THE ATYPICAL CHEMOKINE RECEPTOR ACKR2 IS PROTECTIVE AGAINST SEPSIS

Fernanda Vargas e Silva Castanheira[1]; Vanessa Borges[1]; Fabiane Sônego[1]; Alexandre Kanashiro[1]; Paula BarbimDonate[2];Paulo Henrique Melo [2]; KennethPallas[3]; Remo Castro Russo[4];Flávio Almeida Amaral[5]; Mauro Martins Teixeira[5];Fernando Silva Ramalho [6]; Thiago Mattar Cunha[1]; Foo Y Liew[3,7]; José Carlos Alves-Filho [1];Gerard J Graham[3]; Fernando Queiroz Cunha [1].

Departments of [1] Pharmacology, [2] Biochemistry and Immunology, [6] Pathology and Legal Medicine, RibeirãoPreto Medical School, University of Sao Paulo, Ribeirao Preto, Brazil.

[3]Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, Scotland.

[4] Laboratory of Pulmonary Immunology and Mechanics, Departments of Physiology and Biophysics;[5]Laboratory of Immunopharmacology, Biochemistry and Immunology, Federal University of Minas Gerais/UFMG, Belo-Horizonte, Brazil

[7] School of Biology and Basic Medical Sciences, Soochow University, Suzhou 215006, China.

Correspondingauthor: Prof. Fernando Q. Cunha, PHD. DepartmentofPharmacology, Ribeirao Preto Medical School, UniversityofSao Paulo, Avenida Bandeirantes, 3900, Ribeirao Preto, São Paulo, Brazil. ZIP Code: 14049-900. Tel. 55 16 3315 3324, Fax. 55 16 3633 2301. E-mail: fdqcunha@fmrp.usp.br.

Running Head: ACKR2and sepsis

ABSTRACT

Sepsis is a systemic inflammatory responseas a result of uncontrolled infections. Neutrophils are the first cells to reach the primary sites of infection and chemokinesplay 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 severelesions 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^{+} cellscompared to those of non-septic patients. Our data indicate that ACKR2 may have a protective role during sepsis, and the absence of ACKR2leads to exacerbated chemokine accumulation, neutrophil infiltration and damageto vital organs.

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

INTRODUCTION

Sepsis is life-threatening organ dysfunction causedby 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 processesaremainly mediatedby 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 bindswith high affinity to inflammatory chemokines from the CC subfamilyleading 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 thepathophysiology 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. ACKR2$^{-/-}$ 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 RibeirãoPreto 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, UniãoQuimica, 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, WhitehouseStation, NJ) beginning 6 h after CLP and thenevery 12 h up to day 3. The survival of mice was recordedevery 12 h for 10 days. Mice showing signs of

ataxia, tremor and/or agonal breathing wereeuthanized by overdose of ketamine/xylazine followed by cervical dislocation. At the end of 10 days,surviving mice were euthanized. Some mice were also euthanizedat 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

Theconcentrations of chemokines in the lungs, kidneys and heartwere 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 wasdeterminedby 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, aspreviously 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 performedaccording 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 werestored in paraffin blocks (at the ClinicalHospital, 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 deparaffinizationand hydration, the slides (5 μm)were incubated for 30 min in 0.5% H_2O_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 andthe 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

allothermouse 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 firstcompared the survival rate of WT and ACKR2^{-/-} mice underdifferent 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 modelof 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 shownthat neutrophil migration to the primary site of infection (peritoneal cavity) during CLP is crucial for preventing the spreading of microorganisms to the circulation and thusavoidingthe 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 thereduction 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 accumulationafter 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 afterNS-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 heartof ACKR2$^{-/-}$ mice compared to those of the WT mice.

Consistent with this observation, the number of neutrophils was also significantlyincreased 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 neutrophilswould be expected to lead to exacerbated organ damage. This is shownby a significant increase in theblood 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 or24 h after CLP. *Ackr2* mRNA levels in the lung12 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 expressionreturned to the level of the control mice by 24 h. In addition, the levels of ACKR2 mRNA in thekidney and heartof the micewith 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 expressionis correlated with increasedlevel 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 controlthe infection. Treatment of mice under S-CLP with antibiotics significantly reduced bacteremia andimprovedthe survival of WT animals, as shown previously(22). While none of the WTmice survived past day 2 of S-CLP (without antibiotic), 40% of WT mice treated with the antibiotic survivedS-CLP. In contrast, all the ACKR2$^{-/-}$ mice died from S-CLP by day 2.5whether 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 ofACKR2is 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 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 thattreatment 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 areaswith 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 ofseptic patients had increased frequency of ACKR2^{+} cellsmay 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.

