CD39 limits P2X7 receptor inflammatory signaling and attenuates sepsis-induced liver injury

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Abstract

Background & Aims—The severity of sepsis can be linked to excessive inflammatory responses resulting in hepatic injury. P2X7 receptor activation by extracellular ATP (eATP) exacerbates inflammation by augmenting cytokine production; while CD39 (ENTPD1) scavenges eATP to generate adenosine, thereby limiting P2X7 activation and resulting in A_{2A} receptor stimulation. We aim to determine the functional interaction of P2X7 and A_{2A} receptors on controlling macrophage response, consequently impacting the outcome of sepsis and liver injury.

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Methods—Sepsis was induced by cecal ligation and puncture in C57BL/6 wild-type (WT) and *CD39*^{−/−} mice. Several *in vitro* assays were performed using peritoneal or bone marrow derived macrophages to determine CD39 ectonucleotidase activity and its role in sepsis-induced liver injury.

Results—CD39 expression in macrophages limits ATP-P2X7 receptor pro-inflammatory signaling. P2X7 receptor paradoxically boosts CD39 activity. Inhibition and/or deletion of P2X7 receptor in LPS-primed macrophages attenuates cytokine production and inflammatory signaling as well as preventing ATP-induced increases in CD39 activity. Septic *CD39*^{−/−} mice exhibit higher levels of inflammatory cytokines and show more pronounced liver injury than WT mice. Pharmacological P2X7 blockade largely prevents tissue damage, cell apoptosis, cytokine production, and the activation of inflammatory signaling pathways in the liver from septic WT, while only attenuating these outcomes in *CD39*^{−/−} mice. Furthermore, the combination of P2X7 blockade with adenosine A_{2A} receptor stimulation completely inhibits cytokine production, the activation of inflammatory signaling pathways, and protects septic *CD39*^{−/−} mice against liver injury.

Conclusions—CD39 attenuates sepsis-associated liver injury by scavenging eATP and ultimately generating adenosine. We propose boosting of CD39 would suppress P2X7 responses and trigger adenosinergic signaling to limit systemic inflammation and restore liver homeostasis during the acute phase of sepsis.

Graphical abstract
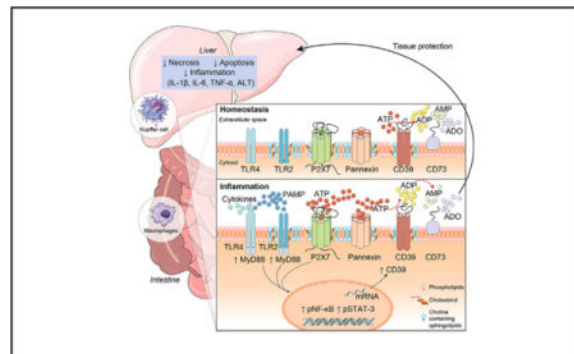
Keywords

Extracellular ATP; Ectonucleotidases; Adenosine; Kupffer cells; Systemic inflammation

Introduction

Sepsis is a leading cause of death in intensive care units worldwide[1] and represents a major public health issue due to high numbers of deaths of economically productive people and major morbidity in survivors. Aspects of the associated morbidity and mortality are related to onset of shock and hemodynamic compromise with multiple organ system dysfunction and failure due to the excessive, unfettered inflammation.[1,2]

The liver plays a central role in metabolic homeostasis and in the host-defense against pathogens; albeit liver function can be acutely compromised by shock as in severe sepsis.[3] Sepsis causes acute liver damage because of hemodynamic compromise but direct hepatocyte and canalicular injury is also feasible. The development of liver failure in sepsis is recognized as a major complication, which contributes to severity of the sepsis and limits positive outcomes.

The inflammatory response in sepsis is dependent upon activation of pathogen recognition receptors (*i.e.* Toll-like receptors [TLRs]) in monocyte/macrophages (Kupffer cells in the liver), which are crucial to the pathogenesis of sepsis and sepsis-associated liver injury.[4,5] The TLR-mediated activation of the MyD88/NF-κB pathway in these cells induces a hyper-inflammatory state mediated by the production of large amounts of tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and IL-1β.[4,6] In turn, TNF-α and IL-1β stimulate hepatocytes and Kupffer cells to produce IL-6, which induces acute-phase protein production via STAT3 activation.[3,7] Therefore, crosstalk between activated macrophages and hepatocytes may be a central component of the pathogenesis of sepsis-associated liver injury.[4]

Purinergic signaling modulates inflammation, coagulation and immune responses.[8,9] Adenosine triphosphate (ATP) may be secreted into the extracellular space as a result of cellular damage or TLR activation, as part of different pathogen-associated molecular patterns (PAMPs) that induce leukocyte activation and migration to the site of infection.[10,11] Through the activation of P2 receptors, extracellular ATP (eATP) can initiate inflammation by acting as a 'danger' signal in the extracellular medium.[9,12] The P2 receptor family comprises of P2Y G-protein coupled receptors and P2X (P2X1-7) ligand-gated ion channels. [13,14] The P2X7 receptor has been the most extensively studied,[9] as activation of this receptor induces maturation and release of pro-inflammatory cytokines, such as IL-1β,[15] together with activation of acid sphingomyelinase[16] and the production of reactive nitrogen and oxygen species.[17] Furthermore, P2X7 receptor signaling is involved in host resistance against a wide variety of microbial pathogens.[9,18,19]

The ATP-dependent pro-inflammatory signaling mediated by the P2X7 receptor is modulated by cell surface ectonucleotidases, such as CD39 also known as ecto-nucleoside triphosphate diphosphohydrolase-1 (ENTPD1) and CD73/ecto-5′-nucleotidase, the major ectonucleotidases expressed in immune and vascular cells.[20] CD39 hydrolyzes extracellular tri- and diphosphonucleo-sides to monophosphonucleosides, whereas the ubiquitously expressed CD73 is responsible for AMP hydrolysis, and ultimately the generation of adenosine, which has anti-inflammatory properties.[21] Interestingly, Cohen *et al.*[11] have reported that ectonucleotidases exert important monocyte-macrophage autoregulatory functions in limiting cellular activation by catalyzing the rapid conversion of ATP, ultimately into adenosine. In addition, the CD39/CD73-axis has been found to decrease mortality and organ damage in sepsis.[21,22] However, the exact cellular mechanisms and signaling pathways underling these observations remains unclear.

We note that: (i) the liver plays a central role in host-defense against pathogens and that hepatic dysfunction contributes to severity and outcome of sepsis;[3] (ii) monocyte-

macrophage responses are crucial to the pathogenesis of abdominal inflammation and liver failure;[4,5] and (iii) P2X7 receptor activation, CD39/CD73-axis, as well as, adenosinergic signaling modulate macrophage responses and plasticity,[11] as eATP-P2X7 receptor mediated immune responses in activated macrophages are counterbalanced by ectonucleotidases, such as CD39, which scavenge ATP. We hypothesized that CD39 ectonucleotidase activity would restore liver homeostasis during sepsis and could be hepatoprotective in such experimental settings.

