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# Pivotal Role of the $\alpha_{2A}$ -Adrenoceptor in Producing Inflammation and Organ Injury in a Rat Model of Sepsis

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## Abstract

**Background:** Norepinephrine (NE) modulates the responsiveness of macrophages to proinflammatory stimuli through the activation of adrenergic receptors (ARs). Being part of the stress response, early increases of NE in sepsis sustain adverse systemic inflammatory responses. The intestine is an important source of NE release in the early stage of cecal ligation and puncture (CLP)-induced sepsis in rats, which then stimulates TNF- $\alpha$  production in Kupffer cells (KCs) through the activation of the  $\alpha_2$ -AR. It is important to know which of the three  $\alpha_2$ -AR subtypes (i.e.,  $\alpha_{2A}$ ,  $\alpha_{2B}$  or  $\alpha_{2C}$ ) is responsible for the upregulation of TNF- $\alpha$  production. The aim of this study was to determine the contribution of  $\alpha_{2A}$ -AR in this process.

**Methodology/Principal Findings:** Adult male rats underwent CLP and KCs were isolated 2 h later. Gene expression of  $\alpha_{2A}$ -AR was determined. In additional experiments, cultured KCs were incubated with NE with or without BRL-44408 maleate, a specific  $\alpha_{2A}$ -AR antagonist, and intraportal infusion of NE for 2 h with or without BRL-44408 maleate was carried out in normal animals. Finally, the impact of  $\alpha_{2A}$ -AR activation by NE was investigated under inflammatory conditions (i.e., endotoxemia and CLP). Gene expression of the  $\alpha_{2A}$ -AR subtype was significantly upregulated after CLP. NE increased the release of TNF- $\alpha$  in cultured KCs, which was specifically inhibited by the  $\alpha_{2A}$ -AR antagonist BRL-44408. Equally, intraportal NE infusion increased TNF- $\alpha$  gene expression in KCs and plasma TNF- $\alpha$  which was also abrogated by co-administration of BRL-44408. NE also potentiated LPS-induced TNF- $\alpha$  release via the  $\alpha_{2A}$ -AR in vitro and in vivo. This potentiation of TNF- $\alpha$  release by NE was mediated through the  $\alpha_{2A}$ -AR coupled G $\alpha_i$  protein and the activation of the p38 MAP kinase. Treatment of septic animals with BRL-44408 suppressed TNF- $\alpha$ , prevented multiple organ injury and significantly improved survival from 45% to 75%.

**Conclusions/Significance:** Our novel finding is that hyperresponsiveness to  $\alpha_2$ -AR stimulation observed in sepsis is primarily due to an increase in  $\alpha_{2A}$ -AR expression in KCs. This appears to be in part responsible for the increased proinflammatory response and ensuing organ injury in sepsis. These findings provide important feasibility information for further developing the  $\alpha_{2A}$ -AR antagonist as a new therapy for sepsis.

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## Introduction

Sepsis and septic shock are complications and considered to be major causes of morbidity and mortality in patients with severe trauma, burns, or blood loss [1]. Tissue-fixed macrophages such as the hepatic Kupffer cells (KCs) are involved in inflammatory and metabolic responses to sepsis [2,3]. The impairment of hepatocellular function observed in early sepsis appears to be due to upregulation of proinflammatory cytokines such as TNF- $\alpha$  [4,5]. We [6] and Kovarik *et al.* [7] reported that systemic levels of norepinephrine (NE) increased significantly after the onset of sepsis, induced by cecal ligation and puncture (CLP). Enterectomy prior to the onset of sepsis markedly reduced circulating levels of NE, showing that the gut is a major source of NE in sepsis [8]. The

catecholamines NE and epinephrine mediate their physiological responses through a group of adrenergic receptor (AR) subtypes [9]. Studies have suggested that NE at concentrations similar to that found in septic animals (~20 nM) mainly stimulate  $\alpha_2$ -adrenergic receptors (ARs). In our previous studies, we reported that gut-derived NE upregulates TNF- $\alpha$  production in KCs through the  $\alpha_2$ -adrenergic pathway [9]. This is a novel finding, since the immunological effect of the sympathetic nerve activity and the adrenal epinephrine was previously considered to be anti-inflammatory through the activation of  $\beta$ -ARs on leukocytes [10].  $\alpha_2$ -ARs are G-protein coupled receptors that mediate the central and peripheral actions of the primary sympathetic neurotransmitter, NE and the adrenal hormone epinephrine through the intracellular suppression of cAMP [11]. However, it remains

unknown which of the three  $\alpha_2$ -AR subtypes (i.e.,  $\alpha_{2A}$ ,  $\alpha_{2B}$  or  $\alpha_{2C}$ ) is responsible for the upregulation of TNF- $\alpha$  production. The aim of this study was therefore to determine the contribution of  $\alpha_{2A}$ -AR in NE-mediated proinflammatory effects in sepsis.

## Materials and Methods

### Experimental model of sepsis

Polymicrobial sepsis was induced in adult male rats by cecal ligation and puncture (CLP). Briefly, rats were fasted overnight prior to the induction of sepsis, but allowed water *ad libitum*. The animals were anesthetized with isoflurane inhalation and a 2-cm ventral midline abdominal incision was made. The cecum was then exposed, ligated just distal to the ileocecal valve to avoid intestinal obstruction, punctured twice with an 18-gauge needle, and returned to the abdominal cavity. The incision was closed in layers and the animals were resuscitated with 3 ml/100 g BW normal saline subcutaneously immediately after CLP [12]. This model of sepsis is associated with an early, hyperdynamic phase (i.e., 2–10 h after CLP; characterized by an increased cardiac output and tissue perfusion, decreased vascular resistance, and hyperglycemia), which is followed by a late, hypodynamic phase (16 h after CLP and later; characterized by reduced cardiac output and tissue perfusion, increased vascular resistance, and hypoglycemia) [5,13,14]. Sham-operated animals underwent the same surgical procedure except that the cecum was neither ligated nor punctured. Studies were then conducted at 2 h (early sepsis) and 20 h (late sepsis) after the induction of sepsis. This project was approved by the Animal Care and Use Committee of the Feinstein Institute for Medical Research and following national guidelines for the use of animals in research.

### Isolation of Kupffer cells

Kupffer cells were isolated from normal and septic rats as previously described elsewhere with some modifications [12]. Briefly, under isoflurane anesthesia, following a midline incision the inferior vena cava was cannulated and the portal vein was severed. The liver was immediately perfused in situ with ~60 ml of Hanks balanced salt solution without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Cellgro, VA) at 37°C at a rate of 15 ml/min. This was followed by perfusion with 120 ml of HBSS containing 0.02% collagenase (Worthington, Lakewood, NJ; Type IV, 180 U/mg) and 100 mM  $\text{CaCl}_2$  solution at the same perfusion rate. The liver was then removed en bloc, rinsed with ~25 ml of HBSS, minced in a petridish containing HBSS with collagenase, and incubated for 20 min at 37°C to further dissociate the cells. The cell suspension was then passed through a 150-mesh, stainless steel screen into cold Dulbecco modified Eagle medium (DMEM; GIBCO Life Technologies, Carlsbad, CA), containing 10% heat-inactivated fetal bovine serum and centrifuged (50 g for 2 min at 4°C) to sediment hepatocytes. The remaining cells in the supernatant were collected by centrifugation (450 g for 10 min at 4°C). The cell pellets resuspended in DMEM. After washing twice, cells were centrifuged on a density cushion of Percoll at 1,000 g for 15 min at 4°C. The buffy coat containing the KCs fraction was collected. The cells were further washed twice. Cell viability as determined by trypan blue exclusion was more than 95%. The yield was at 8–12  $\times 10^6$  KCs/liver with a purity of at least 90%. The isolated KCs were then cultured in DMEM, containing 10% heat-inactivated fetal bovine serum, 10 mM HEPES, 100 U/ml penicillin and 100 g/ml streptomycin at the concentration of  $10^6$  cells/ml overnight with 5%  $\text{CO}_2$  at 37°C. KCs were allowed to adhere to the bottom of a 24-well plate (Costar) overnight and unattached cells were removed by gentle washing.

