 3, C). Additionally, we observed that neutrophils from WT mice had increased expression of the activation marker, CD11b (Fig. 3, D). 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 ($p < 0.05$) as determined by densitometry (Fig. 3, E). 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. 4, A). 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. 4, D). In this study, treatment with
the CB2R-agonist, GP1a, resulted in increased production of TNF-α on a per cell basis and TNF-α accumulation over a 24-h period. Thus, addition of GP1a increased TNF-α 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 μg/day of the CB2R agonist, GP1a. Mice receiving the GP1a as compared with mice receiving the equivalent dose of ethanol vehicle (20 μl/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 Figs. 1A and 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). 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, γδ, or NK T cell as well as macrophage numbers between untreated and GP1a-treated mice (data not shown). However, 6 h

![FIGURE 6.](image)

![FIGURE 7.](image)
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 form 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 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 decreased lung tissue damage and this was associated with decreased neutrophil numbers and oxidative burst.

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 α7 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.

Disclosures

The authors have no financial conflict of interest.

References


Supplemental Figure: Ethanol decreases mortality after CLP. A) Untreated wild-type (n=5) and ethanol-treated mice (n=6) underwent CLP and were monitored for survival for 10 days. Ethanol-treated mice were exposed to 20 µL over a 24-hour period. p=0.003.
Supplemental Figure