We found that CD39 expression in macrophages limits ATP-P2X7 mediated pro-inflammatory responses. Moreover, CD39 deletion exacerbates sepsis-induced liver dysfunction. The combination of P2X7 blockade with an adenosine A_{2A} receptor agonist (ATL146e) completely protects the liver during sepsis, improving experimental outcomes. Therefore, these data indicate that CD39 attenuates sepsis-associated liver injury by both scavenging eATP and ultimately generating adenosine, suggesting innovative therapeutic avenues to explore the possible benefits in clinical sepsis.

Material and methods

Mice and experimental procedures

Male, 8–10-week old wild-type (WT), P2X7 receptor deficient (*P2X7*$^{-/-}$) (originally from the Jackson Laboratory, USA) or CD39 deficient mice (*CD39*$^{-/-}$) C57BL/6 were used. All experiments were approved by the Commission for the Ethical Use of Research Animals (CEUA) from the Federal University of Rio de Janeiro (UFRJ) (approved protocol number: IBCCF138) and by the Institutional Animal Care and Use Committees (IACUC) of Beth Israel Deaconess Medical Center (approved protocol number: 019-2015). Sepsis was induced by cecal ligation and puncture (CLP) as previously described.[23]

Statistical analysis

Results are expressed as means ± standard error of mean (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by a Turkey multiple range tests. Differences between groups were considered statistically significant when $p < 0.05$. The statistical significance of lethality was analyzed using the Kaplan-Meier method and log-rank test.

For further details regarding the materials used, please refer to the Supplementary data and CTAT table.

Results

P2X7 receptor boosts CD39 activity and contributes to NF-κB and STAT3 activation in LPS-primed macrophages

To investigate whether the sepsis-like stimulus (LPS) and P2X7 receptor are involved in the modulation of CD39 activity and inflammatory signaling pathways (MyD88/NF-κB and STAT3), we primed murine peritoneal resident macrophages and BMDM with LPS *in vitro* and treated these cells with P2X7 agonists ATP (500 μM), BzATP (100 μM; a potent P2X7 agonist) or ATPγs (100 μM; a non-hydrolysable ATP analog; data not shown) for 3 h, in the

presence or absence of the specific P2X7 antagonist A740003 (Fig. 1A and B). We noted that P2X7 agonists potentiated ATP hydrolysis in LPS-primed macrophages ($^*p$ <0.05), albeit this effect was reversed by pharmacological blockade of the P2X7 receptor (p >0.05) (Fig. 1A and B). A similar pattern of response was observed in THP-1 derived human macrophages (data not shown). In agreement with the observed changes in biochemical ecto-enzymatic activity, we also noted an associated increase in *CD39* mRNA levels (Fig. 1C), suggesting transcriptional control of CD39 by TLR and P2X7 receptor activation. Indeed, macrophages derived from *P2X7*$^{-/-}$ mice were less able to scavenge ATP after LPS and ATP stimulation (Fig. 1D).

Regarding the possibility that P2X7 triggers intracellular inflammatory pathways, we next observed significant increases in NF-κB and STAT3 activation when ATP stimulation of LPS-primed macrophages was studied (Fig. 1E). Again, these ATP effects on NF-κB activation were not observed in LPS-primed macrophages derived from *P2X7*$^{-/-}$ mice (Fig. 1E). Indeed, LPS-primed *P2X7*$^{-/-}$ macrophages exhibited significantly lower levels of STAT3 activation, when compared to the WT counterparts (Fig. 1E). These data suggest functional links between P2X7 activation and intracellular responses to LPS.

CD39$^{-/-}$ macrophages stimulated with LPS and ATP exhibit an increased NF-κB activation and IL-1β production

In order to test whether CD39 enzyme activity is important to limit P2X7 receptor mediated NF-κB activation in LPS-primed macrophages, we next compared MyD88 and p-NF-κB expression in WT and *CD39*$^{-/-}$ macrophages. These cells were pretreated with P2X7 receptor antagonists or imipramine (operational as an acid sphingomyelinase inhibitor); and then stimulated with LPS and P2X7 agonists. It is already known that P2X7 stimulates acid sphingomyelinase to boost cytokine release;[16] hence inhibition of acid sphingomyelinase might inhibit, at least in part, inflammatory signaling post activation of P2X7 receptor.

The results confirmed that both BzATP and ATP are able to enhance the NF-κB activation induced by LPS *per se*. The pretreatment with P2X7 receptor antagonists (A740003 and oATP); or imipramine attenuate this effect (Fig. 2A). In comparison to the WT group, *CD39*$^{-/-}$ macrophages show increased p-NF-κB activation, both at baseline and after stimulation with LPS and/or with P2X7 agonists (Fig. 2B). Likewise, P2X7 receptor antagonists or imipramine were able to attenuate NF-κB activation. These findings support the notion that CD39 limits P2X7 receptor response. Moreover, CD39 expression is also crucial for controlling P2X7 induced cytokine release. As shown in Fig. 2C and E, *CD39*$^{-/-}$ macrophages showed more IL-1β and generated less IL-10 expression after stimulation with LPS and ATP; in comparison to WT macrophages. Furthermore, both P2X7 receptor antagonists (A740003 and oATP) decreased the production of IL-1β (Fig. 2C), while oATP alone induced a significant decrease in IL-6 and IL-10 levels (Fig. 2D and E) in both groups. Importantly, the same profile of cytokine production was observed after stimulation with LPS + BzATP (data not shown).

Extracellular ATP acting via P2X7 receptor contributes to inflammatory exacerbation in sepsis

Extracellular ATP has been considered an immune adjuvant that can initiate inflammation at sites of infection and/or damage. We studied the CLP model of polymicrobial sepsis in mice to explore the role of ATP-P2X7 receptor signaling in the modulation of immune responses during early phase of abdominal sepsis.

Since neutrophils are rapidly mobilized from the circulation to sites of inflammation during acute infection, we first investigated whether engagement of P2X7 receptor modulates neutrophil infiltration into the peritoneal cavity during sepsis. We observed a significant increase in the number of neutrophils (CD11b$^+$Ly6G$^+$ cells) in the peritoneal cavity 3 h after sepsis induction (Fig. 3A). P2X7 receptor pharmacological inhibition using the antagonist brilliant blue G (BBG) (Fig. 3A) or with genetic deletion of P2X7 (Fig. 3A) significantly (p <0.05) decreased CLP-induced neutrophil recruitment to the peritoneal cavity.

We further observed a significant increase in nitric oxide (NO) production in the peritoneal cavity of septic mice (p <0.001). Genetic deletion or pharmacological inhibition of the P2X7 receptor was able to significantly decrease NO levels in the peritoneum in this setting (Fig. 3C; p <0.001). In accordance with these results, we also found an increased expression of *Nos2* in peritoneal cells obtained from WT mice, but not in cells from the null P2X7$^{-/-}$ (Fig. 3D; p <0.001). Conversely, arginase1 gene expression did not differ among the studied groups at this time point (Fig. 3D; p >0.05). Collectively, these results suggest a role for the ATP-P2X7 signaling in the activation and migration of inflammatory cells to the peritoneal cavity during sepsis.

CD39 activity is increased and modulated by P2X7 receptor in peritoneal cells from septic mice

Extracellular ATP metabolism was evaluated through the activity of CD39 and CD73 enzymes in peritoneal cells at 3 h after CLP-induced sepsis. We observed a significant increase in ATP and ADP hydrolysis – suggestive of an increase in CD39 activity – by peritoneal cells from septic mice (CLP, vehicle-treated or WT) when compared to sham-operated mice (p <0.05) (Fig. 3E, left and middle), whereas the AMP hydrolysis was decreased – suggestive of diminished CD73 activity (Fig. 3E, right). Pharmacological inhibition or genetic deletion of the P2X7 receptor abrogated these sepsis-induced effects (p <0.05).