### Assessment of $\alpha_{2A}$ -AR mRNA

Gene expression of  $\alpha_{2A}$ -AR was assessed by real-time quantitative PCR (Q-PCR). Total RNA was extracted from KCs of CLP and Sham-operated animals as well as from cultured KCs using Tri-reagent (Molecular Research Center, Cincinnati, OH). Q-PCR was carried out on cDNA samples reverse transcribed from 2  $\mu\text{g}$  RNA using murine leukemia virus reverse transcriptase (Applied Biosystems). Using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA), reactions were carried out in 24  $\mu\text{l}$  final volume containing 2 pmol of forward and reverse primers, 12  $\mu\text{l}$  QuantiTect Master Mix, and 1  $\mu\text{l}$  cDNA. Amplification was performed according to Qiagen's recommendations with an Applied Biosystems 7300 real-time PCR. Expression amount of rat GAPDH mRNA was used for normalization of each sample, and analysis of each specific mRNA was conducted in duplicate. Relative expression of mRNA was calculated by the  $\Delta\Delta C_t$  method, and results expressed as fold change with respect to the corresponding experimental control. The following rat primers were used: GAPDH (AF 106860): 5'-ATG ACT CTA CCC ACG GCA AG-3' (forward), 5'-CTG GAA GAT GGT GAT GGG TT-3' (reverse); rat  $\alpha_{2A}$ -AR (NM\_012739), 5'-CGT GTT CGT GGT GTG TTG GT-3' (forward), 5'-GCA GCC GAC CGC TAT GAG-3' (reverse).

### Binding capacity and affinity of KC $\alpha_2$ -adrenoceptors

Freshly isolated KCs ( $10^6$ ) from sham and septic animals at 2 h after CLP were incubated with [ $^3\text{H}$ ]-yohimbine (a radioactively labeled  $\alpha_2$ -AR antagonist; specific activity 79.2 Ci/mmol; Dupont/NEN; final concentration, 2 to 64 nM in a volume of 200  $\mu\text{l}$ ) with or without 10  $\mu\text{M}$  of unlabeled yohimbine for 30 min at 37°C in an assay buffer (40 mM Tris-HCl, 10 mM  $\text{MgCl}_2$ , pH 7.5) [15]. The value of  $B_{\text{max}}$  and  $K_d$  were determined by Scatchard analysis after logarithmic transformation.

### Stimulation of isolated Kupffer cells with the $\alpha_{2A}$ -AR subtype inhibitor BRL-44408

KCs isolated from normal animals were cultured overnight in DMEM medium with 10% heat inactive fetal calf serum, 100 U/ml penicillin/streptomycin, 100 mM HEPES and 100 U/ml L-glutamine at the concentration of  $10^6$  cells/ml. KCs were then stimulated with NE (20 nM) with or without  $\alpha_{2A}$ -AR specific antagonist BRL-44408 maleate (1  $\mu\text{M}$ , Tocris, UK) for 4 h. The supernatant was then collected and TNF- $\alpha$  levels were measured by enzyme-linked immunosorbent assay (ELISA) kit specific for rat TNF- $\alpha$  (Pharmingen, San Diego, CA). The assay was carried out according to the instructions provided by the manufacturer. For additional p38 MAP kinase pathway studies, KCs were cultured in DMEM for an 1 h (p38 phosphorylation) or 24 h (TNF- $\alpha$  release) with the following treatments: NE (20 nM), LPS (100 ng/ml, *E. coli* 055:B5; Sigma, St. Louis, MO), and the inhibitors BRL-44408 (1  $\mu\text{M}$ ), pertussis toxin (PTX, 100 ng/ml), or SB203580 (10  $\mu\text{M}$ ).

### Intraportal administration of NE

Following anesthesia with isoflurane, a 3-cm midline incision was performed. The small intestine was exposed and a branch of the superior mesenteric vein was cannulated with a PE-10 catheter. It should be noted that this procedure did not cause any apparent gut ischemia. NE (20  $\mu\text{M}$  in normal saline containing 0.1% ascorbic acid to prevent NE oxidation) or vehicle was infused into the portal vein at a rate of 13  $\mu\text{l}/\text{min}$  for 2 h using a Harvard pump. Since portal blood flow is ~13 ml/min/liver [16], the above rate of NE infusion would be expected to increase the portal NE level to 20 nM, which is similar to that

observed during sepsis[6]. A third group also received BRL-44408 maleate (1 mM solution at 13  $\mu$ l/min), which was first infused into the portal vein for 15 min and then followed by infusion of 20  $\mu$ M NE in combination with 1 mM BRL-44408 for 2 h at an infusion rate of 13  $\mu$ l/min. After 2 h blood samples were collected by cardiac puncture and KCs were isolated as described above. In additional groups of NE, NE plus BRL-44408, or vehicle-infused animals, LPS (7.5 mg/kg) was administered through intraperitoneal injection at 30 min after the onset of 2-h infusion. At the end of the infusion (i.e., 1.5 h after LPS challenge), blood samples were collected for plasma TNF- $\alpha$  measurement.

### Determination of TNF- $\alpha$ production

Plasma, supernatant from KC culture, and cellular ( $5 \times 10^6$  KCs) TNF- $\alpha$  levels were determined using an ELISA assay kit specific for rat TNF- $\alpha$  (Pharmingen, San Diego, CA). Isolated KCs were also used to determine the TNF- $\alpha$  gene expression by RT-PCR as described previously [17].

### Determination of p38 MAP kinase phosphorylation

KCs were lysed (10 mM Tris saline, pH 7.5 with 1% Triton-100 $\times$ , 1 mM EDTA, 1 mM EGTA, 2 mM Na-orthovanadate, 0.2 mM PMSF, 2  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml aprotinin), centrifuged at 16,000 g for 10 min, the supernatant was collected, and the protein concentration determined. A total of 10  $\mu$ g of protein was loaded on a 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and electrophoretically fractionated in a MES SDS running buffer (Invitrogen). The protein was then transferred to a 0.45- $\mu$ m nitrocellulose membrane, and blocked with 5% bovine serum albumin in 10 mM Tris saline with 0.1% Tween 20, pH 7.6 (TBST). The membrane was incubated with rabbit anti-phosphorylated p38 MAP kinases polyclonal antibodies (1:1000) overnight at 4°C, followed by incubation in 1:20,000 HRP-linked anti-rabbit IgG for 1 h at room temperature. To reveal the reaction bands, the membrane was reacted with ECL Western blot detection system and exposed on X-ray film. The same membranes were stripped and re-blotted with rabbit anti-total-p38 MAP kinases (1:500; please note that stripping and re-blotting may have reduced the signals) to determine the ratio of phosphorylated p38 and total p38. A digital image system was used to determine the density of the bands (Bio-Rad, Hercules, CA).

