ORIGINAL ARTICLE

P2X7 receptor deletion attenuates oxidative stress and liver damage in sepsis

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Abstract

Sepsis is a severe disease characterized by an uncontrolled systemic inflammation and consequent organ dysfunction generated in response to an infection. Extracellular ATP acting through the P2X7 receptor induces the maturation and release of pro-inflammatory cytokines (i.e., IL-1β) and the production of reactive nitrogen and oxygen species that lead to oxidative tissue damage. Here, we investigated the role of the P2X7 receptor in inflammation, oxidative stress, and liver injury in sepsis. Sepsis was induced by cecal ligation and puncture (CLP) in wild-type (WT) and P2X7 knockout (P2X7$^{-/-}$) mice. The oxidative stress in the liver of septic mice was assessed by 2′,7′-dichlorofluorescein oxidation reaction (DCF), thiobarbituric acid-reactive substances (TBARS), and nitrite levels dosage. The status of the endogenous defense system was evaluated through catalase (CAT) and superoxide dismutase (SOD) activities. The inflammation was assessed histologically and by determining the expression of inflammatory cytokines and chemokines by RT-qPCR. We observed an increase in the reactive species and lipid peroxidation in the liver of septic WT mice, but not in the liver from P2X7$^{-/-}$ animals. We found an imbalance SOD/CAT ratio, also only WT septic animals. The number of inflammatory cells and the gene expression of IL-1 β, IL-6, TNF-α, IL-10, CXCL1, and CXCL2 were higher in the liver of WT septic mice in comparison to P2X7$^{-/-}$ septic animals. In summary, our results suggest that the P2X7 receptor might be a therapeutic target to limit oxidative stress damage and liver injury during sepsis.

Keywords Sepsis · Purinergic signaling · ATP · Liver · Oxidative stress

Introduction

Sepsis is a life-threatening organ dysfunction characterized by an uncontrolled and excessive inflammatory response to an

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infection [1, 2]. Although considerable progress has been made in medical assistance over the last decades, sepsis remains a healthcare challenge worldwide [3, 4]. The inflammatory response and organ injury in sepsis initiate when pathogen-associated molecular patterns (PAMPs) bind to pattern recognition receptors (PRR), such as toll-like receptors (TLR), on the surface of immune cells [5, 6]. PRRs trigger activation of inflammatory signaling pathways and the production of inflammatory mediators, including cytokines, chemokines, and oxygen and nitrogen reactive species [7–9]. When the inflammation becomes systemic and excessive, the overproduction of inflammatory mediators affects the functionality of several vital organs, including the liver [10–12].

The liver is the largest gland in the human body. This organ mediates several essential metabolic processes and modulates immunological responses [12–14]. The strategic position of the liver in the bloodstream permanently exposes the organ to several antigens and microbial products with a potential immunostimulatory capacity [14, 15]. Therefore, the liver

plays a central role in the development of systemic inflammation in the initial course of sepsis by sensing danger signals; recognizing pathogens; producing cytokines, chemokines, and acute-phase proteins; and regulating metabolism. Therefore, liver protection is critical for host survival in sepsis [12, 16–18].

Extracellular adenosine triphosphate (eATP) plasma levels increase during sepsis [19]. This nucleotide is an important danger signal that regulates the recruitment and function of inflammatory cells via purinergic receptors [20, 21]. The P2 purinergic family is composed of G protein-coupled P2Y and ionotropic P2X receptors [22]. Among the P2 receptor, the P2X7 receptor has been the most associated with the activation of oxidative and inflammatory mechanisms in several inflammatory and infectious diseases [23–28]. The P2X7 receptor is a critical player in the activation of the NLRP3 inflammasome, acting as a second signal for caspase-1 activation and subsequent release of IL1-β [29, 30]. Besides, this receptor mediates the production of ROS and NO in inflammatory cells [31, 32]. In sepsis, P2X7 genetic deletion improves survival and reduces circulating inflammatory cytokines and systemic inflammation [23, 33]. Moreover, P2X7 receptor pharmacological inhibition reduces sepsis-induced intestinal barrier disruption [27] and brain inflammation and oxidative damage [24, 34]. P2X7 receptor induces inflammation and oxidative damage in a model of carbon tetrachloride-induced steatohepatitis in obese mice by NADPH oxidase-dependent mechanisms [35]. Therefore, here we sought to investigate the role of ATP-gated P2X7 receptor in sepsis-induced inflammation, oxidative damage, and liver injury in sepsis.

Materials and methods

Animals

We used adult male (8–10 weeks old; 25–30 g weight) wild-type (WT) and P2X7 receptor-deficient (P2X7$^{-/-}$) C57BL/6 mice originally obtained from Jackson Laboratories (Bar Harbor, ME, USA). The animals had free access to food and water and were maintained on a 12:12-h light-dark cycle at a temperature of 22 ± 1 °C. Experiments were performed with age- and weight-matched animals, according to the guidelines of the Brazilian College of Animal Experimentation (COBEA). The Commission for the Ethical Use of Research Animals (CEUA) from the Federal University of Rio de Janeiro (UFRJ) approved all experiments (protocol number: IBCCF138).

Sepsis induction by cecal ligation and puncture

Sepsis was induced by cecal ligation and puncture (CLP), a procedure that generates acute polymicrobial peritonitis and,

subsequently, a systemic inflammation, as previously described [36, 37]. Mice were anesthetized by the injection of ketamine (80 mg/kg) and xylazine (5 mg/kg) intraperitoneally, and a 1-cm incision was made on the abdomen. The cecum was exposed and ligated below the ileocecal junction. A double puncture was made using a 21-gauge needle, a small amount of stool was gently squeezed out to induce peritonitis, and the peritoneal cavity and abdominal wall were closed by suture. Sham-operated animals (controls) underwent an identical laparotomy but without ligation and perforation. Animals received 1 mL of 0.9% isotonic NaCl sterile solution subcutaneously to compensate for the third spacing that occurred during the surgery. The liver samples were collected 24 h after the procedure.

Alanine aminotransferase and aspartate aminotransferase dosage

For the determination of ALT and AST, kinetic kits commercially available (Bioclin®, Belo Horizonte, MG, Brazil; cat#K049-6 and K048-6, respectively) were used, and the experiments were carried out according to the manufacturer's instructions. Briefly, 24 h after sepsis induction, the animals were euthanized in a chamber with an atmosphere rich in CO_2, and the blood was collected through cardiac puncture using a 1-mL syringe/27-G needle with sodium citrate (5%). Plasma was obtained through centrifugation ($2000 \times g$ for 10 min) and used for enzymatic analysis. The enzyme reaction was analyzed on a SpectraMax2 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts). The results are expressed in units per liter (U/L).

