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THE ATYPICAL CHEMOKINE RECEPTOR ACKR2 IS PROTECTIVE AGAINST SEPSIS

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Running Head: ACKR2and sepsis
ABSTRACT

Sepsis is a systemic inflammatory response as a result of uncontrolled infections. Neutrophils are the first cells to reach the primary sites of infection and chemokines play a key role in recruiting neutrophils. However, in sepsis chemokines could also contribute to neutrophil infiltration to vital organs leading to multiple organ failure. ACKR2 is an atypical chemokine receptor, which can remove and degrade inflammatory CC chemokines. The role of ACK2 in sepsis is unknown. Using a model of cecal ligation and puncture (CLP), we demonstrate here that ACKR2 deficient (−/−) mice exhibited a significant reduction in the survival rate compared to similarly treated wild type (WT) mice. However, neutrophil migration to the peritoneal cavity and bacterial load were similar between WT and ACKR2−/− mice during CLP. In contrast, ACKR2−/− mice showed increased neutrophil infiltration and elevated CC chemokine levels in the lung, kidney and heart compared to the WT mice. In addition, ACKR2−/− mice also showed more severe lesions in the lung and kidney than those in the WT mice. Consistent with these results, WT mice under non-severe sepsis (90% survival) had higher expression of ACKR2 in these organs than mice under severe sepsis (no survival). Finally, the lungs from septic patients showed increased number of ACKR2+ cells compared to those of non-septic patients. Our data indicate that ACKR2 may have a protective role during sepsis, and the absence of ACKR2 leads to exacerbated chemokine accumulation, neutrophil infiltration and damage to vital organs.

KEYWORDS: Sepsis, atypical chemokine receptor, ACKR2, cecal ligation and puncture, CLP.
INTRODUCTION

Sepsis is life-threatening organ dysfunction caused by dysregulated host response to infection (1). The recruitment of neutrophils to an infected area counters the infection and avoids pathogen spreading to the circulation (2-4). However, the infiltration of neutrophils into vital organs contributes to organ dysfunction during sepsis and, consequently, to death. Both processes are mainly mediated by signaling of the G protein-coupled receptor, including the chemokine receptors (5, 6).

ACKR2 is an atypical chemokine receptor that does not signal via G protein because of a modification in the G protein-binding domain (DRYLAIV motif is altered to DKYLEIV) (7). However, ACKR2 binds with high affinity to inflammatory chemokines from the CC subfamily leading to receptor internalization and CC chemokines degradation (7-9), and contributes to the resolution of several inflammatory conditions (7, 10-13). However, the role of ACKR2 receptor in the pathophysiology of sepsis is hitherto unknown.

In the present study, we hypothesized that ACKR2 could contribute to the degradation of chemokines in the foci of infection or in the vital organs, leading, in the first case, to aggravation of sepsis and, in the second case, to protection. To test these possibilities, we used ACKR2 knockout (−/−) mice in a model of cecal ligation and puncture to induce different severities of sepsis.
MATERIALS AND METHODS

Mice

Experiments were performed on both male and female mice between 6 and 8 weeks old. C57BL/6 (wild-type, WT) mice were obtained from the animal facility of the University of São Paulo, São Paulo, Brazil. ACRK2⁻/⁻ mice on the C57BL/6 background were generated and donated by Professor Sergio Lira (Icahn School of Medicine at Mount Sinai, United States)(10-11). The study was approved by the Ethics Committee for the Use of Animals (CEUA) of Ribeirão Preto Medical School, University of Sao Paulo (protocol number 105/2014). The care and treatment of the animals were based on the Guide for the Care and Use of Laboratory Animals(14).