### Treatment of septic rats with BRL-44408

Rats underwent CLP as described before, the femoral vein was cannulated and connected to a Harvard pump. BRL-44408 (2.5 mg/kg BW) or vehicle was infused for 30 minutes using a Harvard pump. The catheter was tunneled out, and the femoral vein was ligated to stop the bleeding. The animals were allowed to wake up and returned to their cages. 20 h later animals sacrificed and blood collected for the measurement of liver transaminases, creatinine, lactate and TNF- $\alpha$  using commercial assay kits (Pointe Scientific). KC TNF- $\alpha$  mRNA expression was also assessed using the methods described above. The liver enzymes AST and ALT as well as creatinine and lactate levels were measured in blood serum by using commercial assay kits (Pointe Scientific, Canton, MI). In addition we conducted a 10-day survival study was conducted. 20 animals in each group underwent CLP and received either BRL-44408 (2.5 mg/kg BW bolus iv) or the same volume normal saline (Vehicle). 20 h after CLP, the cecum was removed, which based on our experience normally yields a 10-day survival rate of approximately 50%. In additional groups of normal rats, both the left femoral vein and artery were cannulated with a PE-50 tubing. The femoral vein catheter was connected to a Harvard pump for BRL-44408 (2.5 mg/kg BW) or vehicle infusion as described above. The

femoral artery catheter was connected to a blood pressure analyzer (BPA; Digi-Med, Louisville, KY) and mean arterial pressure (MAP) and heart rate were recorded continuously.

### Statistical analysis

All data that passed the normality test are expressed as mean $\pm$ SE and compared by Student's t-test or analysis of variance (ANOVA) using Student-Newman-Keul's post-hoc analysis. Data that are of percentile based nature or failed the normality test are expressed as mean $\pm$ 95% confidence, and compared with ANOVA on Ranks. The binding capacity and affinity was estimated using the Scatchard analysis and curves compared by two-way ANOVA. The survival study was analyzed using the Kaplan-Meier log-rank test. Differences in values were considered significant if  $P < 0.05$ . All statistical analysis was performed using either the SigmaStat<sup>®</sup> or PRISM<sup>®</sup> software.

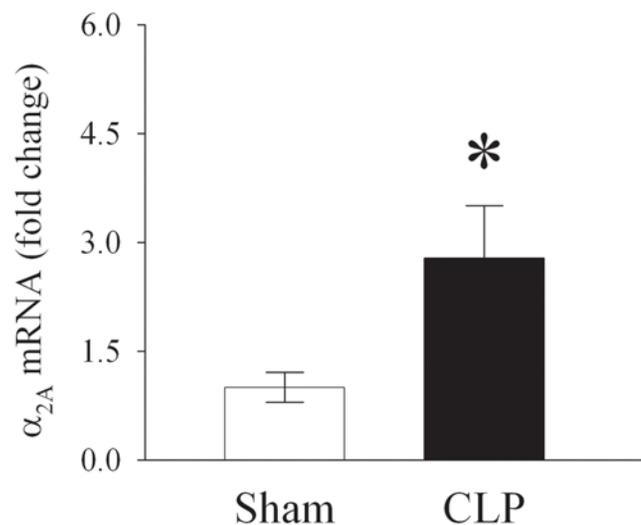
## Results

### Upregulation of KC $\alpha_2$ A-AR expression in CLP-induced sepsis

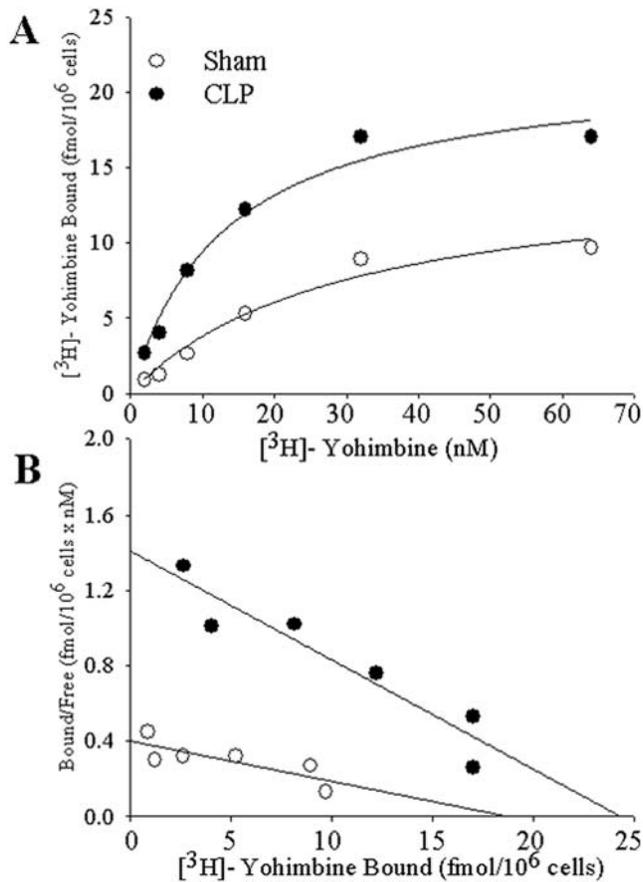
Rats were subjected to sepsis by CLP and KCs were isolated 2 h thereafter. As shown in Figure 1, the gene expression of  $\alpha_2$ A-AR was significantly upregulated by 179% at 2 h post-CLP compared to respective sham-operated animals. In contrast,  $\alpha_2$ B and  $\alpha_2$ C-AR expression did not show any changes after CLP (Data not shown).

### Increased KC $\alpha_2$ -AR binding capacity and affinity in sepsis

To investigate whether increased  $\alpha_2$ -AR expression in sepsis leads to enhanced receptor binding, we incubated KCs isolated from sham-operated or CLP animals with [<sup>3</sup>H]-yohimbine, a radio-labeled  $\alpha_2$ -specific AR antagonist. As shown in Figure 2A, the binding of KC  $\alpha_2$ -AR was saturated at approximately 30 nM [<sup>3</sup>H] yohimbine in both sham-operated and CLP animals. However, maximal binding of the  $\alpha_2$ -specific ligand was much



**Figure 1. Upregulation of KC  $\alpha_2$ A-AR expression in CLP-induced sepsis.** Gene expression of  $\alpha_2$ A-adrenergic receptor (AR) in Kupffer cells isolated from animals at 2 h after cecal ligation and puncture (CLP) or sham-operation. Relative expression of mRNA was calculated by the  $\Delta\Delta$ Ct-method, and results expressed as fold change with respect to the housekeeping gene GAPDH. Values ( $n=6$ /group) are presented as mean $\pm$ SE and compared by Student's t-test. \* $P < 0.05$  vs. Sham. doi:10.1371/journal.pone.0005504.g001