Acknowledgment

REFERENCES

1.   Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM, et al.: The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *Jama* 315, 801-10, 2016.

2.   Alves-Filho JC, de Freitas A, Spiller F, Souto FO, Cunha FQ: The role of neutrophils in severe sepsis. *Shock* 30 Suppl 1, 3-9, 2008.

3.   Shen XF, Cao K, Jiang JP, Guan WX, Du JF: Neutrophil dysregulation during sepsis: an overview and update. *J Cell Mol Med*, 2017.

4.   Sonego F, Castanheira FV, Ferreira RG, Kanashiro A, Leite CA, Nascimento DC, Colon DF, Borges VdeF, Alves-Filho JC, Cunha FQ: Paradoxical Roles of the Neutrophil in Sepsis: Protective and Deleterious. *Front Immunol* 7, 155, 2016.

5.   Bokoch GM: Chemoattractant signaling and leukocyte activation. *Blood* 86, 1649-60, 1995.

6.   Bachelerie F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ, Horuk R, Sparre-Ulrich AH, Locati M, Luster AD, et al.: International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. *Pharmacol Rev* 66, 1-79, 2014.

7.   Graham GJ: D6 and the atypical chemokine receptor family: novel regulators of immune and inflammatory processes. *Eur J Immunol* 39, 342-51, 2009.

8. Nibbs RJ, Wylie SM, Pragnell IB, Graham GJ: Cloning and characterization of a novel murine beta chemokine receptor, D6. Comparison to three other related macrophage inflammatory protein-1alpha receptors, CCR-1, CCR-3, and CCR-5. *J Biol Chem* 272, 12495-504, 1997.

9. Nibbs RJ, Wylie SM, Yang J, Landau NR, Graham GJ: Cloning and characterization of a novel promiscuous human beta-chemokine receptor D6. *J Biol Chem* 272, 32078-83, 1997.

10. Jamieson T, Cook DN, Nibbs RJ, Rot A, Nixon C, McLean P, Alcami A, Lira SA, Wiekowski, M., Graham, G. J.: The chemokine receptor D6 limits the inflammatory response in vivo. *Nat Immunol* 6, 403-11, 2005.

11. Martinez de la Torre Y, Locati M, Buracchi C, Dupor J, Cook DN, Bonecchi R, Nebuloni M, Rukavina D, Vago L, Vecchi A, et al.: Increased inflammation in mice deficient for the chemokine decoy receptor D6. *Eur J Immunol* 35, 1342-6, 2005.

12. Nibbs RJ, Gilchrist DS, King V, Ferra A, Forrow S, Hunter KD, Graham GJ: The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumors. *J Clin Invest* 117, 1884-92, 2007.

13. Vetrano S, Borroni EM, Sarukhan A, Savino B, Bonecchi R, Correale C, Arena V, Fantini M, Roncalli M, Malesci A, et al.: The lymphatic system controls intestinal inflammation and inflammation-associated Colon Cancer through the chemokine decoy receptor D6. *Gut* 59, 197-206, 2010.

14. Clark JD, Gebhart GF, Gonder JC, Keeling ME, Kohn DF: Special Report: The 1996 Guide for the Care and Use of Laboratory Animals. *ILAR J* 38, 41-48, 1997.

15.   Benjamim CF, Silva JS, Fortes ZB, Oliveira MA, Ferreira SH, Cunha FQ: Inhibition of leukocyte rolling by nitric oxide during sepsis leads to reduced migration of active microbicidal neutrophils. *Infect Immun* 70, 3602-10, 2002

16.   Godshall CJ, Scott MJ, Peyton JC, Gardner SA, Cheadle WG: Genetic background determines susceptibility during murine septic peritonitis. *J Surg Res* 102, 45-9, 2002.