Sepsis Model

Cecal ligation and puncture (CLP) was used to induce non-severe (NS-CLP) and severe sepsis (S-CLP). Briefly, the animals were anesthetized with ketamine and xylazine diluted in PBS (100 mg/kg ketamine and 10 mg/kg xylazine, União Quimica, BR). The cecum of the animals was exposed, ligated, and punctured, according with the severity of sepsis (one puncture with 21G needle and one puncture with 18G needle to induce non-severe and severe sepsis, respectively). Next, the cecum was repositioned in the abdomen, and the peritoneal wall was closed. The animals received 1 mL of saline subcutaneously after this procedure to avoid dehydration. Sham-operated mice were under laparotomy without CLP. The group of animals treated with antibiotic received an intraperitoneal injection of ertapenem sodium (20 mg/kg, Merck Research Laboratory, Whitehouse Station, NJ) beginning 6 h after CLP and then every 12 h up to day 3. The survival of mice was recorded every 12 h for 10 days. Mice showing signs of

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ataxia, tremor and/or agonal breathing were euthanized by overdose of ketamine/xylazine followed by cervical dislocation. At the end of 10 days, surviving mice were euthanized. Some mice were also euthanized at different time points after CLP for biochemical and cell migration analysis. It should be noted that the phenotype of ACKR2$^{-/-}$ mice could be variable in this sepsis model. However, when the mice were treated with antibiotics, the ACKR2$^{-/-}$ mice remained consistently more susceptible to CLP. Thus the variability observed was most likely due a change of microbiota in the mice over the years in a conventional SPF environment.

**In vivo determination of Neutrophil Migration**

Neutrophil migration was determined 24 and 36 h after CLP as described previously (15). Briefly, the animals were euthanized and the peritoneal cavity was washed with 1.5 mL of PBS containing 1 mM EDTA. Total cell counts from the lavage were performed with a cell counter (Coulter AC T series analyzer, Coulter Corp., Miami, USA), and differential cell counts were carried out on cyto-centrifuge slides (Cytospin 3, Shandon Southern Products, Astmoore, UK) and stained by the May-Grünwald-Giemsa (Rosenfeld) method.

**Bacterial counts**

Bacterial counts were determined 24 and 36 h after CLP as previously described (16). Briefly, mice were euthanized and peritoneal lavage fluid and blood were harvested, diluted serially with sterile PBS, plated on Muller-Hinton agar dishes (Difco Laboratories, Detroit, USA) and incubated at 37°C for 18 h and colony-forming units (CFU) were visually counted.

**Chemokine assay**
The concentrations of chemokines in the lungs, kidneys and heart were measured 24 h after CLP and in the peritoneal lavage 24 and 36 h after CLP. The lung, kidney and heart tissues were homogenized and the concentration of chemokine in the supernatant was determined by ELISA kits (R&D Systems), as previously described (17).

*Myeloperoxidase activity assay*

Tissue myeloperoxidase (MPO) activity was used as a biochemical index of neutrophil infiltration into the lung, kidney and heart, as previously described (18).

*Assessment of lung resistance*

Mice were anesthetized with a subcutaneous injection of ketamine and xylazine (130 mg/kg ketamine and 8.5 mg/kg xylazine) to maintain spontaneous breathing under anesthesia. Mice were tracheostomized, placed in a body plethysmograph and connected to a computer-controlled ventilator (Forced Pulmonary Maneuver System, Buxco Research Systems, Wilmington, North Carolina USA). This laboratory set-up, specifically designed for mice, has only a canula volume (death space) of 0.8 mL and provides semi-automatically three different maneuvers: Boyle’s Law FRC, quasi-static pressure-volume and fast-flow volume maneuver. First, an average breathing frequency of 160 breaths/min was imposed to the anesthetized animal by pressure-controlled ventilation until a regular breathing pattern and complete expiration at each breathing cycle was obtained. Under mechanical respiration the Lung Resistance (RL) was determined as previously described (19).

*Blood urea nitrogen (BUN) assays*
Animals were euthanized 24 h after CLP and blood was collected to measure renal dysfunction assessed by BUN levels. The assays were performed according to the specification of a commercial kit (Labtest, Brazil).