Oxidative profile

Tissue preparation for oxidative analysis

The liver tissue was homogenized in 10 volumes (1:10 w/v) of 20 mM sodium phosphate buffer (pH 7.4) containing 140 mM KCl and centrifuged at $800 \times g$ for 10 min at 4 °C. The supernatant was used immediately for the determination of oxidative parameters.

2′,7′-Dichlorofluorescein (H₂DCF) oxidation assay

The production of reactive species was measured according to the methodology based on the oxidation of the 2′,7′-dichlorofluorescein (H₂DCF) proposed by LeBel et al. [38]. The samples were incubated in the dark in a 2′,7′-dichlorofluorescein diacetate solution (H₂DCF-DA) for 30 min at 37 °C. H₂DCF-DA is cleaved by cellular esterases, and the resulting H₂DCF is eventually oxidized by the reactive species present in the samples. This last reaction produces a fluorescent product: dichlorofluorescein (DCF), which was

measured at 488-nm excitation and 525-nm emission. Results are presented in nmol DCF per milligram protein [38].

Dosage of thiobarbituric acid-reactive substances

As previously as described by Ohkawa et al. [39], tissue supernatant (200 μL) was mixed with 50 μL 8.1% SDS; 375 μL of 20% acetic acid in aqueous solution (v/v) pH 3.5; and 375 μL of 0.8% thiobarbituric acid and mixed in the vortex. The reaction is produced in a boiling water bath for 1 h. The mixture was cooled in water for 5 min and centrifuged $750 \times g$ for 10 min. TBARS levels were determined by spectrophotometry at 535 nm. A calibration curve with the standard 1,1,3,3-tetrametoxypropane was used. TBARS levels are expressed as TBARS nanomole per milligram protein.

Determination of total sulfhydryl content

The oxidation of thiols in samples leads to the formation of disulfide bonds. Sulfhydryl content is inversely proportional to the oxidative damage in proteins. In this method, 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB) undergoes a chemical modification in the presence of sulfhydryl groups, generating 5-thio-2-nitrobenzoate (TNB). We mixed 50 μL of homogenate with 980 μL of PBS buffer pH 7.4 containing 1 mM EDTA. The reaction was started by the addition of 30 μL of 10 mM DTNB and incubated for 30 min at room temperature in a dark room. The absorbance of this yellow product (TNB) was measured spectrophotometrically at 412 nm. The results were presented as TNB per milligram protein [40].

Nitric oxide production

Nitrite, which is a metabolite of nitric oxide (NO), was evaluated using Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-Naphthyl)ethylenediamine dihydrochloride in water. The samples were incubated 10 min at room temperature in a Griess reagent. Nitrite was used as the standard, and absorbance was measured at 543 nm. The results were expressed as micromole of nitrite per milligram of protein [41].

Catalase assay

CAT assay is based on the consumption of H_2O_2, which is measured by a spectrophotometer at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM phosphate buffer solution pH 7.0, and an amount of protein from 0.1 to 0.3 mg per mL of reaction medium [42]. A unit of CAT is defined as 1 mmol of H_2O_2 consumed per min, and the specific activity was represented as units of CAT/mg of protein.

Superoxide dismutase assay

SOD activity is based on the ability of the pyrogallol compound to self-oxidize in the presence of superoxide, which is also a substrate for the SOD enzyme. The inhibition of the auto-oxidation of this compound occurs in the presence of SOD, whose activity was measured indirectly by spectrophotometry at 420 nm [43]. A calibration curve was made with purified SOD as a standard to calculate SOD activity in the samples. The results were presented as units of SOD per milligram of protein.

Protein quantification

The determination of the total amount of proteins was made using a spectrophotometric method using the Pierce BCA Kit (Thermo Fisher Scientific) and the SpectraMax2 plate reader (Thermo Fisher Scientific) under the manufacturer's instructions. This method combines the reduction of copper (II) to copper (I) cations by proteins in an alkaline medium (the biuret method) with the selective and sensitive colorimetric detection of copper (I) cations. The purple reaction product exhibits an absorbance at 562 nm, whose increase is practically linear with the increase in protein concentration. Protein concentrations were reported on a calibration curve using bovine serum albumin (BSA) absorbance values as standard. The expression of the total protein concentration in the samples is shown in microgram per milliliter.

RNA isolation and real-time quantitative PCR

For the extraction of total RNA from the liver tissue, the TRIzol® reagent (Thermo Fisher Scientific, Somerset, NJ, USA) was used according to the manufacturer's instructions. RNA samples were quantified, and the purity was assessed in a NanoDrop BioChrom apparatus (Harvard Bioscience, Holliston, Massachusetts, USA). The synthesis of cDNA was performed with 1 μg of total RNA using the High Capacity Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific, Somerset, NJ, USA) according to the manufacturer's instructions in a Master Cycler Gradient thermocycler (Eppendorf, Hamburg, Germany).

The real-time quantitative PCR reactions (RT-qPCR) were performed using the Master Mix SYBR Green PCR (Applied Biosystems, Foster City, California, USA) in a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, California, USA). The reactions were performed in a final volume of 10 μL, using 2 μL of diluted cDNA (1:10) and 300 nM of each of the primer forward and reverse. The relative gene expression was calculated using the comparative cycle threshold (Ct) method (ΔΔCt). The β-actin gene (*Actb*) was used as the endogenous control. The sham WT group was chosen as the control group. The primer sequences

are provided in Table 1. The results are presented as relative expression of the gene of interest/*Actb*.

Histological analysis

The liver was harvested 24 h after induced sepsis and fixed with 4% paraformaldehyde, embedded in paraffin, and 5-μm-thick slices were stained with hematoxylin-eosin. A pathologist, who was blinded to the experimental groups, analyzed the histopathology and performed the semiquantitative analyses.

A histological scoring system was applied to evaluate the degree of liver injury associated with sepsis after observation of all liver sections of each animal. The histological parameters steatosis, spotty (focal) necrosis, and ballooning degeneration were considered. The hepatic injury scoring measures features of liver injury induced by sepsis and is represented as the unweighted sum of scores of steatosis (0–3), hepatocellular ballooning or degeneration (0–2), and spotty (focal) necrosis (0–2) with the final score ranging between 0 and 7. This analysis was adapted from the histological scoring system for the nonalcoholic fatty liver disease [44].