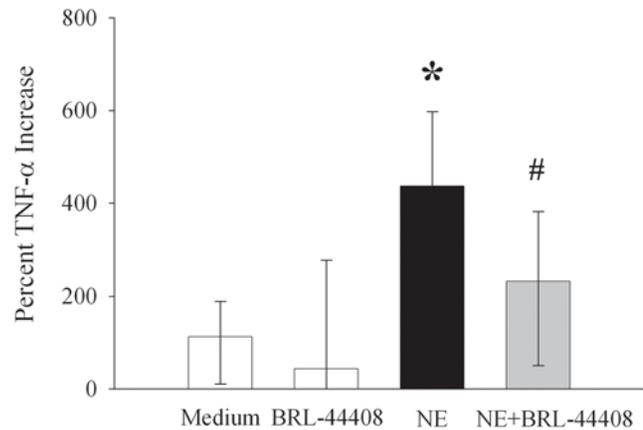


**Figure 2. Increased KC  $\alpha_2$ -AR binding capacity and affinity in sepsis.** Changes in  $\alpha_2$ -receptor binding of the radiolabeled specific ligand [ $^3$ H]-yohimbine in rat Kupffer cells obtained from sham-operated and cecal ligation and puncture (CLP) animals (A).  $B_{max}$  and  $K_d$  were estimated using the the Scatchard analysis (B). The data points represent the average of three different experiments. Best fit analysis and two-way ANOVA showed that curves are different for Sham and CLP ( $P=0.0036$ ). doi:10.1371/journal.pone.0005504.g002

higher in KCs from CLP animals (Fig. 2A). Data transformation to a Scatchard plot yielded linear regression lines consistent with a single class of antagonist binding capacity (Fig. 2B). Scatchard analysis revealed a 28% increase in maximal binding capacity with an average 24.3 fmol/ $10^6$  cells 2 h after CLP compared to a  $B_{max}$  of 19.0 fmol/ $10^6$  cells in sham animals (Fig. 2B). Similarly, the average  $K_d$  decreased by 64% from 47.6 nM in sham animals to 17.2 nM 2 h after CLP (Fig. 2B), indicating increased affinity in septic animals. In addition, we discovered a 50% reduction in cAMP levels in KCs that decreased from  $7.31 \pm 0.14$  pmol/ $5 \times 10^6$  cells in sham-operated animals to  $3.82 \pm 1.70$  pmol/ $5 \times 10^6$  cells at 2 h after CLP (decreased by 48%,  $n = 4-5$ ;  $P < 0.05$ ).

**Stimulation with NE increases TNF- $\alpha$  release from KCs via  $\alpha_2$ A-ARs**

Isolated KCs were stimulated with NE (20 nM) with or without BRL-44408 maleate (1  $\mu$ M) for 4 h. While TNF- $\alpha$  release increased by 440% after NE-stimulation, its increase was completely suppressed after  $\alpha_2$ A-AR blockade by BRL-44408 maleate (Fig. 3). BRL-44408 alone did not have any measurable effects on TNF- $\alpha$  release in the absence of NE-stimulation (Fig. 3).



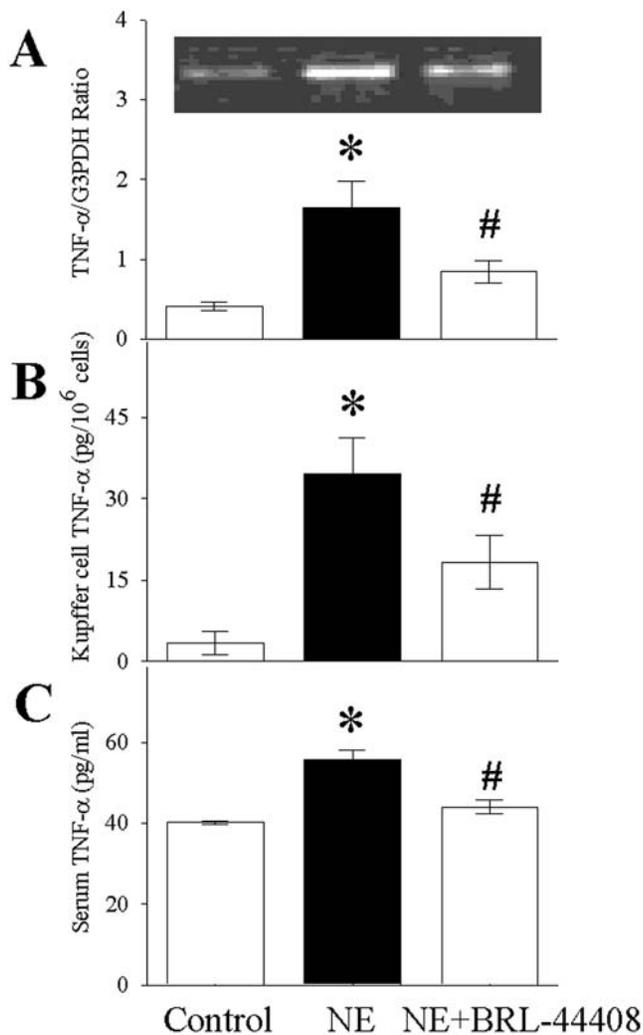
**Figure 3. Stimulation with NE increases TNF- $\alpha$  release from KCs via  $\alpha_2$ A-ARs.** Alterations of TNF- $\alpha$  after stimulation of isolated KCs with NE (20 nM) with or without BRL-44408 maleate (1  $\mu$ M), a specific  $\alpha_2$ A-AR antagonist, for 4 h. Percentage values ( $n = 6$ /group) are presented as mean  $\pm$  95% confidence and compared by ANOVA on Ranks and Student-Newman-Keul's method. \* $P < 0.05$  vs. Medium; # $P < 0.05$  vs. NE. doi:10.1371/journal.pone.0005504.g003

**Portal infusion of NE induces  $\alpha_2$ A-AR-dependent TNF- $\alpha$  production in KCs**

To elucidate whether the  $\alpha_2$ A-AR is also responsible for TNF- $\alpha$  upregulation in vivo, we administered NE through the portal vein in normal animals for 2 h and isolated KCs for analysis. TNF- $\alpha$  gene expression was upregulated by 4-fold in KCs from animals that were subjected to intraportal infusion of NE as compared to vehicle-treated animals (Fig. 4A). BRL-44408 pretreatment prevented the upregulation of TNF- $\alpha$  gene expression. Similarly, TNF- $\alpha$  protein levels increased after intraportal infusion of NE by 10-fold and co-administration of NE with BRL-44408 maleate reduced cellular TNF- $\alpha$  levels by 47% (Fig. 4B). Serum levels of TNF- $\alpha$  also increased after intraportal infusion of NE from  $40.2 \pm 0.8$  pg/ml to  $55.7 \pm 5.2$  pg/ml (Fig. 4C). BRL-44408 significantly suppressed plasma TNF- $\alpha$  levels by 25% to blood TNF- $\alpha$  concentrations found in sham operated animals (Fig 4C). These results underline the crucial role of the  $\alpha_2$ A-AR in the proinflammatory response of Kupffer cells after NE-stimulation under in vivo conditions.

**NE-mediated potentiation of LPS-mediated TNF- $\alpha$  release through the  $\alpha_2$ A-AR**

To investigate the role of the  $\alpha_2$ A-AR in NE-mediated potentiation of a proinflammatory response to endotoxin, we studied the response of KCs to NE and LPS in the presence or absence of the  $\alpha_2$ A-specific inhibitor BRL-44408 in vitro (Fig. 5A) and in vivo (Fig. 5B). Cultured KCs were responsive to endotoxin and TNF- $\alpha$  levels increased by over 16-fold after stimulation with 100 ng/ml LPS ( $P < 0.05$ , Fig. 5A). The simultaneous treatment with NE+LPS caused TNF- $\alpha$  levels increased by an additional 63% ( $P < 0.05$  vs. LPS alone, Fig. 5A), indicating a potentiation of LPS-induced TNF- $\alpha$  release from cultured KC. Concurrent inhibition of the  $\alpha_2$ A-AR using BRL-44408 (1  $\mu$ M) significantly attenuated TNF- $\alpha$  release by over 60% (Fig. 5A). To verify the crucial role of the  $\alpha_2$ A-AR in the proinflammatory response to NE in endotoxemia, we measured plasma levels of TNF- $\alpha$  after intraperitoneal injection of LPS (7.5 mg/kg) and systemic intravenous administration of NE. LPS alone resulted in a 10-fold increase of TNF- $\alpha$  plasma levels after 4 h (Fig. 5B) and the co-administration of NE resulted a marked enhancement in plasma