17.   Cunha TM, Verri WA Jr, Schivo IR, Napimoga MH, Parada CA, Poole S, Teixeira MM, Ferreira SH, Cunha FQ: Crucial role of neutrophils in the development of mechanical inflammatory hypernociception. *J Leukoc Biol* 83, 824-32, 2008.

18.   Moreno SE, Alves-Filho JC, Bertozi G, Alfaya TM, Theze J, Ferreira SH, Vargaftig BB: Systemic administration of interleukin-2 inhibits inflammatory neutrophil migration: role of nitric oxide. *Br J Pharmacol* 148, 1060-6, 2006.

19.   Nogueira DS, Gazzinelli-Guimaraes PH, Barbosa FS, Resende NM, Silva CC, de Oliveira LM, AmorimCC, Oliveira FM, Mattos MS, Kraemer LR, et al.: Multiple Exposures to Ascaris suum Induce Tissue Injury and Mixed Th2/Th17 Immune Response in Mice. *PLoS Negl Trop Dis* 10, e0004382, 2016.

20.   Benjamim CF, Ferreira SH, Cunha FQ: Role of nitric oxide in the failure of neutrophil migration in sepsis. *J Infect Dis* 182, 214-23, 2000.

21.   Souto FO, Alves-Filho JC, Turato WM, Auxiliadora-Martins M, Basile-FilhoA, Cunha FQ: Essential role of CCR2 in neutrophil tissue infiltration and multiple organ dysfunction in sepsis. *Am J Respir Crit Care Med* 183, 234-42, 2011.

22.   Nascimento DC, Alves-Filho JC, Sonego F, Fukada SY, Pereira MS, Benjamim C, Zamboni DS, Silva JS, Cunha FQ: Role of regulatory T cells in long-term immune dysfunction associated with severe sepsis. *Crit Care Med* 38, 1718-25, 2010.

23.   Ayres JS and Schneider DS: Tolerance of infections. *Annu Rev Immunol* 30, 271-94, 2012.

24.   Medzhitov R: Septic shock: on the importance of being tolerant. *Immunity* 39, 799-800, 2013.

25.   Figueiredo N, Chora A, Raquel H, Pejanovic N, Pereira P, Hartleben B, Neves-Costa A, Moita C, Pedroso D, Pinto A, et al.: Anthracyclines induce DNA damage response-mediated protection against severe sepsis. *Immunity* 39, 874-84, 2013.

26.   Larsen R, Gozzelino R, Jeney V, Tokaji L, Bozza FA, Japiassu AM, Bonaparte D, Cavalcante MM, Chora A, Ferreira A, et al.: A central role for free heme in the pathogenesis of severe sepsis. *Sci Transl Med* 2, 51ra71, 2010.

27.   Vermont CL, Hazelzet JA, de Kleijn ED, van den Dobbelsteen GP, de Groot R: CC and CXC chemokine levels in children with meningococcal sepsis accurately predict mortality and disease severity. *Crit Care* 10, R33, 2006.

28.   Singh MD, King V, Baldwin H, Burden D, Thorrat A, Holmes S, McInnes IB, Nicoll R, Shams K, Pallas K, et al.: Elevated expression of the chemokine-scavenging receptor D6 is associated with impaired lesion development in psoriasis. *Am J Pathol* 181, 1158-64, 2012.

29.   Bazzan E, Saetta M, Turato G, Borroni EM, Cancellieri C, Baraldo S, Savino B, Calabrese F, Ballarin A, Balestro E, et al.: Expression of the atypical chemokine receptor D6 in human alveolar macrophages in COPD. Chest 143, 98-106, 2013.