The degree of steatosis (steatosis identified as homogeneous lipid droplet in the hepatocyte cytoplasm present as microvesicular or macrovesicular) was graded and scored as

follows: 0, none; 1, ≤ 30% hepatocytes containing fat; 2, 30%–70% hepatocytes containing fat; 3, ≥ 70% hepatocytes containing fat. Ballooning degeneration was graded and scored as follows: 0, normal hepatocytes with cuboidal shape and pink eosinophilic cytoplasm; 1, presence of clusters of hepatocytes with a rounded shape and pale, reticulated cytoplasm and the same size of normal ones; 2, clusters of rounded, enlarged hepatocytes. The grade of spotty necrosis considered was as follows: 0, lack of focal necrosis; 1, one focus viewed with a 10× objective lens; 2, more than two foci viewed with a 10× objective lens.

Lobular inflammation was evaluated in histological sections of the liver stained with HE and represented the presence of polynucleated cells (neutrophils) or mononucleate cells (macrophages/monocytes or lymphocytes) in the sinusoidal lumen. Fifteen photomicrographs were obtained from each animal using a light microscope Eclipse E800 (Nikon, Japan) assisted with a digital camera Evolution VR Cooled Color 13 bits (Media Cybernetics, Bethesda, USA). The software Q-Capture 2.95.0, version 2.0.5 (Silicon Graphics Inc., EUA), was utilized to obtain high-resolution images (2048 X1536 pixels buffer) using the 20× objective lens. Results were expressed as the total number of inflammatory cells/ histological field.

Statistical analysis

The Kolmogorov-Smirnov statistical test was used to verify whether the data obtained in this work has a normal distribution. The results were analyzed through a two-way analysis of variance (two-way ANOVA) followed by Tukey's multiple tests, considering $p < 0.05$ as a statistical difference. The GraphPad Prism Version 5 program (GraphPad Software Incorporation) was used for data analysis.

Results

P2X7 receptor expression increases in the liver of septic mice

ATP-P2X7 receptor signaling seems to be relevant for the excessive inflammation in sepsis. Initially, we found that P2X7 receptor expression significantly increases in the liver of WT septic animals 24 h after sepsis induction (Fig. 1; $p < 0.05$), suggesting the participation of this receptor in hepatic dysfunction in sepsis.

P2X7 receptor genetic deletion attenuates liver injury in septic mice

Hepatocyte lesions were determined by quantifying the activity of liver enzymes (ALT and AST) in the mice serum 24 h

Table 1 PCR primer sequences

Gene		Sequence (5′–3′)
P2rX7	Sense	AAT CGG TGT GTT TCC TTT GG
P2rX7	Antisense	CCG GGT GAC TTT GTT TGT CT
Nox1	Sense	TAA ATT GCC CCT CCA TTT CC
Nox1	Antisense	CCC TGC TGC TCG AAT ATG AA
Nox2	Sense	CCC TTT GGT ACA GCC AGT GAA GAT
Nox2	Antisense	CAA TCC CGG CTC CCA CTA ACA TCA
Nos2	Sense	ACA TCG ACC CGT CCA CAG TAT
Nos2	Antisense	CAG AGG GGT AGG CTT GTC TC
Il1β	Sense	TTC AGG CAG GCA GTA TCA CTC
Il1β	Antisense	CCA CGG GAA AGA CAC AGG TAG
Il6	Sense	CTG CAA GAG ACT TCC ATC CAG
Il6	Antisense	AGT GGT ATA GAC AGG TCT GTT GG
Tnf	Sense	GGT CCC CAA AGG GAT GAG AAG TTC
Tnf	Antisense	CCA CTT GGT GGT TTG CTA CGA CG
Il10	Sense	GCT GGA CAA CAT ACT GCT AAC C
Il10	Antisense	ATT CCG ATA AGG CTT GGC AA
Cxcl1	Sense	ACT GCA CCC AAA CCG AAG TC
Cxcl1	Antisense	TGG GGA CAC CTT TTA GCA TCT T
Cxcl2	Sense	CCA ACC ACC AGG CTA CAG G
Cxcl2	Antisense	GCG TCA CAC TCA AGC TCT G
Actb	Sense	TAT GCC AAC ACA GTG CTG TCT GG
Actb	Antisense	ACT CCT GCT TGC TGA TCC ACA T
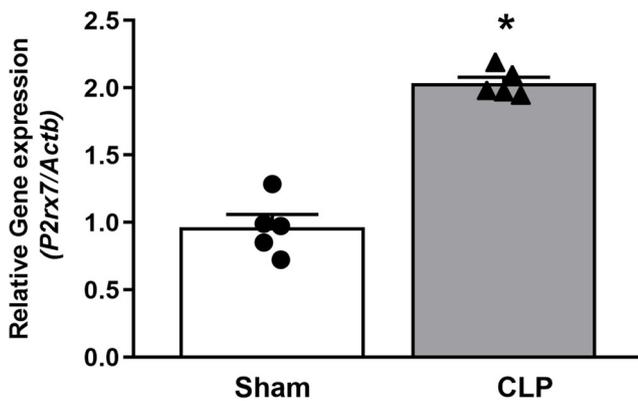
Fig. 1 P2X7 receptor gene expression in the liver of septic mice. P2X7 gene expression was analyzed by RT-qPCR in the liver from septic mice 24 h after surgery. Data are expressed as mean ± S.E.M. ($n = 5$ for both sham and CLP groups). Data were analyzed by t test. Statistically significant differences between sham and CLP groups are represented by asterisks (*$p < 0.05$)

after sepsis induction. The presence of these enzymes in the circulation provides evidence of the extent and magnitude of the hepatic lesion. An increase in AST and ALT was observed in the serum from both WT and P2X7$^{-/-}$ septic animals when compared to sham groups ($p < 0.05$) (Fig. 2 a and b, respectively). However, this increase in both enzymes was significantly lower in P2X7$^{-/-}$ septic animals in comparison to WT septic mice ($p < 0.05$) (Fig. 2). Thus, the absence of the P2X7 receptor seems to be important to protect liver integrity during the initial 24 h of sepsis.