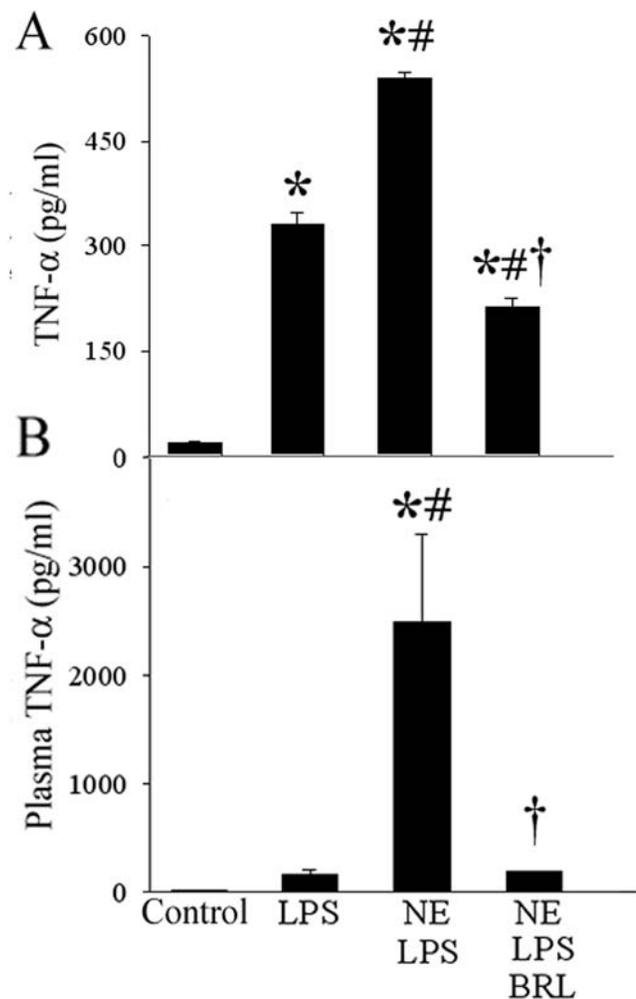


**Figure 4. Portal infusion of NE induces  $\alpha_2A$ -AR-dependent TNF- $\alpha$  production in KCs.** *In vivo* Alterations in Kupffer cell gene expression (A) and protein levels of TNF- $\alpha$  (B) as well as serum TNF- $\alpha$  levels (C) after administration of NE, NE combined with BRL-44408 maleate for 2 h through the portal vein. A representative gene and ratio of TNF- $\alpha$  and housekeeping gene G3PDH are presented in panel (A). Values (n=5/group) are presented as mean $\pm$ SE and compared by one-way ANOVA and Student-Newman-Keul's method. \*P<0.05 vs. Control; #P<0.05 vs. NE. doi:10.1371/journal.pone.0005504.g004

TNF- $\alpha$  release by 155-fold (P<0.05; Fig. 5B). Administration of the  $\alpha_2A$ -AR antagonist BRL-44408 completely blocked the LPS/NE-induced TNF- $\alpha$  release (Fig. 5B). Thus, NE-mediated potentiation of LPS-induced TNF- $\alpha$  release is  $\alpha_2A$ -AR-dependent even after systemic application of NE.

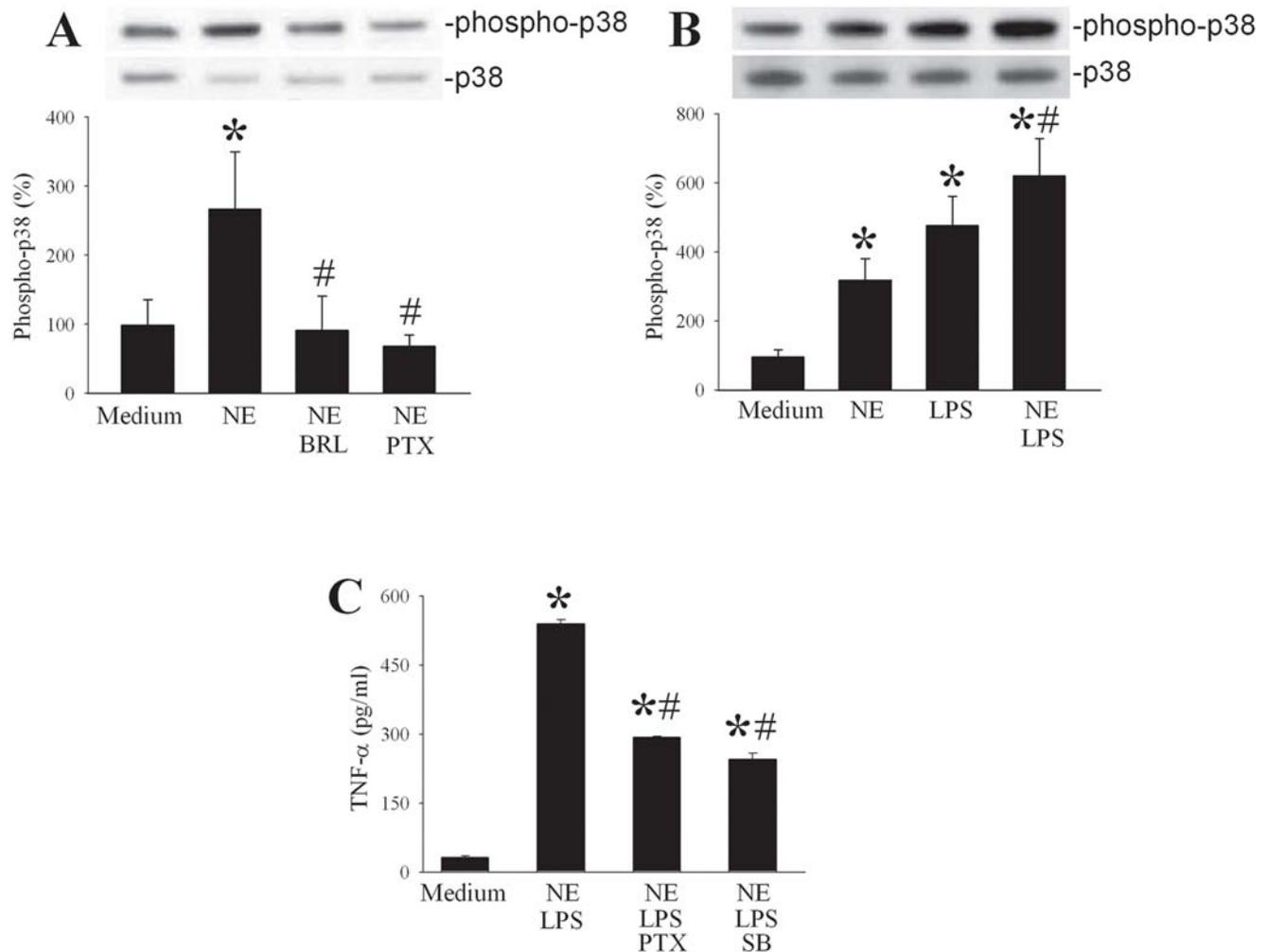
#### $\alpha_2A$ -AR-dependent activation of p38 MAP kinase