FIGURE LEGENDS

Figure 1. ACKR2$^{-/-}$ mice are more susceptible to CLP-induced sepsis. WT and ACKR2$^{-/-}$ miceunderwent 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 theperitoneal 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.
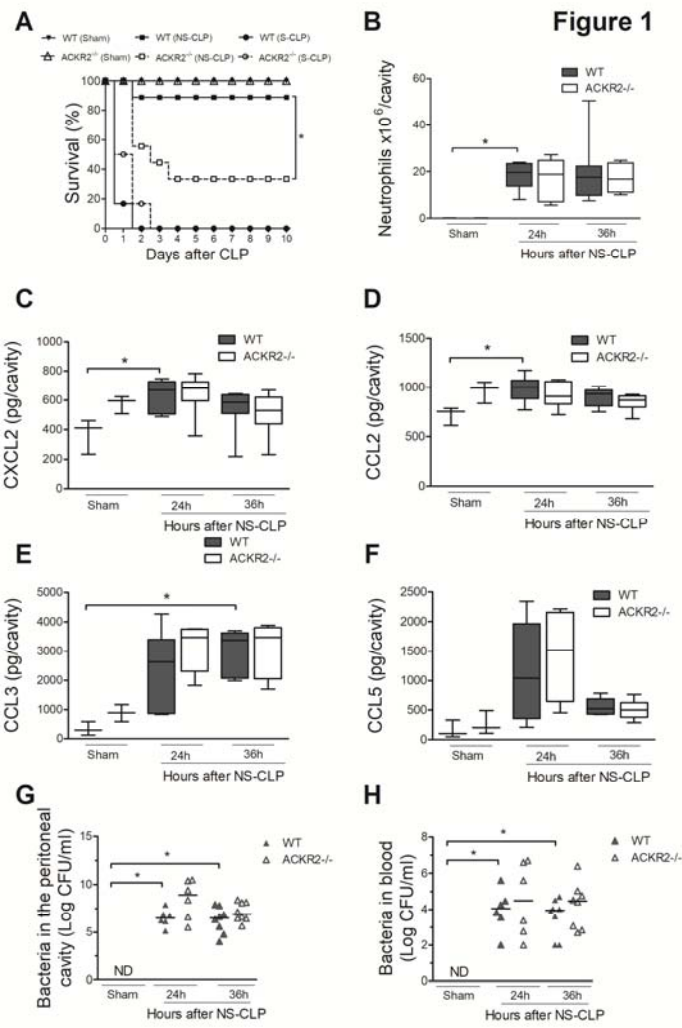
Figure 1

Figure 2. CC chemokine levels are increased in secondary organs of ACKR2^{-/-} mice after CLP. WT and ACKR2^{-/-} 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.
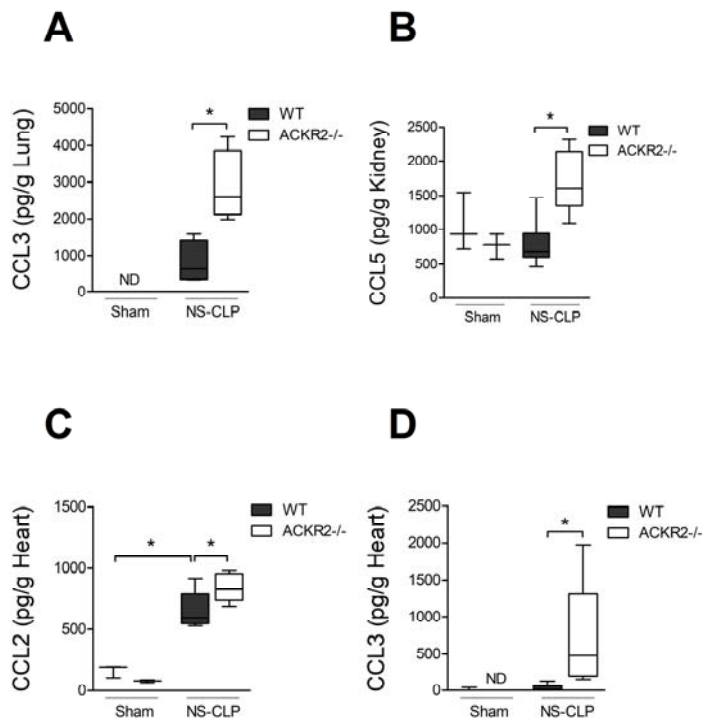