P2X7 receptor genetic deletion reduces sepsis-induced oxidative stress in mice liver

We observed a significant increase ($p < 0.05$) in the production of reactive species in septic WT animals when compared to the WT sham group, confirming the well-reported oxidative stress-induced by sepsis (Fig. 3a, left). P2X7-deficient septic mice did not show an increase in DCF oxidation when compared to their respective sham group ($p > 0.05$). Therefore, the genetic deletion of P2X7 blocked the increase in DCF oxidation in the liver homogenates from septic animals compared to the septic WT group ($p < 0.05$) (Fig. 3a, right). In addition to the increase in free radical production, we observed an increase ($p < 0.05$) in lipid peroxidation and nitrite content in WT septic animals in comparison with the WT sham group (Fig. 3 b and d, left). No increases ($p > 0.05$) were observed in P2X7$^{-/-}$ septic mice when compared to the P2X7$^{-/-}$ sham group (Fig. 3 b and d, right). No differences were detected in the sulfhydryl content (Fig. 3c; $p > 0.05$). Our results confirm the oxidative stress induced by sepsis in the liver and show that P2X7-deficient animals were protected, suggesting that the P2X7 receptor may be necessary for the production of reactive species in the liver of septic animals 24 h after surgery.
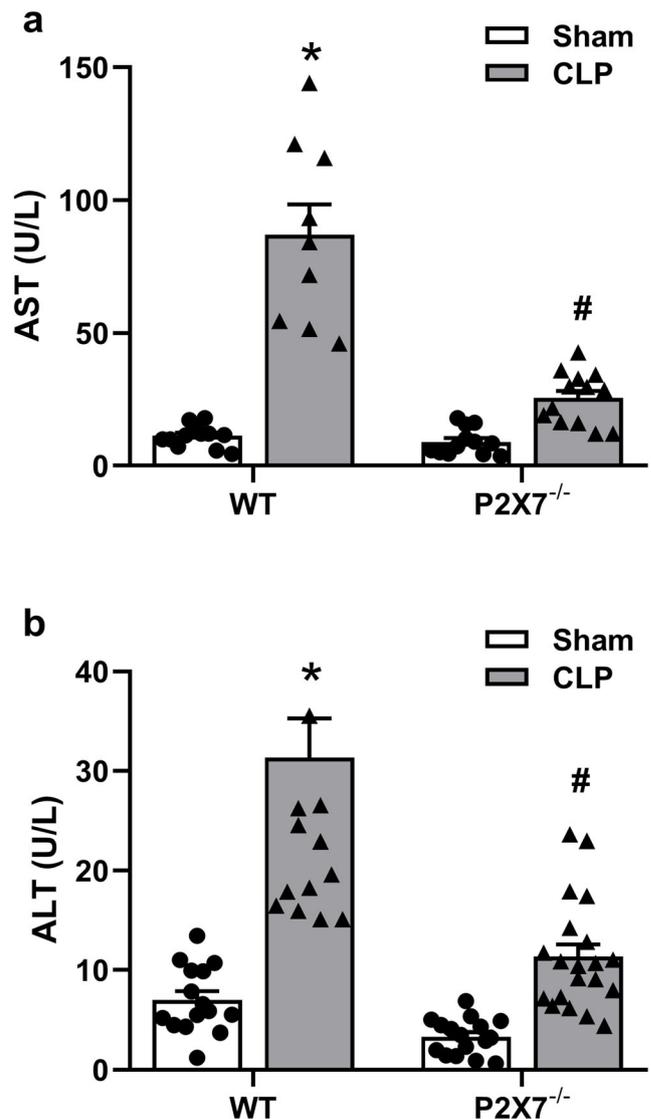
Fig. 2 AST and ALT in the serum of WT and P2X7$^{-/-}$ animals 24 h after sepsis induction. Serum AST in WT (left (**a**)) and P2X7$^{-/-}$ (right (**a**)) mice. Serum ALT in WT (left (**b**)) P2X7$^{-/-}$ (right (**b**)) mice. The asterisk represents statistical difference (*$p < 0.05$) when compared to sham groups with their respective CLP group, while the number sign represents statistical difference (#$p < 0.05$) when compared to CLP groups (WT CLP vs. P2X7$^{-/-}$ CLP). Data were analyzed by two-way ANOVA and they are expressed as mean + S.E.M. ($n \geq 10$ animals per group)

P2X7 receptor contributes to an imbalance in the activities of antioxidant enzymes (SOD/CAT) in the liver septic animals

SOD and CAT activities were analyzed in the liver of septic mice. A decrease in CAT activity was observed (Fig. 4b, left) in septic WT animals ($p < 0.05$), but no statistical differences were found in SOD activity in CLP WT animals when compared with their respective sham group (Fig. 4a; $p > 0.05$). When we evaluated the P2X7$^{-/-}$ animals, we did not verify
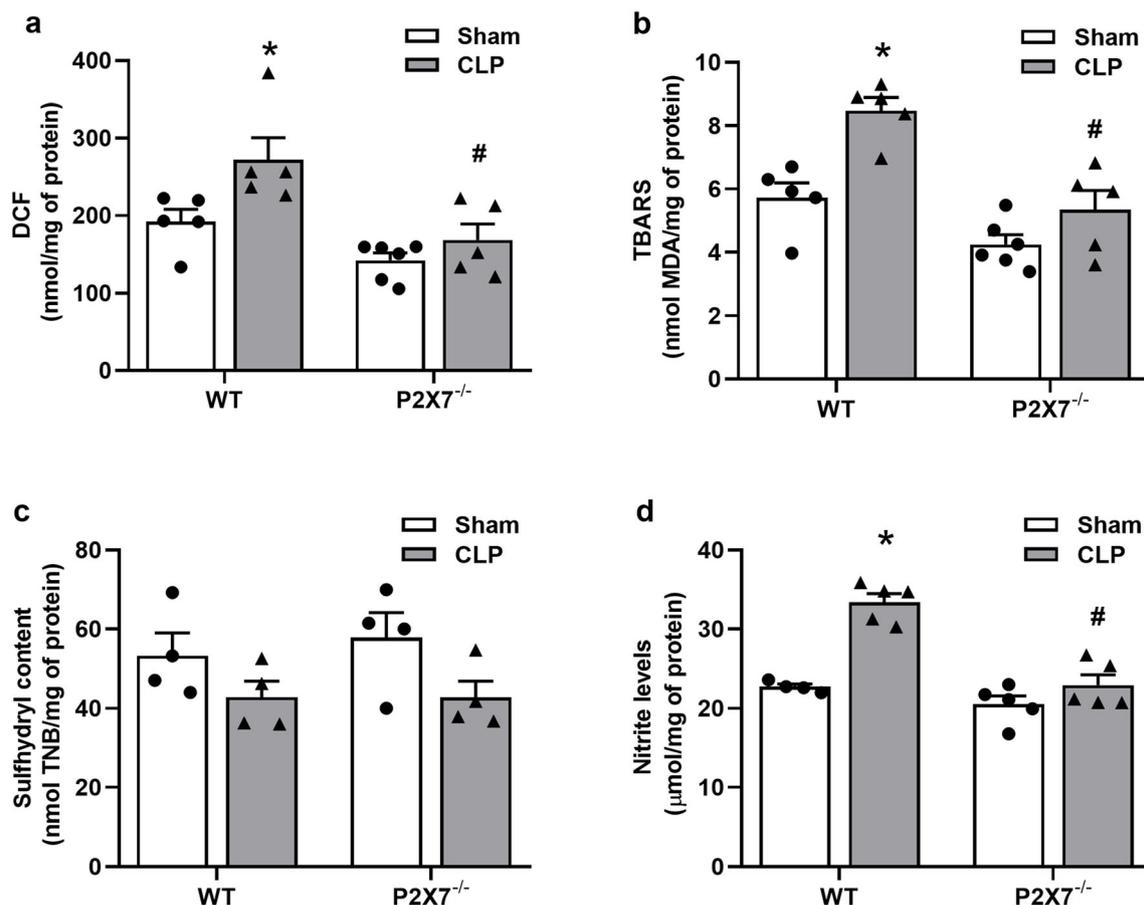
Fig. 3 The genetic deletion of the P2X7 receptor blocks the production of free radicals in the liver of septic mice. **a** The production of reactive species as measured by the oxidation of 2′,7′-dichlorofluorescein (DCF), **b** lipid peroxidation measured by substances reactive to thiobarbituric acid (TBARS), **c** total sulfhydryl content, and **d** nitrite levels in the liver of WT and P2X7$^{-/-}$ mice 24 after sepsis induction.