LPS induces the activation of intracellular pathways, including the p38 MAP kinase by its phosphorylation, which plays a crucial role in the proinflammatory response of macrophages [18]. To determine, whether  $\alpha_2A$ -AR activation affects the p38 pathway, we stimulated cultured KCs with NE (20 nM) and blocked either the  $\alpha_2A$ -AR with BRL-44408 or its coupled  $G_{\alpha_i}$ -protein with pertussis toxin (PTX). Both BRL-44408 (1  $\mu$ M) and pertussis toxin (PTX, 100 ng/ml) prevented the NE-induced phosphorylation of the p38 MAP kinase (Fig. 6A). This indicates that NE acts through the



**Figure 5. NE-mediated potentiation of LPS-mediated TNF- $\alpha$  release through the  $\alpha_2A$ -AR.** BRL-44408 maleate blocks TNF- $\alpha$  production in LPS-stimulated KCs after co-incubation with NE *in vitro* and *in vivo*. (A) Alterations in TNF- $\alpha$  release from cultured KCs 24 h after stimulation with NE (20 nM) and LPS (100 ng/ml), with or without BRL-44408 (BRL, 1  $\mu$ M). Data are presented as mean $\pm$ SE (n=8) and compared by one-way ANOVA and Student-Newman-Keuls test. \*P<0.05 vs. Control; #P<0.05 vs. LPS alone; †P<0.05 vs. NE+LPS. (B) Alterations in plasma levels of TNF- $\alpha$  after administration of LPS (7.5 mg/kg, intra-peritoneal) and NE (20  $\mu$ M NE for 2 h at 13  $\mu$ l/min), with or without BRL-44408 (2.5 mg/kg BW, intra-portal). Data are presented as mean $\pm$ SE (n=4-6) and compared by one-way ANOVA and Student-Newman-Keul's method. \*P<0.05 vs. Control; #P<0.05 vs. LPS alone; †P<0.05 vs. NE+LPS. doi:10.1371/journal.pone.0005504.g005

$\alpha_2A$ -AR and  $G_{\alpha_i}$  protein to activate the p38 MAP kinase. To verify this effect under inflammatory conditions, we assessed NE-mediated phosphorylation of the p38 MAP kinase in cultured KC at 1 h after stimulation with LPS (100 ng/ml). Either agent by itself induced the phosphorylation of p38 compared to control, however, the combination of NE and LPS showed the strongest activation (over 7-fold induction) of p38 in cultured KCs (Fig. 6B). This indicates that LPS-induced p38-activation can be potentiated by simultaneous NE stimulation. To determine the role of this pathway in the  $\alpha_2A$ -AR-mediated proinflammatory effect of NE, additional experiments were performed to measure TNF- $\alpha$  release after NE+LPS stimulation with or without inhibition of the  $G_{\alpha_i}$  or the p38 MAP kinase. Both PTX (100 ng/ml) and SB203580



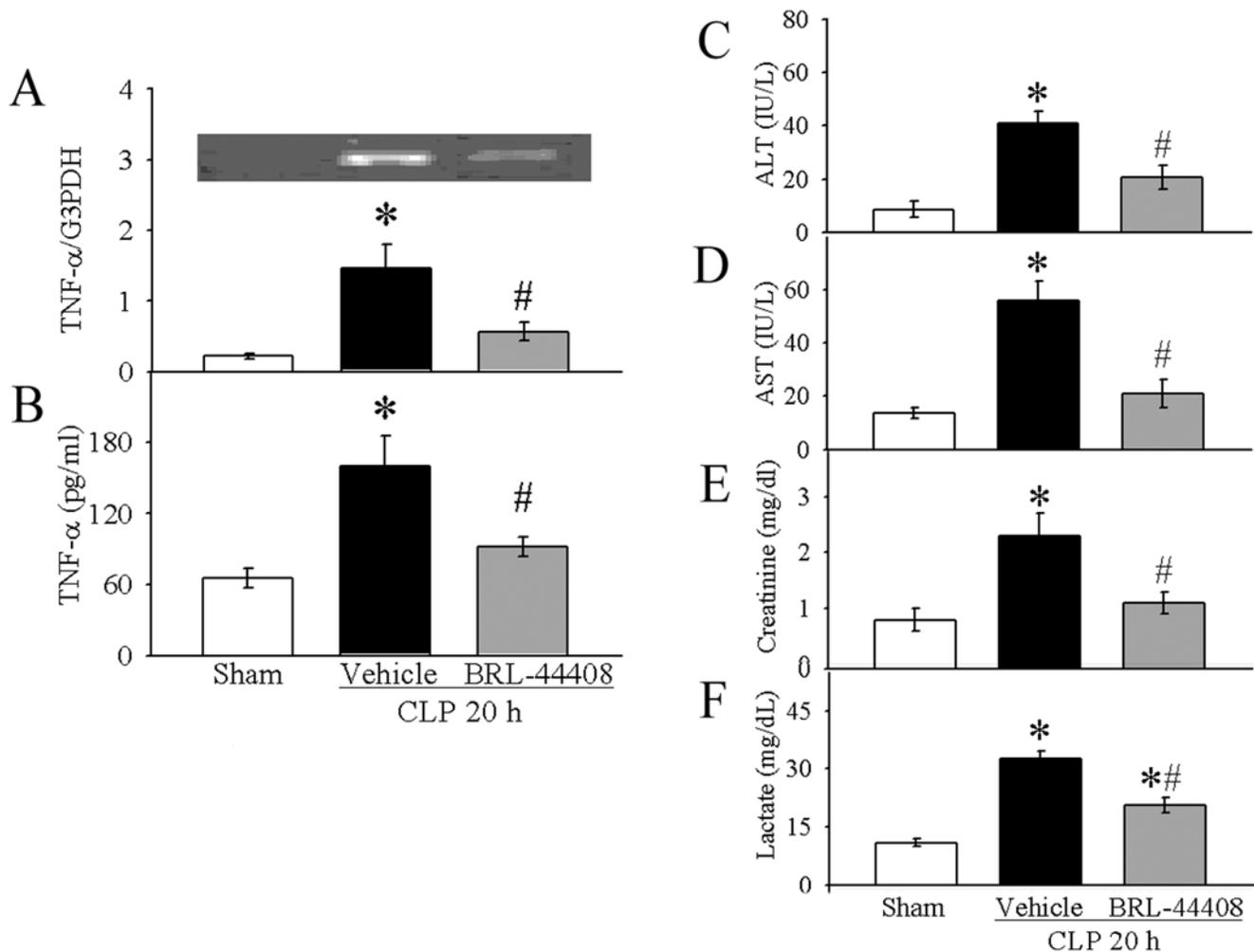
**Figure 6. NE-mediated potentiation of p38 MAP kinase phosphorylation via  $\alpha_{2A}$ -ARs.** (A) Alterations in p38 MAP kinase after 1 h culture with NE alone (20 nM), NE+BRL-44408 (BRL, 1  $\mu$ M), or NE+pertussis toxin (PTX, 100 ng/ml). The relative percentage of phosphorylated p38/total p38 MAP kinase is presented as mean  $\pm$  95% confidence (n = 4–6) and compared by ANOVA on Ranks and Student-Newman-Keul's method. The Medium group is considered as 100%. \*P < 0.05 vs. Medium; #P < 0.05 vs. NE alone. Representative gels are presented. (B) Enhanced activation of p38 MAP kinase after 1 h culture with a combination of NE (20 nM) and LPS (100 ng/ml). The relative percentage of phosphorylated p38/total p38 MAP kinase ratio is presented as mean  $\pm$  95% confidence (n = 4–6) and compared by ANOVA on Ranks and Student-Newman-Keul's method. Medium group is considered as 100%. \*P < 0.05 vs. Medium; #P < 0.05 vs. LPS alone. Representative gels are also presented. (C) Suppression of TNF- $\alpha$  release from cultured KCs after stimulation with NE (20 nM)+LPS (100 ng/ml) with or without pertussis toxin (PTX, 100 ng/ml), a  $G_{\alpha_i}$ -protein inhibitor, or SB203580 (10  $\mu$ M), a p38 MAP kinase inhibitor. Data are presented as mean  $\pm$  SE (n = 4–6) and compared by one-way ANOVA and Student-Newman-Keul's method. \*P < 0.05 vs. Medium; #P < 0.05 vs. LPS+NE. doi:10.1371/journal.pone.0005504.g006

(10  $\mu$ M; a p38 MAP kinase inhibitor) attenuated the NE-mediated increase in TNF- $\alpha$  release by 46% and 55% respectively (Fig. 6C). This study further confirms that NE potentiates LPS-induced TNF- $\alpha$  through the  $\alpha_{2A}$ -AR coupled  $G_{\alpha_i}$  protein and the activation of the p38 MAP kinase.