Moreover, a subpopulation of monocytes/macrophages expressing CD39 (CD11b$^+$Ly6G$^-$CD39$^+$), but not neutrophils (CD11b$^+$Ly6G$^+$CD39$^+$) in the peritoneal cavity appeared responsible for the enhanced extracellular nucleotide phosphohydrolysis, associated with sepsis (Fig. 3B, p >0.05). Indeed, BBG pretreatment prevented sepsis-induced increase in a number of these CD39$^+$ monocyte/macrophage (Fig. 3B), further supporting a role for P2X7 in controlling CD39 activity in this setting.

CD39 deletion contributes to excessive cytokine production after CLP-induced sepsis

Levels of IL1-β, IL-6, and IL-10 were significantly increased in both serum and peritoneal fluid from WT and $CD39^{-/-}$ mice after 3 h of CLP model induction (p <0.05; Fig. 3F). Notably, IL-10 levels in peritoneal lavage fluid obtained from $CD39^{-/-}$ mice did not change despite sepsis induction (p >0.05; Fig. 3F). Moreover, septic $CD39^{-/-}$ mice had significantly higher levels of IL-1β, and lower levels of IL-10 when compared to septic WT mice (p <0.05; Fig. 3F). Furthermore, the inhibition of P2X7 receptor with BBG attenuated cytokine production in all septic mice samples (p <0.05; Fig. 3F). Altogether, these results implicate a role for CD39 in limiting the eATP-P2X7 pro-inflammatory effects and immune responses during sepsis and peritoneal inflammation. Of note, imipramine treatment (20 mg/kg) did not show any significant protective effects in septic WT or $CD39^{-/-}$ mice (data not shown).

Simultaneous P2X7 receptor blockade and adenosine A$_{2A}$ receptor activation completely protects from liver injury during sepsis

While exploring the role of purinergic signaling in sepsis progression and acute liver injury, histological analysis indicated foci of liver necrosis in tissues obtained from WT and $CD39^{-/-}$ mice 24 h after sepsis induction (Fig. 4A and B). Moreover, $CD39^{-/-}$ animals had enlarged pathological areas of cell death, in comparison to WT mice (Fig. 4A and B). Pre-treating these animals with BBG completely protected the liver in WT mice, but only attenuated the liver injury in $CD39^{-/-}$ animals (Fig. 4A and B).

Given these data, we therefore hypothesized that CD39 is important not only for the removal of extracellular ATP, but also for providing substrate (AMP) for ultimately generating adenosine by the enzyme CD73. We, therefore, tested this assumption by pre-treating $CD39^{-/-}$ mice with BBG, inducing the CLP model of polymicrobial sepsis and injecting an A$_{2A}$ adenosine receptor agonist right after the surgery.

The association of P2X7 receptor inhibition with adenosine A$_{2A}$ receptor activation was able to completely protect $CD39^{-/-}$ animals from liver injury during sepsis. In agreement with these findings, serum levels of alanine aminotransferase (ALT) were increased in septic WT and $CD39^{-/-}$ mice, suggestive of liver injury, and these levels were significantly higher in septic $CD39^{-/-}$ mice (Fig. 4C). Pretreatment with BBG could prevent ALT increases in WT mice, but not in septic $CD39^{-/-}$ mice (Fig. 4C). However, the combination of BBG (P2X7 blockade) and ATL146e (adenosine A$_{2A}$ receptor agonist) abrogated the serum ALT enzyme increase in septic $CD39^{-/-}$ animals (Fig. 4C), suggesting that both P2X7 receptor blockade and adenosine generation are pivotal to maintain liver integrity during sepsis. Of note, the administration of A$_{2A}$ agonist alone did not confer any protective effect in WT or $CD39^{-/-}$ animals (data not shown).

P2X7 receptor inhibition and adenosine A$_{2A}$ receptor activation decreased the number of apoptotic cells in the liver from septic mice

Tissues from both WT and $CD39^{-/-}$ mice presented a significant increase in the number of TUNEL-positive cells, with morphology indicative of sinusoidal cells, 24 h after sepsis induction (Fig. 5A and B; p <0.05). Once more, pretreatment with BBG significantly decreased the number of apoptotic cells in the liver of septic WT and $CD39^{-/-}$ mice (Fig. 5A

and B; $p < 0.05$), but a complete rescue was obtained only in the latter when animals were treated with BBG + ATL146e.

P2X7 receptor blockade and adenosine A2A receptor activation reduces the cytokine production and NF-κB and STAT3 activation in the liver of septic mice

Increased levels of IL-1β, IL-6, TNF-α, and IL-10 were found in liver samples from both septic WT and *CD39*^{−/−} mice (Fig. 6A respectively; $p < 0.05$). IL-1β production was significantly higher in the liver from septic *CD39*^{−/−} mice when compared to the septic WT mice (Fig. 6A, $p < 0.05$). Pretreatment with BBG attenuated the production of all cytokines in septic WT mice (Fig. 6A; $p < 0.05$), while this effect was less pronounced in the *CD39*^{−/−} group (note the elevated production of IL-1β and IL-6 in relation to the corresponding sham group) (Fig. 6A). However, associating P2X7 receptor blockade with adenosine A$_{2A}$ receptor activation completely inhibited sepsis-induced cytokine production in the liver from these *CD39*^{−/−} animals (Fig. 6A). Of note, the same cytokine pattern was observed in blood and peritoneal lavage fluid from WT and *CD39*^{−/−} mice fluid 24 h after sepsis induction and pharmacological treatments (Fig. S1).

Further, we also detected an increase in NF-κB and STAT3 activation in the liver from septic WT and *CD39*^{−/−} mice. Indeed, the p-NF-κB increase was higher in *CD39*^{−/−} compared to septic WT mice (Fig. 6B). Again, pretreatment with BBG attenuated the increase in the levels of p-STAT3 in WT, but not in septic *CD39*^{−/−} animals. Combined treatment with BBG and adenosine A$_{2A}$ receptor agonist significantly decreased NF-κB and STAT3 activation in mouse liver from septic *CD39*^{−/−} mice. The expression of MyD88 was increased in both septic WT and *CD39*^{−/−} mice, but no level reversion was seen despite the pharmacological treatments (Fig. 6B).

Finally, we observed that BBG and BBG + ATL146e treatments prolonged the survival time of the septic WT mice by approximately 40% (p = 0.08), and 55% (p = 0.02), respectively (Fig. 7).