The asterisk represents statistical difference (*$p < 0.05$) when compared to sham groups with their respective CLP group, while the number sign represents statistical difference (#$p < 0.05$) when compared to CLP groups (WT CLP vs. P2X7 CLP$^{-/-}$). Data were analyzed by two-way ANOVA and they are expressed as mean ± S.E.M. ($n \geq 4$ animals per group)

significant differences in SOD or CAT activities when compared with their sham group (Fig. 4 a and b; $p > 0.05$). These data show an imbalance in SOD/CAT activity in the liver of septic WT animals ($p < 0.05$), which can result in an accumulation of H_2O_2 (Fig. 4c, left). This imbalance between the ratio of SOD/CAT activities was not observed in the liver P2X7$^{-/-}$ septic animals (Fig. 4c, right).

Gene expression of iNOS and NADPH oxidases increase in the liver of septic animals

Biomarkers of redox status were assessed in the liver of septic animals 24 h after sepsis. Besides, we investigated the role of the P2X7 receptor in modulating the expression of enzymes involved in oxidative metabolism, such as NADPH oxidases and iNOS in liver tissue. Our results show an increase ($p < 0.05$) in the relative expression of *Nox1*, *Nox2*, and *Nos2* in WT septic animals (Fig. 5a–c, left). The P2X7 receptor deletion prevented these increases since no differences in

Nox1, *Nox2* ($p > 0.05$), and a partial increase in *Nos2* expression was observed in P2X7$^{-/-}$ septic mice in comparison to the P2X7$^{-/-}$ sham group (Fig. 5a–c right). Taken together, these results indicate that the P2X7 receptor contributes to the activation of NADPH oxidases and iNOS in liver tissue during sepsis.

P2X7 receptor functionality is crucial to upregulate cytokine and chemokine expression in the liver of septic animals

The liver is one of the foremost vital organs severely affected during sepsis, and the macrophages Kupffer cells (KC) residing in the liver produce pro-inflammatory cytokines that contribute to the liver damage in sepsis. Thus, we investigated the contribution of the P2X7 receptor to IL-1β, IL-6, TNF-α, and IL-10 expression in the liver of septic animals. As depicted in Fig. 6, both IL-1β, IL-6, TNF-α, and IL-10 increase in the liver of WT septic animals when compared to the sham group
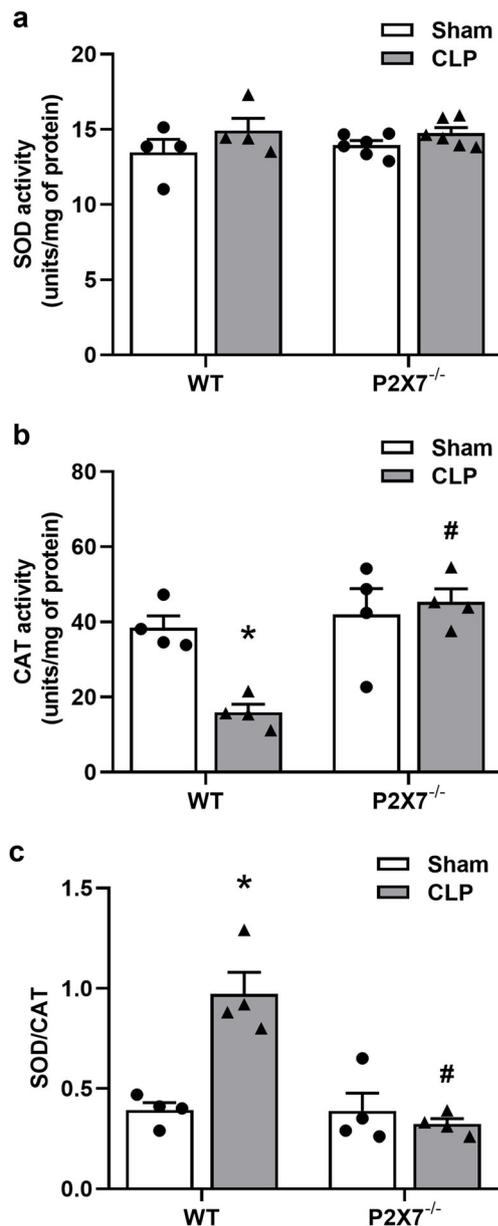
Fig. 4 P2X7 receptor induces an imbalance in SOD/CAT activities in the liver from septic animals. **a** SOD activity was measured by auto-oxidation of the pyrogallol compound and **b** CAT activity was assessed by the disappearance of H_2O_2 liver homogenates from WT and P2X7$^{-/-}$ septic mice. **c** The ratio between SOD/CAT activities. The asterisk represents statistical difference (*$p < 0.05$) when compared to sham groups with their respective CLP group, while the number sign represents statistical difference (#$p < 0.05$) when compared to CLP groups (WT CLP vs. P2X7 CLP$^{-/-}$). Data were analyzed by two-way ANOVA and they are expressed as mean + S.E.M. ($n \geq 4$ animals per group)