#### BRL-44408 is beneficial in experimental sepsis

Using the above *in vitro* and *in vivo* systems, we were able to show that BRL-44408 can attenuate the proinflammatory effect of NE either alone or in conjunction with LPS. To investigate the beneficial effect of BRL-44408 in sepsis, we used an experimental sepsis model of CLP in rats receiving BRL-44408. 20 h later we measured cytokine levels in KCs, plasma and injury parameters for liver (AST & ALT), kidney (creatinine) and general oxygenation (lactate). As expected, TNF- $\alpha$  mRNA levels in KCs as well as plasma levels were increased 20 h after CLP by 6.6-fold and 2.4-

fold, respectively (Figs. 7A–B). BRL-44408 treatment completely inhibited TNF- $\alpha$  production and release (Figs. 7A–B). Similarly, CLP-induced increases in surrogate markers for liver and kidney injury (AST, ALT and creatinine by 4.8-, 4.1-, and 2.9-fold, respectively) were completely blocked after treatment with BRL-44408 (Figs. 7C–E). Lactate, a marker for tissue perfusion and oxygenation, that was increased by 3-fold after CLP and was significantly suppressed by 37% after BRL-44408 infusion (Fig. 7F). These above results indicate that BRL-44408 confers an anti-inflammatory effect and protects from organ injury and tissue malperfusion in CLP-induced sepsis in rats. To show that these beneficial effects translate into an improved outcome, we conducted a survival study. As shown in Figure 8, CLP and vehicle treatment resulted in a 55% mortality rate over a 10-day period. Treatment with BRL-44408, however, protected over 55% of animals at risk, resulting in an overall survival rate of 75%. To



**Figure 7. Beneficial effects of  $\alpha_2A$ -AR inhibition in sepsis.** (A–B) BRL-44408 mediated suppression of TNF- $\alpha$  gene expression in Kupffer cells (A) and plasma concentrations (B) 20 h after CLP. (C–F) BRL-44408 mediated improvement of organ damage parameters. Rats underwent CLP and 20 h later ALT (C), AST (D), creatinine (E) and lactate (F) levels were measured as described in the methods. Values (n=5/group) are presented as means $\pm$ SE and compared by one-way ANOVA and Student-Newman-Keul's method. \*P<0.05 vs. Sham; #P<0.05 vs. CLP+Vehicle. doi:10.1371/journal.pone.0005504.g007

determine the effect of the  $\alpha_2A$ -AR blockade on other organ system, we monitored MAP and heart rates during BRL-44408 administration in normal rats. As shown in **Figure 9**, intravenously infusion of BRL-44408 at the dose of 2.5 mg/kg BW had no measurable effects on MAP and heart rates.

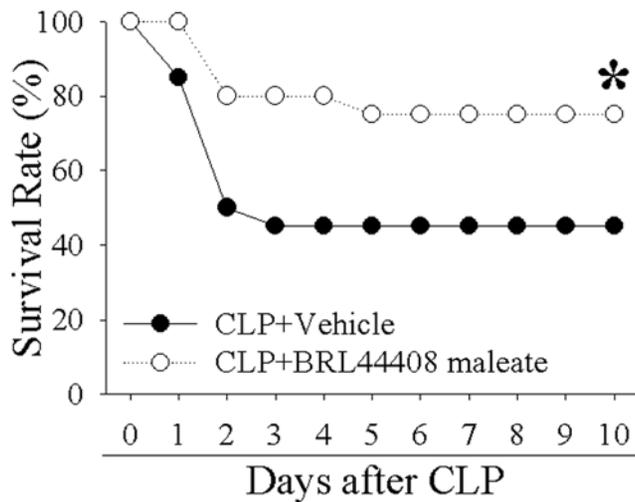
## Discussion

Gut-derived NE has been shown to play a critical role in inducing hepatocellular dysfunction in early sepsis, exerting its effect through the non-synaptic, high-affinity  $\alpha_2$ -AR [19]. Kotanidou *et al.* have shown that urethane, a general anesthetic with  $\alpha_2$ -AR blocking properties, protects against LPS partly by reducing TNF- $\alpha$  release [20]. We have previously reported that TNF- $\alpha$  secretion from NE stimulated Kupffer cells can be inhibited by the general  $\alpha_2$ -AR inhibitor yohimbine, suggesting that  $\alpha_2$ -ARs on Kupffer cells are particularly responsible for the upregulation of TNF- $\alpha$  release [21]. The presence of  $\alpha_2$ -ARs on macrophages has been previously confirmed by receptor binding assays and *in situ* hybridization [22,23]. Studies by other groups have shown that TNF- $\alpha$  upregulation can be mediated by the stimulation of  $\alpha_2$ -ARs [11,24]. Here we show that it is the  $\alpha_2A$ -AR

that is upregulated in KCs leading to enhanced receptor binding and proinflammatory cytokine release in sepsis.

We have previously shown that TNF- $\alpha$  is significantly increased by incubation with NE (20 nM) for 4 h [21]. Since KCs are a major source of proinflammatory cytokines [25], intraportal infusion of NE appears to have a direct measurable effect on TNF- $\alpha$  release *in vivo*. Infusion of NE through the femoral vein may reduce active NE levels reaching the liver compared to direct intraportal injection. Hence, one may expect a diminished proinflammatory response of NE after systemic administration. We have shown however, that even after peripheral intravenous administration of NE, the LPS-induced TNF- $\alpha$  increase becomes dramatically potentiated through an  $\alpha_2A$ -AR-dependent pathway, possibly through the involvement of other tissue macrophages.