**Real Time q-PCR**

Lung, kidney and heart were collected in trizol® (Sigma) 12 or 24 h after CLP and macerated for extraction of total RNA. After extraction, an aliquot of 1μL was used to determine the RNA concentration by reading in NanoDrop (Thermo Fisher Scientific) and 1 μg of the extracted RNA was used for the cDNA conversion using the High-Capacity cDNA Reverse transcription kit (Life Technologies) in a thermal cycler. The cDNA was used for the quantification of the Ccbp2 (ACKR2) gene expression (TaqMan® Assay: Mm00445551_m1) by real-time PCR using TaqMan® Universal Master Mix II, at the UNG, according to the manufacturer's instruction (Life Technologies). Gapdh gene (TaqMan® Assay: Gapdh-Mm99999915_g1) was used as a control for normalization of expression levels. The real-time quantitative reaction was performed in the Viia ™ 7 Real-Time PCR System (Applied Biosystems). The results were analyzed using the 2-ΔΔCT method and are expressed in relation to the reference group (sham).

**Patient samples**

Lung samples from patients who died from sepsis (n = 11) and controls (n = 11, individuals who died from other causes), that were stored in paraffin blocks (at the Clinical Hospital, Department of Pathology, Ribeirao Preto Medical School) were used in this study. In addition, sample from 4 patients with Chronic Obstructive Pulmonary Disease (COPD) were used as control for the antibody. The median of the age of the individuals included in the
study was 36 for the septic patients and 38 for the control patients. 27% and 54% of the controls
and septic patients, respectively, were female. The procedures performed with these samples were
approved by the Committee of Ethics in Research with Human Subjects (CEP), Certificate of
Presentation for Ethical Assessment (CAAE) 54775416.5.0000.5440 and official approval
number 1579/2016.

**Immunohistochemistry for ACKR2**

After deparaffinization and hydration, the slides (5 µm) were incubated for 30 min in 0.5%
H\(_2\)O\(_2\) diluted in methanol. The slides were then washed in TBST/Tween (0.05%) for further
incubation in 0.01M citrate buffer (pH = 6.0) for 5 min at 100° C. After washing in
TBST/Tween, the sections were incubated with Fc block for 30 min and in 2.5% horse serum for
a further 30 min. The slides were then labeled with anti-ACKR2 polyclonal rabbit antibody
(Sigma) overnight, at 4° C. The slides were then washed and incubated with an anti-rabbit
secondary antibody (Vector Labs, Peterborough, UK) for 1 h at room temperature. After
washing, peroxidase-conjugated streptavidin (Vector Labs, Peterborough, UK; 30 minutes, room
temperature) was added and the slides were incubated with DAB for 2 min. After hydration, the
slides were stained with hematoxylin and assembled with mounting medium. ACKR2 expression
analyzes were performed at 400X magnification, and 20 fields were evaluated per slide. The
number of positive cells for ACKR2+ cellswas counted visually and the results are expressed as
mean number of ACKR2 positive cells per field.

**Statistical analyzes**

The statistical analyzes were performed using GraphPad Prism. Survival rates were
analyzed by Mantel-Cox log-rank test. Bacterial counts are expressed as medians and
all other mouse data are expressed as box-and-whiskers (Min to Max). The human data are expressed as dot-plot and mean. The human data were analyzed by *t*-test and all the others by ANOVA followed by Bonferroni.

**RESULTS**

*ACKR2-deficient mice are more susceptible to CLP-induced sepsis*

To investigate the potential role of ACKR2 in the pathogenesis of sepsis, we first compared the survival rate of WT and ACKR2−/− mice under different severities of CLP-induced sepsis. In the severe model of CLP (S-CLP), all mice died within 3 days after CLP and there was no difference in the survival rate between the WT and ACKR2−/− mice. However, in the non-severe model of sepsis (NS-CLP), ACKR2−/− mice were significantly more susceptible than the WT mice (survival rate: 88% in WT animals versus 33% in the ACKR2−/− group) (Fig. 1A). These results indicate that ACKR2 plays a significant role in protecting mice against sepsis.