Fig. 5 Gene expression of iNOS and NADPH oxidases increases in the liver of WT but not in P2X7-deficient septic mice. The relative expression of the **a** Nox1, **b** Nox2, and **c** Nos2 genes was analyzed by RT-qPCR in the liver of septic mice 24 h after surgery. The asterisk represents statistical difference (*$p < 0.05$) when compared to sham groups with their respective CLP group, while the number sign represents statistical difference (#$p < 0.05$) when compared to CLP groups (WT CLP vs. P2X7 CLP$^{-/-}$). Data were analyzed by two-way ANOVA and they are expressed as mean ± S.E.M. ($n \geq 7$ animals per group)

($p < 0.05$) (Fig. 6 a, b, c, and d respectively, left). In the absence of the P2X7 receptor, no significant increases in the expression of these cytokines were observed in the organ 24 h after CLP induction compared to the sham group (Fig. 6 a, b, c, and d, right) ($p > 0.05$).

Chemokines, such as CXCL1 and CXCL2, are small molecular weight proteins that are important for acute liver damage, and hepatocytes, KC, HCS, and LSEC produce them in response to injury. The expression of CXCL1 and CXCL2 by these cells generates a chemoattractive gradient that promotes the infiltration of monocytes, macrophages, NK cells, and neutrophils from the circulation to the inflammation site. To determine whether the P2X7 receptor is involved in mechanisms that upregulate the expression of these proinflammatory chemokines, we also evaluated the relative gene
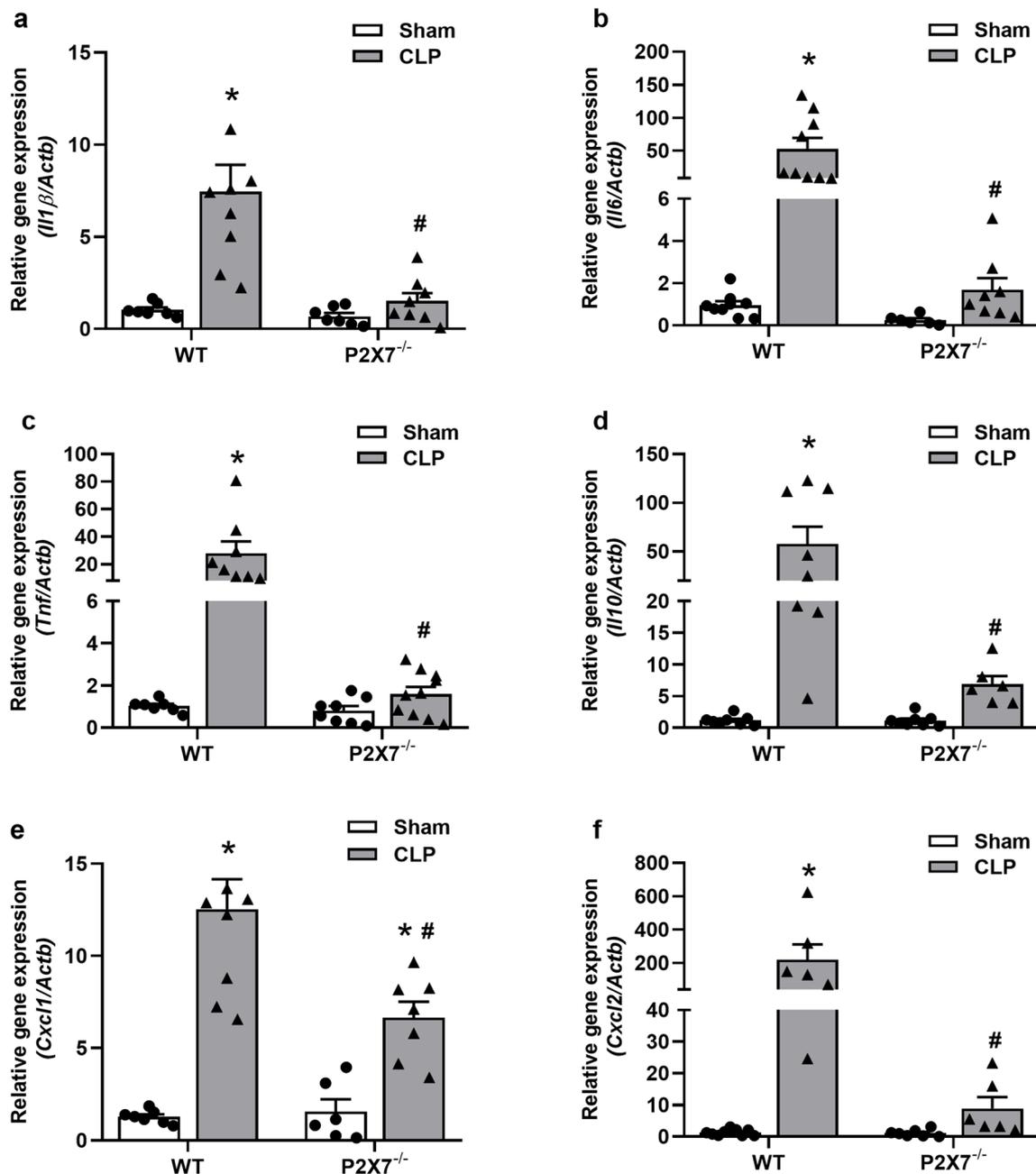
Fig. 6 Gene expression of inflammatory cytokines and chemokines in the liver of septic mice. The relative gene expression of the genes of **a** *Il1β*, **b** *Il6*, **c** *Tnf*, **d** *Il10*, **e** *Cxcl1*, and **f** *CxCl2* was analyzed by RT-qPCR in the liver of septic mice 24 h after surgery. The asterisk represents statistical difference (*$p < 0.05$) when comparing the sham WT groups with their respective CLP group, while the number sign represents statistical difference (#$p < 0.05$) when comparing the CLP P2X7$^{-/-}$ groups with CLP WT. Data were analyzed by two-way ANOVA and they are expressed as mean ± S.E.M. of 3 independent experiments ($n \geq 6$ animals per group)

expression of the *Cxcl1* and *Cxcl2* genes in the liver of septic mice 24 h after surgery. We observed an increase ($p < 0.05$) in the relative expression of *Cxcl1* in both septic WT and P2X7$^{-/-}$ animals (Fig. 6e), while *Cxcl2* increases ($p < 0.05$) only in WT septic mice (Fig. 6f, left). However, *Cxcl1* expression was higher ($p < 0.05$) in WT than P2X7$^{-/-}$ septic animals (Fig. 6e). These results suggest that the P2X7 receptor is important to

the production of cytokines and chemokines in the liver during the acute phase of sepsis.