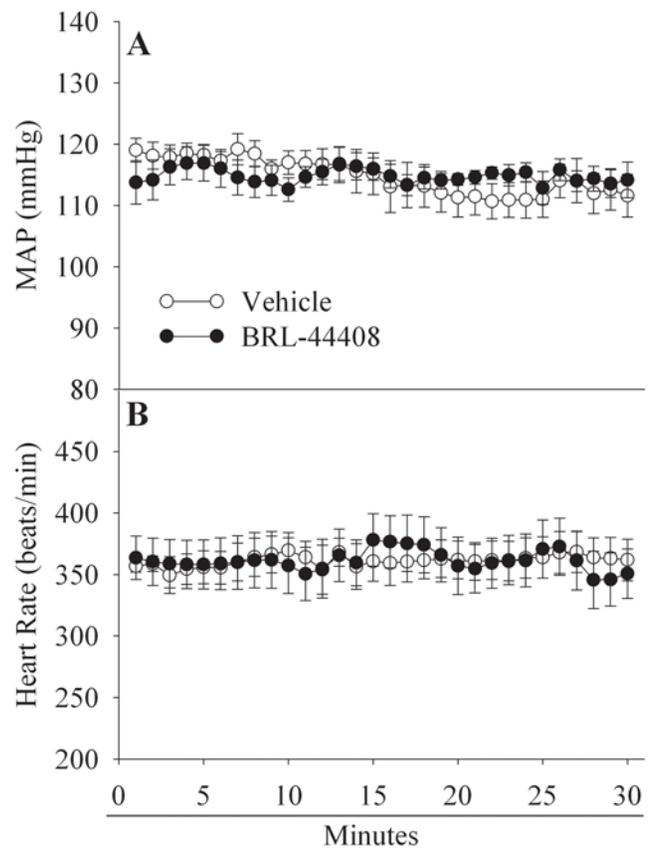
Adrenergic receptors are subdivided into three major subtypes  $\alpha_1$ ,  $\alpha_2$ , and  $\beta$ , which are then subdivided into  $\alpha_{1A}$ ,  $\alpha_{1B}$ , and  $\alpha_{1C}$ ,  $\alpha_{2A}$ ,  $\alpha_{2B}$ , and  $\alpha_{2C}$  and  $\beta_1$ ,  $\beta_2$ , and  $\beta_3$ . Upon binding of  $\beta_2$ -ARs for example, epinephrine and high doses of NE through increasing intracellular cAMP levels [26].  $\alpha_2$ -adrenoceptors are G $_i$ - and G $_o$ -protein coupled receptors that decrease intracellular cAMP, open K $^+$  channels, and inhibit voltage gated Ca $^{2+}$  channels, all of which lead to hyperpolarization of neurons and activation of immune



**Figure 8. The  $\alpha_2$ A-AR inhibitor BRL-44408 improves survival in septic rats.** Rats underwent CLP (n=20/group) and received either Vehicle treatment or BRL-44408 maleate iv 2.5 mg/kg BW. Cecums were removed 20 h later and animals observed for up to 10 days. \*P<0.05 vs. Vehicle, Kaplan-Meier logrank test. doi:10.1371/journal.pone.0005504.g008

cells [26]. In the CNS,  $\alpha_2$ -adrenoceptors are predominantly presynaptic. They regulate the release of neurotransmitters through a negative feedback. Functional studies of the genetic receptor subtypes have linked the  $\alpha_2$ B-AR to peripheral vasoconstriction and analgesic effects of N<sub>2</sub>O (nitrous oxide) and other anesthetic agents. The  $\alpha_2$ A-AR, either alone or with  $\alpha_2$ C-AR co-activation, is involved in the central inhibition of sympathetic activities, modulation of neurotransmitter release, sedation, and anti-epileptic effects. As we have shown here, the pro-inflammatory action of NE, mediated by the  $\alpha_2$ A-receptor subtype expressed on hepatic macrophages (i.e., KCs) can now be added to this list.

So far, however, we could find only one report regarding the  $\alpha_2$ -AR subtypes responsible for TNF- $\alpha$  upregulation in pulmonary inflammation and none in sepsis itself. In their work by Flierl *et al.*, the authors focused on the phagocyte-derived catecholamines that boost inflammatory responses via the  $\alpha_2$ -AR [27]. Although this report indicated similar increases in the  $\alpha_2$ A-AR subtype in alveolar macrophages and neutrophils after LPS-stimulation, the role of individual subtypes in the proinflammatory response was not addressed [27]. In our study we have assessed the influence of these subtypes in hepatic macrophages, i.e., KCs. We have shown that the gene expression of  $\alpha_2$ A-AR has significantly increased 2 h after CLP, while no significant changes in  $\alpha_2$ B and  $\alpha_2$ C-AR could be observed. Kupffer cells stimulated with NE in combination with the  $\alpha_2$ A-AR inhibitor BRL-44408 maleate reduced TNF- $\alpha$  release, while the  $\alpha_2$ B-AR inhibitor imiloxan hydrochloride increased TNF- $\alpha$  levels (data not shown). The difference between those two receptor subtypes may lie in the intracellular signaling pathways. While all  $\alpha_2$ -ARs suppress intracellular cAMP levels through its G $\alpha_i$  coupled protein, they also change intracellular calcium and potassium levels to a different degree, which may influence intricate signaling pathways and eventually cellular response. As opposed to *in vivo* findings that showed no differences, our *in vitro* results show that  $\alpha_2$ B-AR inhibition is able to inhibit TNF- $\alpha$  release from cultured KCs, which is further complicating the role of  $\alpha_2$ B-ARs in the inflammatory response. *In vivo*, intraportal administration of NE significantly increased serum and Kupffer cell levels of TNF- $\alpha$ , and only the  $\alpha_2$ A-AR specific antagonist, BRL-44408 could significantly reduce TNF- $\alpha$  plasma levels and



**Figure 9. BRL-44408 has no effects on MAP and heart rates in normal rats.** Effects of  $\alpha_2$ A-AR inhibitor BRL-44408 on mean arterial pressure (MAP, **A**) and heart rates (**B**) in normal rats. Normal rats received either Vehicle treatment or BRL-44408 maleate iv 2.5 mg/kg BW over a period of 30 min. Data are presented as mean $\pm$ SE (n=5/group), and compared by Student's *t*-test. No statistical difference was found. doi:10.1371/journal.pone.0005504.g009

Kupffer cell TNF- $\alpha$  release. Recent reports show that not only sympathetic mediators, but also the cholinergic pathways modulate the systemic inflammatory response. Thus TNF- $\alpha$  release can be reduced by increasing efferent vagus nerve activity and acetylcholine release [28]. In this regard, the nicotinic acetylcholine receptor  $\alpha_7$  subunit appears to be responsible for the inhibition of macrophage TNF- $\alpha$  release by acetylcholine [29]. The role of the  $\alpha_2$ A-AR in the regulation of vagus nerve activity is a possibility *in vivo*, but our *in vitro* results indicate that the TNF- $\alpha$  suppressive effect of BRL-44408 is independent of a parasympathetic influence. Our present work and others' studies also show that this tremendous activation of the sympathetic and parasympathetic nervous system during sepsis is not only a result of these devastating conditions but also influences of inflammatory responses itself by regulating proinflammatory cytokines via adrenergic and cholinergic receptors.

$\alpha_2$ A-ARs also have effects on the cardiovascular and nervous systems [30–32]. Since changes in MAP and heart rates reflect the activity of both the cardiovascular and nervous systems, we monitored MAP and heart rates during BRL-44408 administration in normal rats. Our results indicate that intravenously infusion of BRL-44408 at the dose of 2.5 mg/kg BW had no measurable effects on the MAP and heart rate. In this regard, the beneficial effect of  $\alpha_2$ A-AR blockade in sepsis is unlikely due to its direct effects on the cardiovascular and nervous systems.

In summary, our results suggest that hyperresponsiveness to  $\alpha$ <sub>2</sub>-AR stimulation observed in sepsis is primarily due to an increase in  $\alpha$ <sub>2A</sub>-AR expression in KCs. This appears to be in-part responsible for the increased proinflammatory response and ensuing organ injury in sepsis. These findings provide important feasibility information for further developing the  $\alpha$ <sub>2A</sub>-AR antagonist as a new therapy for sepsis.

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## Author Contributions

Conceived and designed the experiments: RW SMG TSR PW. Performed the experiments: PD MZ WD YJ. Analyzed the data: MM PD MZ RW. Wrote the paper: MM PW.