We have previously shown that neutrophil migration to the primary site of infection (peritoneal cavity) during CLP is crucial for preventing the spreading of microorganisms to the circulation and thus avoiding the aggravation of sepsis (15, 20). We therefore determined neutrophil migration and the bacterial load in the peritoneal cavity and in the blood. Surprisingly, there was no significant difference in the number of neutrophils in the peritoneal cavity between the WT and ACKR2−/− mice under the NS-CLP (Fig. 1B). Consistent with this observation, the level of chemokines (CXCL2, CCL2, CCL3, and CCL5) in the peritoneal cavity was also similar in WT and ACKR2−/− mice after NS-CLP (Figs. 1C-F). In addition, the bacterial loads in the peritoneal cavity (Fig. 1G) and in the circulation (Fig. 1H) were also similar between WT and ACKR2−/− animals. These results indicate that the increased severity of the ACKR2−/− mice was
likely to be due to factors other than the reduction of neutrophil migration and bacterial load in the peritoneal cavity.

**ACKR2 controls chemokines accumulation and neutrophil infiltration in secondary organs during sepsis**

We and others have shown that CC chemokines are increased in secondary organs and induce neutrophil accumulation after sepsis induction (21). We therefore investigated the possible role of ACKR2 in the control of the systemic inflammatory response. Lung, kidney and heart were collected 24 h after NS-CLP and the concentrations of chemokine in the tissue homogenates were determined by ELISA. There was a significant increase in the level of CCL3 in the lung (Fig. 2A), CCL5 in the kidney (Fig. 2B) and CCL2 (Fig. 2C) and CCL3 (Fig. 2D) in the heart of ACKR2−/− mice compared to those of the WT mice.

Consistent with this observation, the number of neutrophils was also significantly increased in the lung, kidney and heart of the ACKR2−/− mice compared to those of the WT mice (Fig. 3A-C). The increase in the number of neutrophils would be expected to lead to exacerbated organ damage. This is shown by a significant increase in the blood urea nitrogen (BUN, a measurement of kidney damage) levels (Fig. 3D) and by a tendency to increase in the lung resistance (Fig. 3E) in the ACKR2−/− mice compared to those of the WT mice under NS-CLP.

**Ackr2 mRNA is induced in vital organs after sepsis**

We then investigated whether the expression of ACKR2 is affected by sepsis induction. WT mice were given either non-severe sepsis (NS-CLP) or severe sepsis (S-CLP) and their organs harvested 12 or 24 h after CLP. Ackr2 mRNA levels in the lung 12 h (Fig. 4A) and 24 h (Fig. 4B) after CLP, kidney 24 h after CLP (Fig. 4C) and heart 24 h after CLP (Fig. 4D) were
significantly increased in the mice with NS-CLP compared to those of the control sham-operated mice. In contrast, although *Ackr2* expression was also increased in the lungs of mice under S-CLP 12 h after surgery, its expression returned to the level of the control mice by 24 h. In addition, the levels of ACKR2 mRNA in the kidney and heart of the mice with S-CLP were also not significantly different from those of the control mice 24 h after sepsis induction. These results indicate that the sustained higher level of *Ackr2*mRNA expression is correlated with increased level of survival of septic mice, and hence support the notion that ACKR2 are protective against sepsis through its elevated levels in the vital organs.

**The effect of antibiotics on the sepsis in ACKR2−/− mice**

To confirm that the protective role of ACKR2 during sepsis is due to the participation of this receptor in the systemic inflammatory response, rather than in the control of the local response against the infection, we next used a lethal sepsis model (S-CLP), which induces an exacerbated inflammatory response, and then treat the animals with antibiotics to control the infection. Treatment of mice under S-CLP with antibiotics significantly reduced bacteremia and improved the survival of WT animals, as shown previously (22). While none of the WT mice survived past day 2 of S-CLP (without antibiotic), 40% of WT mice treated with the antibiotic survived S-CLP. In contrast, all the ACKR2−/− mice died from S-CLP by day 2.5 whether treated or not with antibiotics (Fig. 5A). Additionally, the ACKR2−/− mice showed higher levels of myeloperoxidase (MPO) in the lung, kidney and heart than those of the WT mice following S-CLP and antibiotic treatment (Fig. 5B-D). These results therefore confirm that the higher mortality rate in the ACKR2−/− mice was not due to local infection. Instead, the high susceptibility of the ACKR2−/− mice was likely to be due to inflammatory response in the vital organs.
**ACKR2 expression in patients with sepsis**