Discussion

Severe sepsis induces hepatic dysfunction and acute liver injury and indicates a poor prognosis with deleterious outcomes in clinically ill patients. Monocyte/macrophages are key players in the pathogenesis of acute liver injury in sepsis as well as in other liver related illnesses.[4,5] TLR-mediated NF-κB activation in monocyte-macrophages induces the production of inflammatory cytokines, such as TNF-α and IL-1β.[4,6] In turn, TNF-α and IL-1β stimulate hepatocytes and Kupffer cells to produce IL-6, which induces acute-phase protein production via STAT3 activation, further contributing to systemic activation of immune responses.[3,7] In addition, NF-κB and STAT3 activation may also increase CD39 expression.[24]

Here we show that NF-κB and STAT3 phosphorylation is potentiated by P2X7 receptor activation in LPS primed-macrophages, suggesting a contribution for this receptor in a model of sepsis related inflammation and liver injury. TLR4 and P2X7 receptor activation both induce increases in the activity and gene expression of CD39 in macrophages, in a

putative negative feed-back loop, to scavenge extracellular ATP and thereby limit inflammation and sepsis-associated liver damage.[11]

CD39 is the rate-limiting enzyme in the extracellular nucleotide to adenosine phosphohydrolytic cascade, thereby modulating the activation of purinergic receptors and inflammation in the liver.[20,25] CD39 *per se* is able to reduce cellular responses triggered by P2X7, such as apoptosis and release of pro-inflammatory cytokines in macrophages.[26] Our data show that CD39 limits the P2X7-mediated increased p-NF-κB levels and controls cytokines released by macrophages. Further, the lack of CD39 boosts IL-1β levels, probably as a consequence of increased levels of extracellular ATP (second signal to the inflammasome activation).[15]

We also note that IL-10 production is decreased in *CD39*^{−/−} macrophages, which can be explained by the fact that absence of CD39 would compromise adenosine generation and might limit A_{2B} receptor activation, which otherwise would lead to IL-10 production.[27] Moreover, *CD39*^{−/−} macrophages treated with P2X7 antagonists decrease IL-1β release, reinforcing a link between CD39 and P2X7 receptor in controlling cell signaling transcription and translation of inflammatory cytokines. Considering monocyte-macrophage responses dictate the severity of liver injury, it is feasible that CD39 could be a potential therapeutic target to limit P2X7-dependent inflammatory responses, thereby protecting the liver and improving outcomes in sepsis.

CD39-P2X7 receptor modulatory effects have also been demonstrated *in vivo* during hepatic ischemia reperfusion injury,[28] acetaminophen acute liver failure,[29] and in polymicrobial sepsis.[18,22] P2X7 receptor activation is important in providing a protective mechanism by enhancing macrophage bacterial killing ability,[19] and increasing secretion of IgM by B1 cells in sepsis.[30] However, P2X7 receptor activation might contribute to the production of NO, inflammatory cytokines and exacerbate inflammatory response in sepsis.[18,31] Similarly, CD39 genetic deletion increases the mortality rate and the production of cytokines in septic mice, operational through purinergic mechanisms.[22]

Here, we show that P2X7 receptor pharmacological inhibition with BBG decreases the production of inflammatory cytokines (IL1-β, IL-6, and IL-10), the NO production, and the neutrophil recruitment to peritoneal cavity 3 h after induction of sepsis. NO may enhance bacterial killing by inducing neutrophil activation and migration to the sites of infection.[32] On the other hand, NO induces vasodilatation, activation of inflammatory pathways, and reduction of cardiac function, which is likely to increase sepsis severity.[18,32] In addition to NO, excessive cytokine production is related to poor outcomes in sepsis.

Therefore, our data reinforce the idea that eATP acting through P2X7 receptor contributes to inflammatory response in early phases of sepsis. In addition, the inhibition of this receptor with BBG could be a therapeutic strategy to limit sepsis progression and treat established inflammatory disease.

BBG has already been tested in clinical trials and no toxicity or adverse effects were reported in humans.[33,34] In addition, BBG treatment has protective effects in different animal models of inflammatory and liver disease.[31,35] The AZD9056 – a selective orally

active inhibitor of the purinergic receptor P2X7 – has shown beneficial effects in patients with moderate-to-severe Crohn's Disease.[36] We also show that pharmacological inhibition of P2X7 has a protective effect, decreasing liver damage, the number of apoptotic cells, the cytokine production, and the activation of inflammation signaling pathways (NF-κB and STAT3) in the liver of septic WT mice, while minimally attenuated those effects in the *CD39*^{−/−} group. This result might be explained by the fact that septic *CD39*^{−/−} mice present with exacerbated inflammatory processes, generated by the lack of ectonucleotidases. We propose that this lack of CD39 results in ATP/adenosine imbalance and favors the pro-inflammatory response over the anti-inflammatory one. This assumption was confirmed by our data showing that a combination of P2X7 blockade with the specific A_{2A} receptor agonist ATL146e was able to completely protect *CD39*^{−/−} mice from harmful effects of sepsis, such as liver injury and progression of the inflammatory process. In addition, BBG + ATL146e treatment was able to improve the survival of septic WT mice by approximately 55%. Our investigations support previous studies showing that stimulation of A_{2A} receptor by specific agonists (ALT146e or ATL313) significantly decreases animal mortality in sepsis[37,38] and attenuates hepatic ischemia reperfusion injury.[39] Although animal models do not completely reflect the complexity and heterogeneity of sepsis in humans, these data have clinical relevance since they suggest that targeting this system by removing the extracellular ATP and increasing adenosine levels may be a new strategy to prevent sepsis progression, hepatotoxicity, and death.

In summary, we show that P2X7 receptor may modulate the functionality of CD39, increasing the associated ecto-enzymatic activity. CD39 functions as an extracellular ATP scavenger, thus limiting P2X7 activation and inhibiting pro-inflammatory macrophage responses. Moreover, during the progression of sepsis, the functionality of CD39 is simultaneously crucial for the generation of adenosine, which has anti-inflammatory, immunosuppressive, and protective effects in sepsis-associated liver injury (Fig. 8). Thus, these results support the involvement of the purinergic signaling in macrophage activation and sepsis-induced liver injury. Furthermore, these data suggest possible therapeutic approaches based on the administration of soluble apyrases or truncated forms of CD39, which mimic ectonucleotidase activity. These approaches might be combined with the administration of P2X7 receptor blockers to limit monocyte-macrophage responses and liver injury during the acute phase of systemic and abdominal sepsis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit Care Med. 2001; 29:1303–1310. [PubMed: 11445675]

2. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The third international consensus definitions for sepsis and septic shock (Sepsis-3). JAMA. 2016; 315:801–810. [PubMed: 26903338]

3. Nesseler N, Launey Y, Aninat C, Morel F, Malledant Y, Seguin P. Clinical review: The liver in sepsis. Crit Care. 2012; 16:235. [PubMed: 23134597]

4. Possamai LA, Thursz MR, Wendon JA, Antoniades CG. Modulation of monocyte/macrophage function: a therapeutic strategy in the treatment of acute liver failure. J Hepatol. 2014; 61:439–445. [PubMed: 24703954]

5. Antoniades CG, Khamri W, Abeles RD, Taams LS, Triantafyllou E, Possamai LA, et al. Secretory leukocyte protease inhibitor: a pivotal mediator of anti-inflammatory responses in acetaminophen-induced acute liver failure. Hepatology. 2014; 59:1564–1576. [PubMed: 24282114]

6. Osuchowski MF, Welch K, Siddiqui J, Remick DG. Circulating cytokine/inhibitor profiles reshape the understanding of the SIRS/CARS continuum in sepsis and predict mortality. J Immunol. 2006; 177:1967–1974. [PubMed: 16849510]