Figure 2

Figure 3. ACKR2$^{-/-}$ mice have increased neutrophils and lesion in peripheral organs after CLP. WT and ACKR2$^{-/-}$mice underwent sham orNS-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 arewhiskers (min to max), and were analyzed by ANOVA followed by Bonferroni, n=5-7,*p < 0.05. Data are representative of 2experiments (A-C, E).
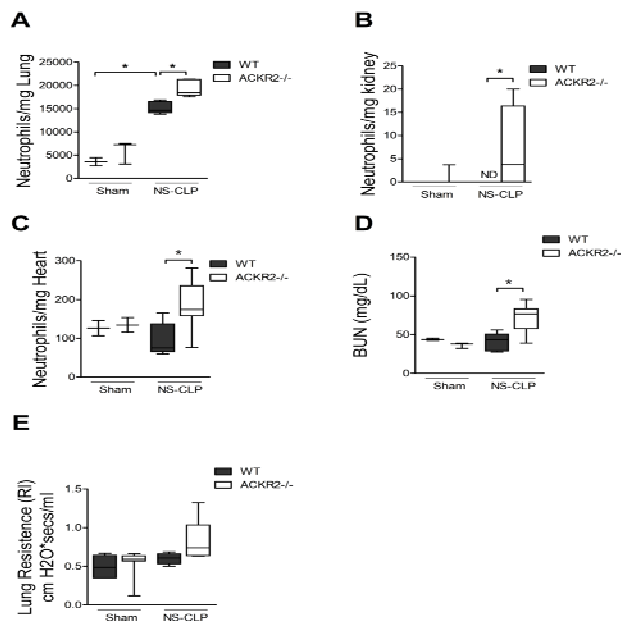

Figure 3

Figure 4. ACKR2is increasedin peripheral organs after CLP. WT mice underwent sham,NS-CLPorS-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 arewhiskers (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.
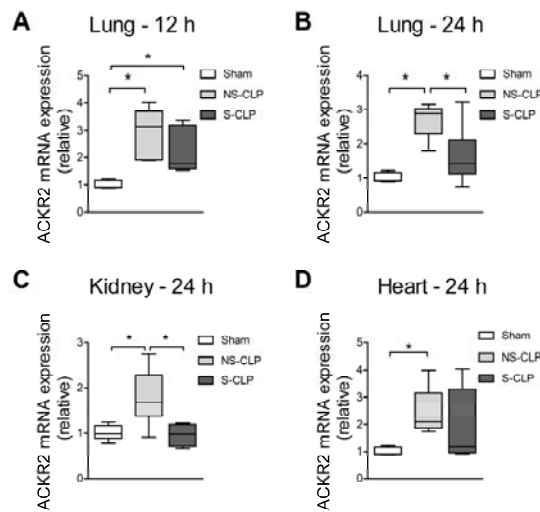

Figure 4

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 arewhiskers (min to max), and were analyzed by ANOVA followed by Bonferroni, n=8,*p < 0.05.
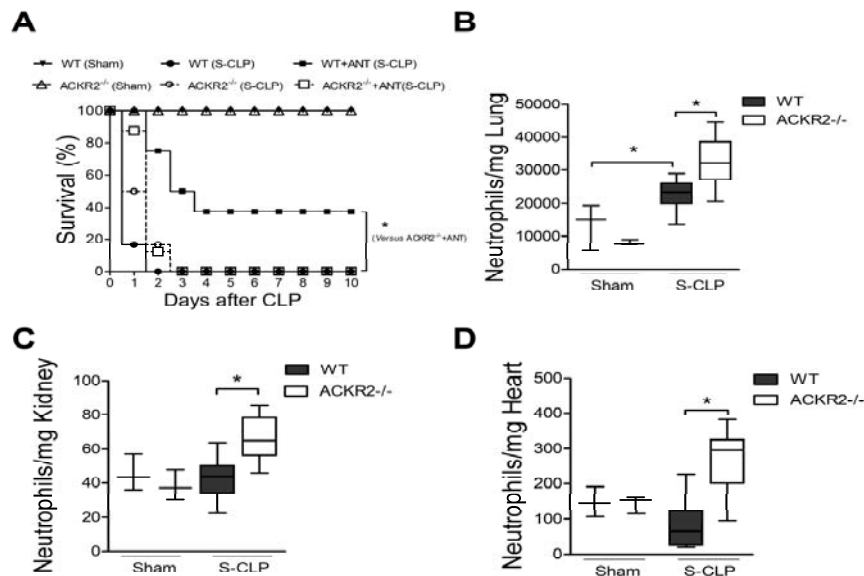

Figure 5

Figure 6. ACKR2expression is increased in the lung of septic patients. Lung tissues of 11 patients with sepsis and 11 control samples from non-septic patientswere 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.

Figure 6