Finally, we investigated whether the expression of ACKR2 is also altered in the lungs of patients with sepsis. We stained for ACKR2 positive cells in the lung tissues of 11 patients with sepsis and 11 control samples from non-septic patients. The analysis of the expression of ACKR2+ cells in the lung of septic patients showed clear and markedly elevated number of ACKR2+ cells in comparison with control patients (Fig. 6A-C). As a positive control for the antibody used, we also stained ACKR2 in patients with Chronic Obstructive Pulmonary Disease (COPD) (Fig. 6D). Morphological analysis indicates that the ACKR2+ cells are macrophage-like cells. These results are consistent with the elevated levels of Ackr2 mRNA expression in the lungs of mice during the early stage of severe sepsis (Fig. 4A).

**Discussion**

Data reported here demonstrate a hitherto unrecognized protective role of the atypical chemokine receptor, ACKR2, in sepsis. ACKR2 is induced during sepsis and it attenuates the damage to the vital organs caused by sepsis, most likely by reducing the level of local CC chemokines, which are potent mediators in recruiting activated neutrophils to these organs and hence causing damage to the organs. Interestingly, ACKR2 had little or no effect on the levels of chemokines and the number of neutrophils in the primary foci of infection (peritoneal cavity). Consequently, ACKR2 also had no effect on the bacterial load in the peritoneal cavity or in the peripheral blood.

The dichotomy effect of ACKR2 seen here is consistent with the emerging concept separating the control of bacteremia from limiting inflammatory organ damage in the management of sepsis (23). Resistance to pathogens does not always confer protection from
multiple organ damage (24). This is clearly borne out in our study showing that the control of bacterial load by antibiotics did not rescue the ACKR2−/− mice from multiple organ damage caused by the high level of chemokines and neutrophil infiltration in the lung, kidney and heart. This observation is also consistent with a recent observation that treatment of mice under CLP-induced sepsis with anthracycline, a compound used for the treatment of cancer, improved the survival of mice by increasing autophagy and reducing lung injury without affecting the bacterial load, suggesting that anthracycline treatment protected the organ damage during sepsis (25). In addition, animals deficient in the enzyme hemeoxygenase are more susceptible to sepsis, due to increased organ damage with no change in the pathogen load (26).

The levels of systemic cytokines and chemokines vary with the severity of the disease during sepsis. Clinically, the levels of CCL2 and CCL3 are elevated systemically during severe sepsis or septic shock compared with non-severe sepsis (27). One possible explanation is that during severe sepsis the further spread of the pathogens into the bloodstream leads to increased activation of the host cells, culminating in the increased production of the inflammatory mediators. Our data showing that ACKR2 expression is increased in the lungs, kidney and heart of WT mice after the induction of non-severe sepsis, but not in severe sepsis offers an alternative mechanism. Thus, the higher tissue levels of inflammatory CC chemokines during severe sepsis could be also due to the failure to remove and degradate these chemokines by the reduced levels of ACKR2 in these organs. In psoriasis, it was demonstrated that ACKR2 expression is increased in uninvolved skin regions of patients. However, the expression of ACKR2 is reduced or absent in damaged regions. Associated with this, the skin inflammation is increased in regions with injuries, or in areas with low ACKR2 expression (28). In addition, transgenic expression of ACKR2 in keratinocytes prevented the growth of skin tumors in mice by the reduction of skin
inflammation (12). However, the mechanism by which ACKR2 is modulated during sepsis and in these other pathologies is currently unknown and warrants further investigation.

Our clinical data showing that the lungs of septic patients had increased frequency of ACKR2+ cells may at first seem counter intuitive. However, our data from murine sepsis show that both severe and non severe septic mice exhibited elevated Ackr2 mRNA at 12 h time point suggest that the observed increased frequency of ACKR2+ cells in the lungs of the sepsis patients is not inconsistent with a role of ACKR2 in clinical sepsis. Thus, the observed elevated levels of ACKR2 in the patients who succumbed to sepsis may represent a failed-attempt by these patients to control the disease. However, this hypothesis is ethically untestable. In corroboration, alveolar macrophages from patients with chronic COPD also express ACKR2 and may, in same way, controls the acute lung injury during this disease (29).