7. Bode JG, Albrecht U, Haussinger D, Heinrich PC, Schaper F. Hepatic acute phase proteins–regulation by IL-6- and IL-1-type cytokines involving STAT3 and its crosstalk with NF-kappaB-dependent signaling. Eur J Cell Biol. 2012; 91:496–505. [PubMed: 22093287]

8. Bours MJ, Swennen EL, Di VF, Cronstein BN, Dagnelie PC. Adenosine 5′-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacol Ther. 2006; 112:358–404. [PubMed: 16784779]

9. Morandini AC, Savio LE, Coutinho-Silva R. The role of P2X7 receptor in infectious inflammatory diseases and the influence of ectonucleotidases. Biomed J. 2014; 37:169–177. [PubMed: 25116711]

10. Kawamura H, Kawamura T, Kanda Y, Kobayashi T, Abo T. Extracellular ATP-stimulated macrophages produce macrophage inflammatory protein-2 which is important for neutrophil migration. Immunology. 2012; 136:448–458. [PubMed: 22564028]

11. Cohen HB, Briggs KT, Marino JP, Ravid K, Robson SC, Mosser DM. TLR stimulation initiates a CD39-based autoregulatory mechanism that limits macrophage inflammatory responses. Blood. 2013; 122:1935–1945. [PubMed: 23908469]

12. Di VF. Purinergic signalling in the immune system. A brief update. Purinergic Signal. 2007; 3:1–3. [PubMed: 18404413]

13. Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev. 1998; 50:413–492. [PubMed: 9755289]

14. Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, et al. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev. 2006; 58:281–341. [PubMed: 16968944]

15. Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M, et al. The P2X7 receptor: a key player in IL-1 processing and release. J Immunol. 2006; 176:3877–3883. [PubMed: 16547218]

16. Bianco F, Perrotta C, Novellino L, Francolini M, Riganti L, Menna E, et al. Acid sphingomyelinase activity triggers microparticle release from glial cells. EMBO J. 2009; 28:1043–1054. [PubMed: 19300439]

17. Hewinson J, Mackenzie AB. P2X(7) receptor-mediated reactive oxygen and nitrogen species formation: from receptor to generators. Biochem Soc Trans. 2007; 35:1168–1170. [PubMed: 17956304]

18. Santana PT, Benjamim CF, Martinez CG, Kurtenbach E, Takiya CM, Coutinho-Silva R. The P2X7 Receptor Contributes to the Development of the Exacerbated Inflammatory Response Associated with Sepsis. J Innate Immun. 2015; 7:417–427. [PubMed: 25675986]

19. Csoka B, Nemeth ZH, Toro G, Idzko M, Zech A, Koscso B, et al. Extracellular ATP protects against sepsis through macrophage P2X7 purinergic receptors by enhancing intracellular bacterial killing. FASEB J. 2015; 29:3626–3637. [PubMed: 26060214]

20. Robson SC, Sevigny J, Zimmermann H. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. Purinergic Signal. 2006; 2:409–430. [PubMed: 18404480]

21. Hasko G, Csoka B, Koscso B, Chandra R, Pacher P, Thompson LF, et al. Ecto-5′-nucleotidase (CD73) decreases mortality and organ injury in sepsis. J Immunol. 2011; 187:4256–4267. [PubMed: 21918191]

22. Csoka B, Nemeth ZH, Toro G, Koscso B, Kokai E, Robson SC, et al. CD39 improves survival in microbial sepsis by attenuating systemic inflammation. FASEB J. 2015; 29:25–36. [PubMed: 25318479]

23. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA. Immunodesign of experimental sepsis by cecal ligation and puncture. Nat Protoc. 2009; 4:31–36. [PubMed: 19131954]

24. Chalmin F, Mignot G, Bruchard M, Chevriaux A, Vegran F, Hichami A, et al. Stat3 and Gfi-1 transcription factors control Th17 cell immunosuppressive activity via the regulation of ectonucleotidase expression. Immunity. 2012; 36:362–373. [PubMed: 22406269]

25. Vaughn BP, Robson SC, Burnstock G. Pathological roles of purinergic signaling in the liver. J Hepatol. 2012; 57:916–920. [PubMed: 22709619]

26. Levesque SA, Kukulski F, Enjyoji K, Robson SC, Sevigny J. NTPDase1 governs P2X7-dependent functions in murine macrophages. Eur J Immunol. 2010; 40:1473–1485. [PubMed: 20201036]

27. Nemeth ZH, Lutz CS, Csoka B, Deitch EA, Leibovich SJ, Gause WC, et al. Adenosine augments IL-10 production by macrophages through an A2B receptor-mediated posttranscriptional mechanism. J Immunol. 2005; 175:8260–8270. [PubMed: 16339566]

28. Beldi G, Banz Y, Kroemer A, Sun X, Wu Y, Graubardt N, et al. Deletion of CD39 on natural killer cells attenuates hepatic ischemia/reperfusion injury in mice. Hepatology. 2010; 51:1702–1711. [PubMed: 20146261]

29. Hoque R, Sohail MA, Salhanick S, Malik AF, Ghani A, Robson SC, et al. P2X7 receptor-mediated purinergic signaling promotes liver injury in acetaminophen hepatotoxicity in mice. Am J Physiol Gastrointest Liver Physiol. 2012; 302:G1171–G1179. [PubMed: 22383490]

30. Proietti M, Cornacchione V, Rezzonico JT, Romagnani A, Faliti CE, Perruzza L, et al. ATP-gated ionotropic P2X7 receptor controls follicular T helper cell numbers in Peyer's patches to promote host-microbiota mutualism. Immunity. 2014; 41:789–801. [PubMed: 25464855]

31. Savio, LE., Andrade, MG., de Andrade, MP., Santana, PT., Moreira-Souza, AC., Kolling, J., et al. P2X7 receptor signaling contributes to sepsis-associated brain dysfunction. Mol Neurobiol. 2016. http://dx.doi.org/10.1007/s12035-016-0168-9

32. Fortin CF, McDonald PP, Fulop T, Lesur O. Sepsis, leukocytes, and nitric oxide (NO): an intricate affair. Shock. 2010; 33:344–352. [PubMed: 19789465]

33. Remy M, Thaler S, Schumann RG, May CA, Fiedorowicz M, Schuettauf F, et al. An in vivo evaluation of Brilliant Blue G in animals and humans. Br J Ophthalmol. 2008; 92:1142–1147. [PubMed: 18653608]

34. Totan Y, Guler E, Guragac FB, Uzun E, Dogdu E. Brilliant blue G assisted macular surgery: the effect of air infusion on contrast recognisability in internal limiting membrane peeling. Br J Ophthalmol. 2015; 99:75–80. [PubMed: 25091954]

35. Tung HC, Lee FY, Wang SS, Tsai MH, Lee JY, Huo TI, et al. The beneficial effects of P2X7 antagonism in rats with bile duct ligation-induced cirrhosis. PLoS One. 2015; 10:e0124654. [PubMed: 25933224]

36. Eser A, Colombel JF, Rutgeerts P, Vermeire S, Vogelsang H, Braddock M, et al. Safety and efficacy of an oral inhibitor of the purinergic receptor P2X7 in adult patients with moderately to severely active crohn's disease: a randomized placebo-controlled, double-blind, phase IIa study. Inflamm Bowel Dis. 2015; 21:2247–2253. [PubMed: 26197451]