P2X7 receptor deletion reduces the number of inflammatory cells in liver tissue from septic mice

Liver histology demonstrated significant liver injury 24 h after sepsis induction in both WT and P2X7$^{-/-}$ mice when

compared to their respective sham groups (Fig. 7a–g). Our histological analysis also shows an increased number of inflammatory cells in the liver parenchyma 24 h after sepsis induction in both septic WT and P2X7$^{-/-}$ animals ($p < 0.05$) (Fig. 7a–d). The number of inflammatory cells was higher in WT septic mice in comparison to P2X7$^{-/-}$ septic animals ($p < 0.05$) (Fig. 7e). Therefore, the P2X7 receptor seems to be involved in the recruitment of cells to liver parenchyma, promoting inflammation and tissue damage.

Steatosis, ballooning degeneration, focal necrosis, and inflammation constituted the morphological patterns of liver injury in sepsis. Therefore, an index of liver injury (Fig. 7h) and a histological score of liver inflammation (Fig. 7e). Histological analysis of septic animals demonstrated that hepatic steatosis—characterized by the presence of cytoplasm vacuolization—was observed in both WT and P2X7$^{-/-}$ CLP mice, albeit slightly pronounced in WT animals (Fig. 7f–h). Indeed, P2X7$^{-/-}$ mice have an abnormal liver structure in which steatosis and cytoplasm vacuolization is already present independent of sepsis induction (Fig. 7 b and h), as reported by Arguin et al. [45].

Discussion

Sepsis is initiated by an infection that generates a systemic inflammatory response disrupting the immune system and triggering a cascade of events that can progress to multiple organ failure and host death [1]. Sepsis is the leading cause of death in non-cardiac intensive care units worldwide and represents a significant public health concern [4, 46]. P2X7 receptor deletion attenuates the systemic inflammatory responses and improves outcomes in a murine model of sepsis [33]. This receptor aggravates inflammation and tissue damage in different organs during sepsis, including the lungs, intestine, and brain [24, 27, 33, 34]. Here, we show that the P2X7 receptor deletion attenuates inflammation and oxidative damage in the liver from septic mice.

The P2X7 receptor mediates liver oxidative stress, inflammatory mechanisms [23, 47], and fibrogenesis [48]. This receptor triggers Kupffer cell inflammatory cellular responses and hepatocyte damage in liver diseases [20, 23, 49]. Indeed, we found that P2X7 receptor expression increases in the liver of septic mice. Moreover, AST and ALT enzymes released by
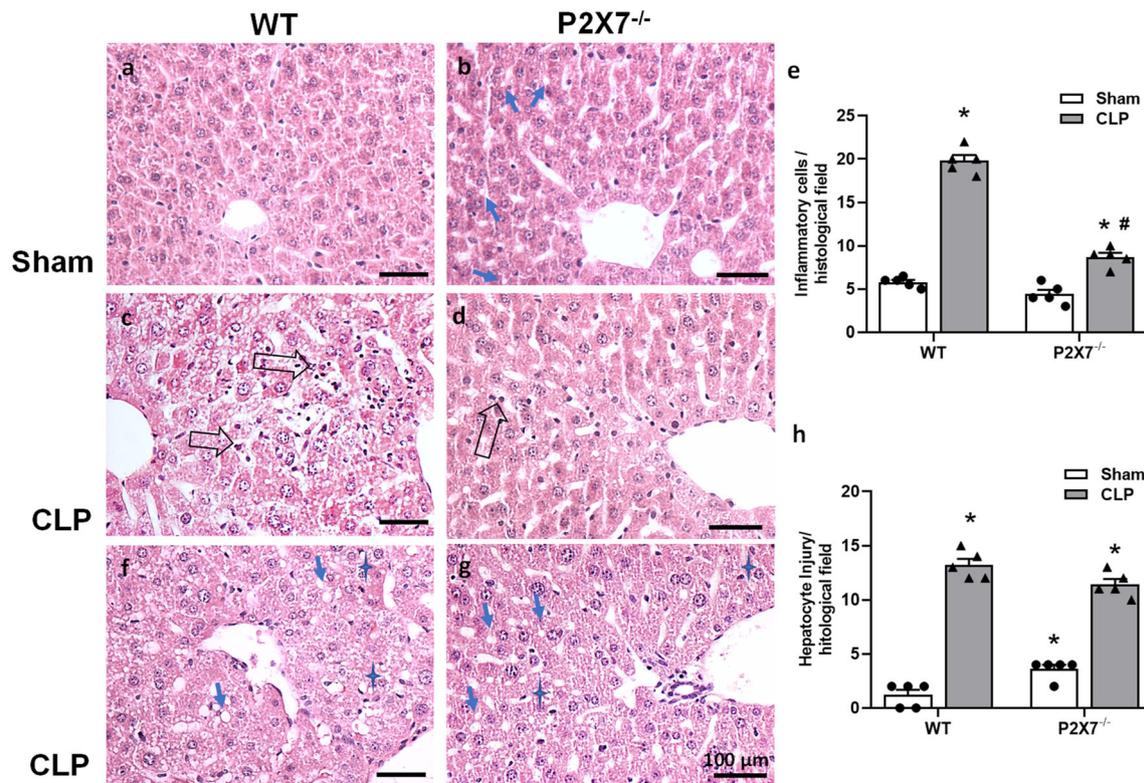
Fig. 7 P2X7 genetic deletion reduces the number of inflammatory cells in the liver from septic mice. Representative photomicrographs of hepatic parenchyma of WT and P2X7$^{-/-}$ mice before (a and b, respectively) and 24 after surgery (c and f WT; and d and g P2X7$^{-/-}$). c Liver parenchyma from CLP WT mice showing numerous inflammatory cells inside the sinusoids (arrows). d Less number of inflammatory cells in liver parenchyma of CLP P2X7$^{-/-}$ mice. e Quantitative analysis of the number of inflammatory cells per field in the liver parenchyma. Representative images of hepatocyte alterations in WT (f) and P2X7$^{-/-}$ (g) septic mice. Blue arrows indicate steatosis in hepatocytes, and blue stars indicate swelling/ballooning. h Liver injury score. The asterisk represents statistical difference (*$p < 0.05$) when compared to sham groups with their respective CLP group, while the number sign represents statistical difference (#$p < 0.05$) when compared to CLP groups (WT CLP vs. P2X7$^{-/-}$ CLP). Data were analyzed by two-way ANOVA and they are expressed as mean ± S.E.M. ($n \geq 5$ animals per group). Twenty fields were analyzed by histological section for WT and P2X7$^{-/-}$ animals. Scale bar = 100 μm