Sepsis is a complex condition likely involved the interplay of multiple regulatory factors and mediators. Our finding that ACKR2 plays a substantive protective role reducing remote organ injury in sepsis suggests that ACKR2 may be a potential therapeutic agent in this important and clinically unmet condition.

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REFERENCES


FIGURE LEGENDS

Figure 1. ACKR2^-/- mice are more susceptible to CLP-induced sepsis. WT and ACKR2^-/- mice underwent sham, non-severe (NS-CLP) or severe (S-CLP) sepsis surgery. (A) Survival of mice with CLP-induced sepsis. Results are % of survival, analyzed by Mantel-Cox log-rank test, n=6-9 animals/group, from 3-5 experiments. This graph is representative of one experiment. * p<0.05. (B) Neutrophil migration to the peritoneal cavity. The levels of CXCL2 (C), CCL2 (D), CCL3 (E) and CCL5 (F) were measured in the peritoneal exudate. The results are whiskers (min to max), n=6-8 animals/group. (G) Colony-forming units (CFU) in peritoneal exudates and (H) in blood. Horizontal bars are median values, each symbol represents individual mouse.
Figure 2. CC chemokine levels are increased in secondary organs of ACKR2<sup>−/−</sup> mice after CLP. WT and ACKR2<sup>−/−</sup> mice underwent sham or non-severe CLP and the level of CCL3 in the lung (A), CCL5 in the kidney (B), and CCL2 (C) and CCL3 (D) in the heart were measured. The results are whiskers (min to max), and were analyzed by ANOVA followed by Bonferroni, n=4-6 animals/group,*p < 0.05. Data are representative of 2 experiments.

**Figure 2**
Figure 3. ACKR2<sup>−−</sup> mice have increased neutrophils and lesion in peripheral organs after CLP. WT and ACKR2<sup>−−</sup>mice underwent sham or NS-CLP. The neutrophil infiltration in the lung (A), kidney (B) and heart (C) were measured by myeloperoxidase activity (MPO). (D) BUN (blood urea nitrogen) and (E) Lung resistance were measured 24 h after CLP. The results are whiskers (min to max), and were analyzed by ANOVA followed by Bonferroni, n=5-7, *p < 0.05. Data are representative of 2 experiments (A-C, E).
Figure 4. ACKR2 is increased in peripheral organs after CLP. WT mice underwent sham, NS-CLP or S-CLP. ACKR2 mRNA at 12 h (A) and 24 h (B) in the lungs, kidney at 24 h (C) and heart at 24 h (D) were analyzed by Real Time qPCR. The results are whiskers (min to max), and were analyzed by ANOVA followed by Bonferroni, n=5-6 animals/group, * p < 0.05. Data are representative of 2 experiments.
Figure 5. ACKR2 controls systemic inflammation during sepsis. WT and ACKR2−/− mice underwent sham or severe sepsis (S-CLP). Some of the mice were treated with antibiotics (WT + ANT and ACKR2−/− + ANT) after S-CLP. (A) Survival of mice, n=6-8 animals/group, *P<0.05. Neutrophils in the lung (B), kidney (C) and heart (D) were determined 24 h after CLP, by myeloperoxidase activity (MPO). The results are whiskers (min to max), and were analyzed by ANOVA followed by Bonferroni, n=8,*p < 0.05.
Figure 6. ACKR2 expression is increased in the lung of septic patients. Lung tissues of 11 patients with sepsis and 11 control samples from non-septic patient were stained with anti-ACKR2 antibody for immunohistochemistry. (A-B) Representative images of ACKR2 expression in control (A) and in septic patients (B) are shown. Arrows indicate ACKR2 positive cells. (C) Number of ACKR2 positive cells per field in the lung of control and septic patients. Twenty fields were analyzed per slide. *P<0.05 by Student’s t test. (D) Representative image of ACKR2 expression inpatient with Chronic Obstructive Pulmonary Disease (COPD). Arrows indicate ACKR2 positive cells.