37. Sullivan GW, Fang G, Linden J, Scheld WM. A2A adenosine receptor activation improves survival in mouse models of endotoxemia and sepsis. J Infect Dis. 2004; 189:1897–1904. [PubMed: 15122527]

38. Moore CC, Martin EN, Lee GH, Obrig T, Linden J, Scheld WM. An A2A adenosine receptor agonist, ATL313, reduces inflammation and improves survival in murine sepsis models. BMC Infect Dis. 2008; 8:141. [PubMed: 18937852]

39. Lappas CM, Day YJ, Marshall MA, Engelhard VH, Linden J. Adenosine A2A receptor activation reduces hepatic ischemia reperfusion injury by inhibiting CD1d-dependent NKT cell activation. J Exp Med. 2006; 203:2639–2648. [PubMed: 17088433]

Lay summary

CD39 expression in macrophages limits P2X7-mediated pro-inflammatory responses, scavenging extracellular ATP and ultimately generating adenosine. CD39 genetic deletion exacerbates sepsis-induced experimental liver injury. Combinations of a P2X7 antagonist and adenosine A2A receptor agonist are hepatoprotective abdominal sepsis.

Highlights

- CD39 expression by monocyte-macrophages limits P2X7-mediated inflammatory responses.

- CD39 genetic deletion exacerbates sepsis-induced liver injury and systemic outcomes.

- P2X7 blockade and concurrent adenosine A_{2A} receptor activation in sepsis provide substantive hepatoprotective effects.

- Simultaneous P2X7 blockade and adenosine A_{2A} receptor activation improve survival in sepsis.
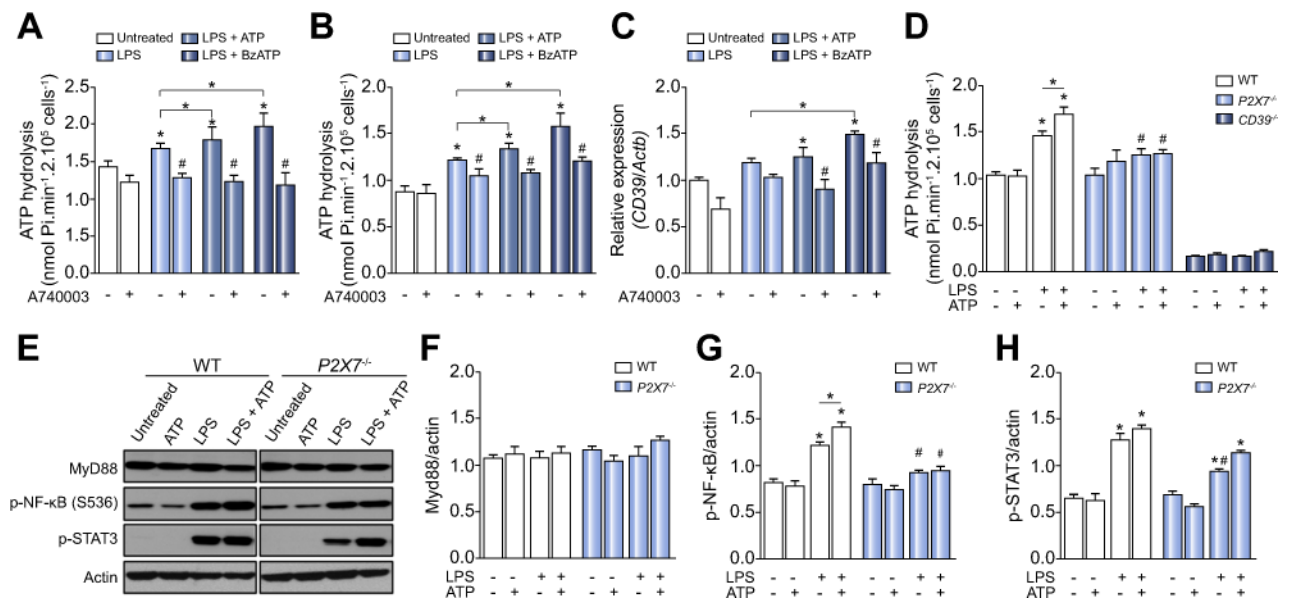
Fig. 1. P2X7 receptor boosts CD39 activity and contributes to NF-κB and STAT3 activation in LPS-primed macrophages

(A) ATP hydrolysis by LPS-primed peritoneal macrophages and (B) BMDM stimulated with P2X7 receptor agonists pretreated or not with A740003. (C) *CD39* gene expression in LPS-primed BMDM. (D) ATP hydrolysis by P2X7 and CD39 deficient LPS-primed macrophages stimulated with ATP. (E) Representative Western blot membranes with densitometric analysis of (F) MyD88, (G) p-NF-κB, and (H) p-STAT3 protein expression in LPS-primed macrophages derived from WT or *P2X7*^{−/−} mice stimulated with 500 μM ATP. Data are expressed as mean ± SEM of three independent experiments performed in triplicates analyzed by one-way ANOVA, Turkey tests. Asterisks represent statistically significant differences ($p < 0.05$; *) compared to unstimulated control group. The number signs (#) represent statistically significant difference ($p < 0.05$) when comparing groups of animals and WT vs. *P2X7*^{−/−}.
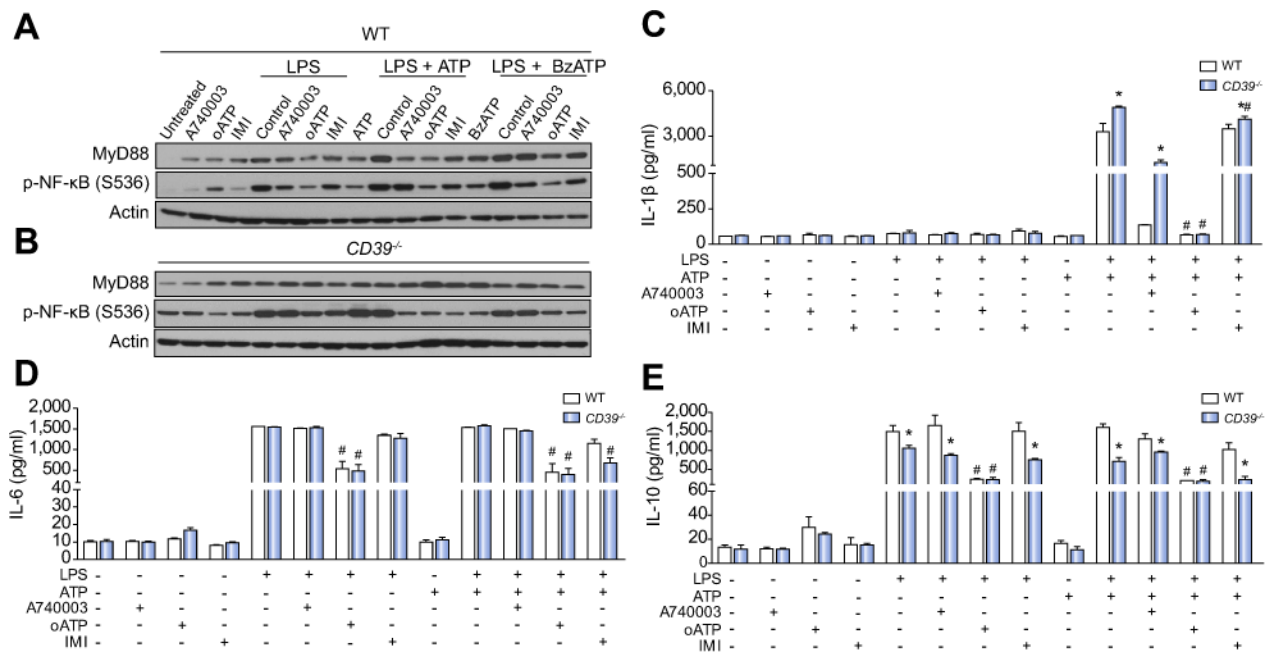
Fig. 2. P2X7 receptor blockade decreases LPS-mediated NF-κB activation and cytokine production in WT and *CD39*$^{-/-}$ BMDM