hepatic cell disruption are reduced in the serum of septic P2X7-deficient mice, providing evidence for the relevance of this receptor in sepsis-associated liver injury. Accordingly, P2X7-deficient mice showed a significant reduction in serum ALT in experimental models of acetaminophen-induced acute liver injury [49] and nonalcoholic steatohepatitis [47]. Nevertheless, our histological analysis showed that P2X7-deficient mice have basal hepatic steatosis, and because of that, no differences were observed between WT and P2X7$^{-/-}$ in the hepatic liver score. These results are in accordance with a previous study that showed a disturbed glucose metabolism with consequent higher glycemia, dyslipidemia, increased susceptibility to glucose intolerance, and insulin resistance, with consequent hepatic steatosis in P2X7-deficient mice [45]. Therefore, the reduction in serum hepatic enzymes and oxidative stress in P2X7$^{-/-}$ septic mice suggest that these alterations are secondary to sepsis, while hepatocyte injury score in these animals depends on the altered glucose metabolism with basal hepatic steatosis, as detected in sham mice.

Oxidative damage contributes to metabolic and inflammatory liver diseases [50]. Oxidative stress represents an imbalance between oxidizing and antioxidant agents. Cellular proteins, membrane lipids, and DNA are cellular structures that are primarily affected by ROS and RNS [51]. The oxidative imbalance results in structural and functional abnormalities in the liver. One of the main characteristics of sepsis pathophysiology is the activation of tissue-resident immune cells (i.e., Kupffer cells) and the infiltration of phagocytic cells in liver parenchyma (i.e., PMN and monocytes). These cells respond to an infection producing superoxide, hydrogen peroxide, and nitric oxide inducing tissue damage [50, 52, 53]. Our results show an increase in the production of reactive species in the liver of septic WT animals, as already reported in sepsis [52, 54].

Interestingly, the P2X7 genetic deletion blocked this effect 24 h after surgery, indicating a lower production of reactive species in these animals. In addition to the increase in free radical production, we observed an increase in lipid peroxidation and nitrite content in septic WT animals, but not in P2X7-deficient septic animals. These results are in accordance with previous reports showing P2X7-dependent mechanisms for the generation of ROS and RNS by murine macrophages [28, 31, 32, 55] and in the brain of septic mice [24].

In an experimental model of nonalcoholic steatohepatitis, the P2X7 receptor triggers oxidative stress, inflammation, and fibrosis [47, 56]. *P2X7* receptor-selective antagonist Brilliant Blue G also prevents acetaminophen hepatotoxicity by reducing tissue oxidative stress and inflammation [57]. Indeed, Chatterjee et al. [35] showed that the P2X7 receptor induces the production of reactive species through NADPH oxidase-dependent mechanisms in a model of carbon tetrachloride-induced steatohepatitis. In this study, the P2X7 receptor

increases the expression of the p47 phox subunit that binds to membrane subunit gp91 phox producing reactive species. We found an increased expression of *Nox1* and *Nox2* in the liver of WT but not in the liver of P2X7$^{-/-}$ mice. Therefore, the P2X7 receptor seems to be involved in the activation of oxidative mechanisms in the liver, contributing to sepsis pathogenesis.

The antioxidant enzymes SOD and CAT have a protective role in several diseases [51, 58]. Our results evaluating the activity of endogenous antioxidant enzymes in the hepatic tissue showed a decrease in CAT activity in WT septic animals but not in SOD activity. The ratio between the enzymatic activities of SOD/CAT in septic WT animals indicates an accumulation of H_2O_2. This imbalance was not observed in P2X7$^{-/-}$ septic animals, providing evidence that the functionality of this receptor also alters antioxidant mechanisms. These data are relevant since the antioxidant capacity in patients with sepsis correlates with the severity of the disease and outcomes [59].

Activated KCs have the ability to produce NO through the induction of iNOS expression [60]. The NO produced reacts with ROS to form a highly reactive and toxic compound, the ONOO$^-$. In our settings, nitrite levels and the *Nos2* expression are significantly attenuated in P2X7$^{-/-}$ septic animals when compared with WT septic mice. Several factors modulate the iNOS gene expression pathway. The cytokines TNF-α and IL-1β can induce the expression of *NOS2* in the liver [60]. We also found a significantly diminished expression of IL-1β, IL-6, TNF-α, IL-10, CXCL, and CXCL2 in the liver of P2X7$^{-/-}$ septic mice. We and others have shown that genetic deletion or pharmacological inhibition of the P2X7 receptor decreases the production of inflammatory cytokines (IL-1β, IL-6, and IL-10), NO production, and the recruitment of neutrophils into the peritoneal cavity after sepsis induction [23, 27, 33]. In this line of evidence, our histological analysis showed a reduced number of inflammatory cells in the liver P2X7$^{-/-}$ septic mice, suggesting that the activity of this receptor is relevant for recruiting inflammatory cells to the liver during sepsis progression. These results provide evidence for a critical role of the P2X7 receptor in the development of liver inflammation and tissue damage in sepsis. Such findings are also in accordance with studies in humans that identified P2X7 receptor single-nucleotide polymorphism of gain-of-function related with an increased sepsis severity [61].

Conclusion

The present study shows that P2X7 receptor functionality contributes to sepsis-associated liver oxidative damage and inflammation favoring the progression of the disease. Therefore, these findings suggest possible therapeutic approaches based on P2X7 genotyping and administration of

P2X7 receptor blockers to limit oxidative damage, inflammation, and liver injury during the acute phase of sepsis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Experiments were performed with age- and weight-matched animals, according to the guidelines of the Brazilian College of Animal Experimentation (COBEA). The Commission for the Ethical Use of Research Animals (CEUA) from the Federal University of Rio de Janeiro (UFRJ) approved all experiments (protocol number: IBCCF138).

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