(A) MyD88 and p-NF-κB protein expression (B) IL-1β, (C) IL-6, and (D) IL-10 production in WT and *CD39*$^{-/-}$ LPS-primed BMDM stimulated with ATP (500 μM) or BzATP (100 μM). For P2X7 receptor inhibition, samples were pretreated with the P2X7 receptor antagonists oATP (300 μM, for 2 h) or A740003 (0.1 μM, for 30 min) or with imipramine (IMI, 30 μM for 30 min) before priming. Data are expressed as mean ± SEM of three independent experiments analyzed by one-way ANOVA, Turkey tests. Asterisks represent statistically significant differences (*p* <0.05; *) when comparing WT to *CD39*$^{-/-}$ or groups. Number signs (#) represent statistically significant differences (*p* <0.05) when the group pretreated with antagonists were compared to the same stimulated group non-pretreated.
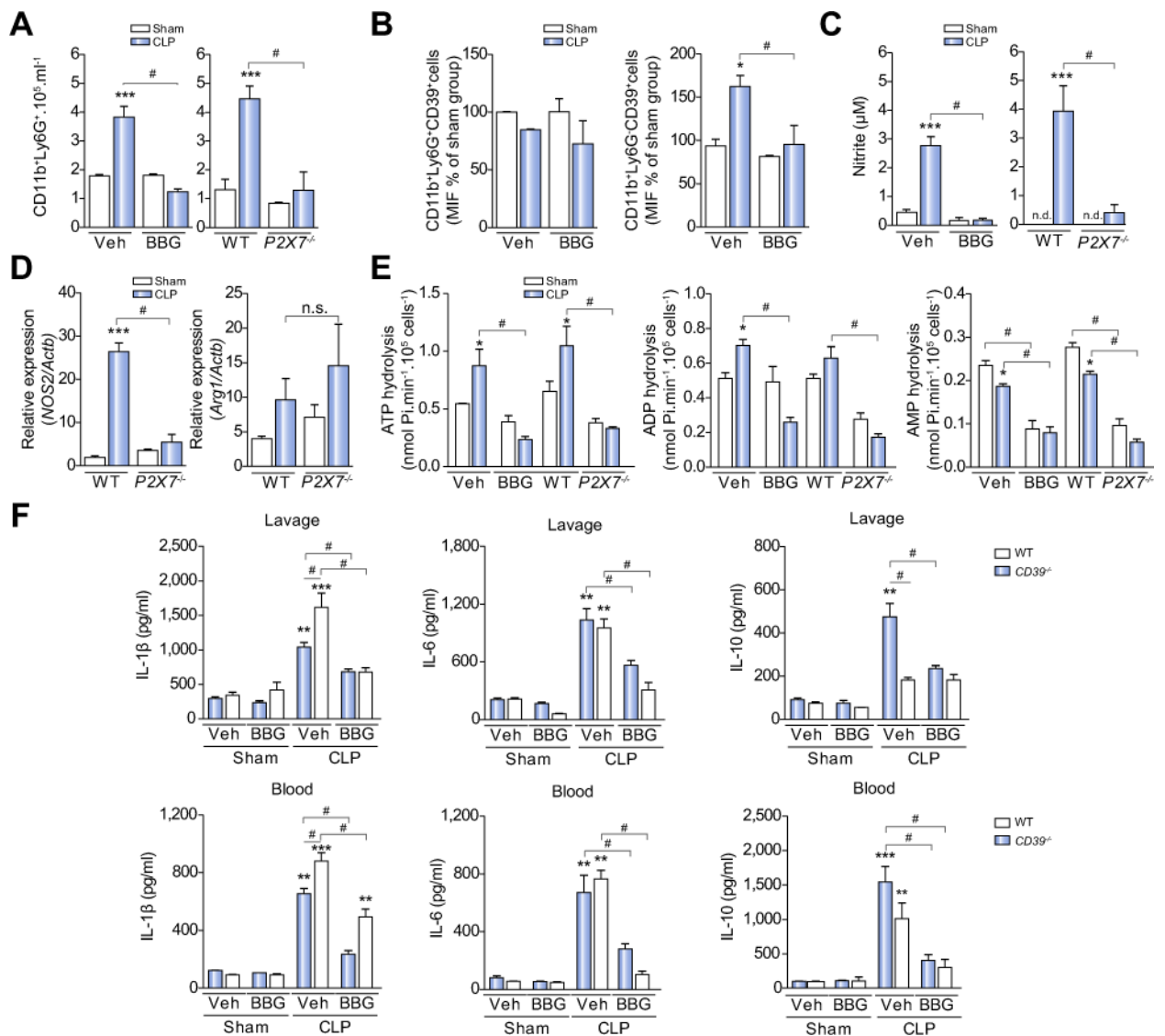
Fig. 3. P2X7 receptor blockade attenuates systemic inflammatory responses in septic WT and *CD39*$^{-/-}$ mice

Peritoneal lavage fluid was collected 3 h after surgery and the (A) number of neutrophils (CD11b$^+$Ly6G$^+$) and monocyte/macrophages (CD11b$^+$Ly6G$^-$) was quantified by flow cytometry, as well as, (B) CD39 expression in these cells. (C) Nitric oxide production and (D) *Nos2* expression in peritoneal cavity cells from WT or *P2X7*$^{-/-}$ or BBG-pretreated septic mice. (E) ATP, ADP, and AMP hydrolysis by peritoneal cavity cells from WT or *P2X7*$^{-/-}$ or BBG-pretreated septic mice. (F) Cytokine production in peritoneal lavage fluid and blood from WT or *CD39*$^{-/-}$ mice. Data are expressed as mean ± SEM of four independent experiments (min. n = 3 mice/experiment) analyzed by one-way ANOVA, Turkey tests. Statistically significant differences between Sham and CLP, and between CLP groups (CLP vehicle vs. BBG, CLP WT *vs. P2X7*$^{-/-}$ or *CD39*$^{-/-}$) are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) and by the number sign (#, $p < 0.05$), respectively.
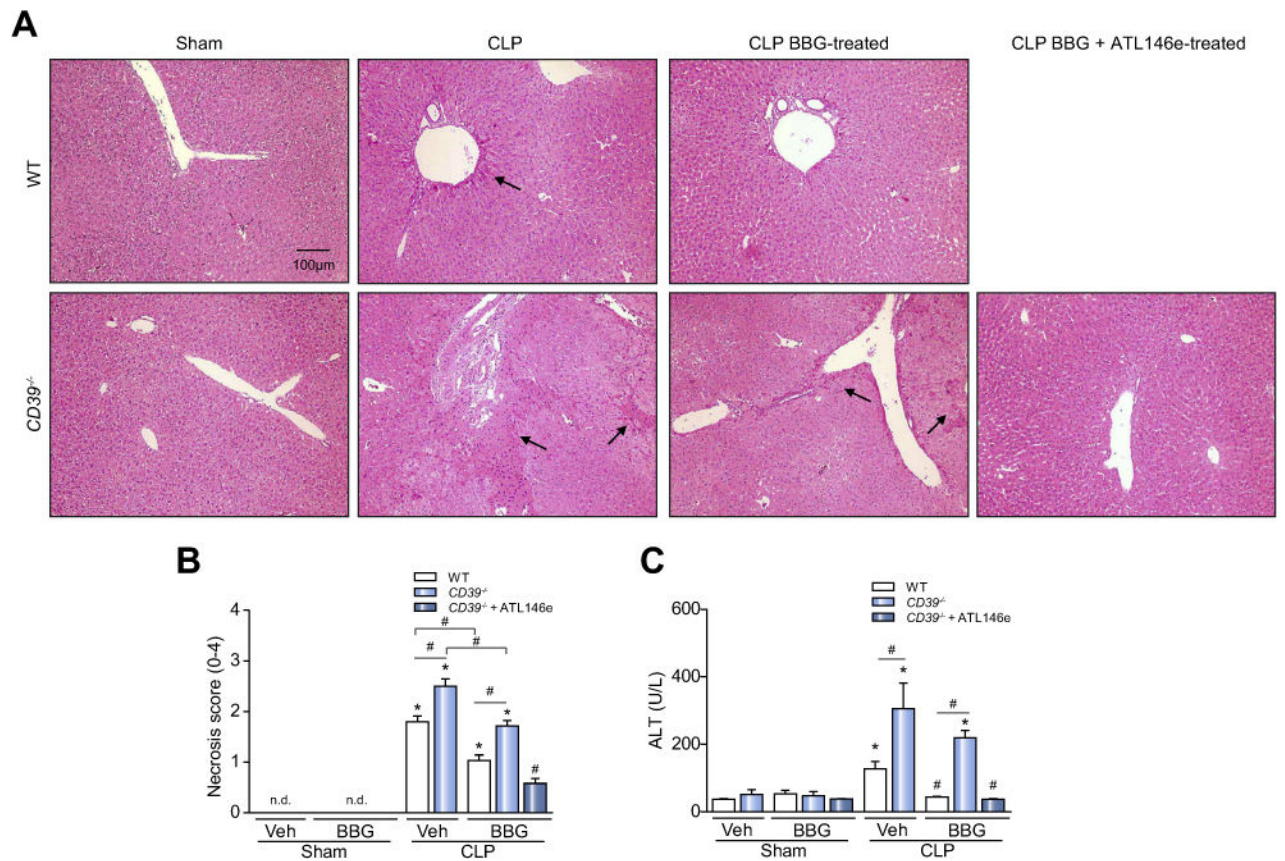
Fig. 4. P2X7 receptor pharmacological inhibition and adenosine A$_{2A}$ receptor activation completely protects the liver from WT and $CD39^{-/-}$ mice during sepsis

The liver was harvested 24 h after surgery for the evaluation of histopathological changes. Representative photomicrographs of hepatic parenchyma in (A) WT and $CD39^{-/-}$ mice are shown. Arrows show the necrotic areas. (B) Necrosis score; values represent mean ± SEM of four animals per group, considering 10 random fields per mice. (C) Alanine aminotransferase (ALT) levels in septic mice. Data are expressed as mean ± SEM of two independent experiments (n = 6) analyzed by one-way ANOVA, Turkey tests. Statistically significant differences between Sham and CLP, and between CLP groups (CLP vehicle vs. CLP BBG, CLP vehicle vs. CLP BBG + ATL146e) are indicated by asterisks (*, $p < 0.05$) and by number sign (#, $p < 0.05$), respectively.
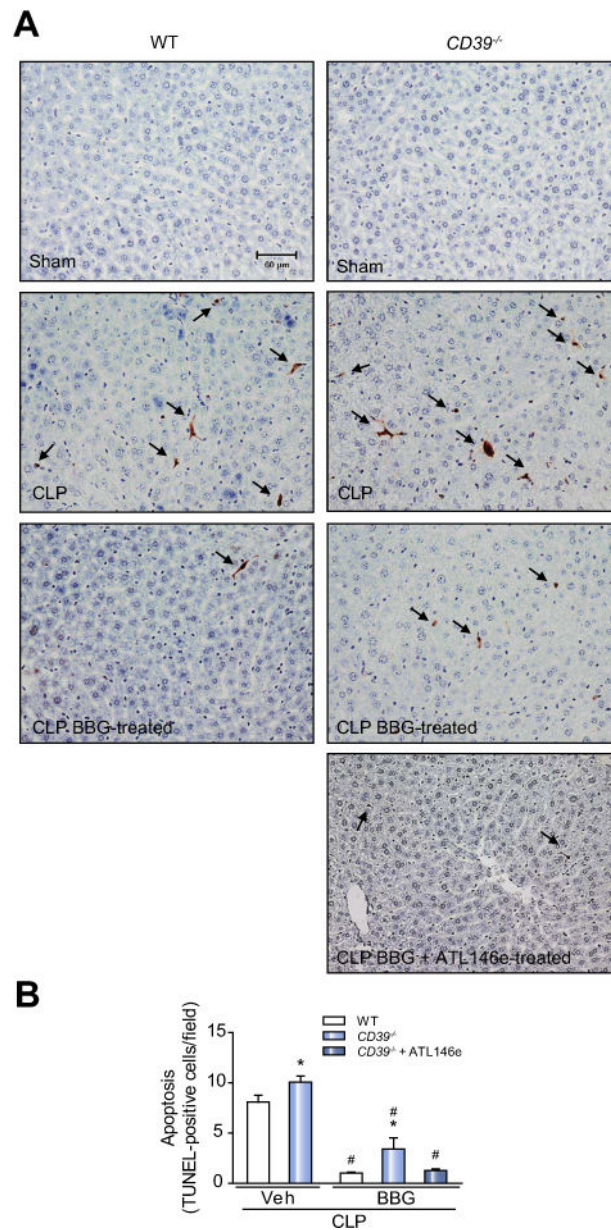
Fig. 5. P2X7 receptor inhibition decreases the number of apoptotic cells in the liver from septic WT or CD39$^{-/-}$ mice

The liver was harvested 24 h after surgery. (A) Photomicrographs show the hepatic TUNEL-positive cells (arrows) in septic WT and CD39$^{-/-}$ mice. (B) Number of TUNEL-positive cells per field. Values represent mean ± SEM of four animals per group, considering five random fields per mice. Data are expressed as mean ± SEM of two independent experiments (n = 6) analyzed by one-way ANOVA, Turkey tests. Statistically significant differences between Sham and CLP, and between CLP groups (CLP vehicle *vs.* CLP BBG, CLP vehicle *vs.* CLP BBG + ATL146e) are indicated by asterisks (*, $p < 0.05$) and by the number sign (#, $p < 0.05$), respectively.
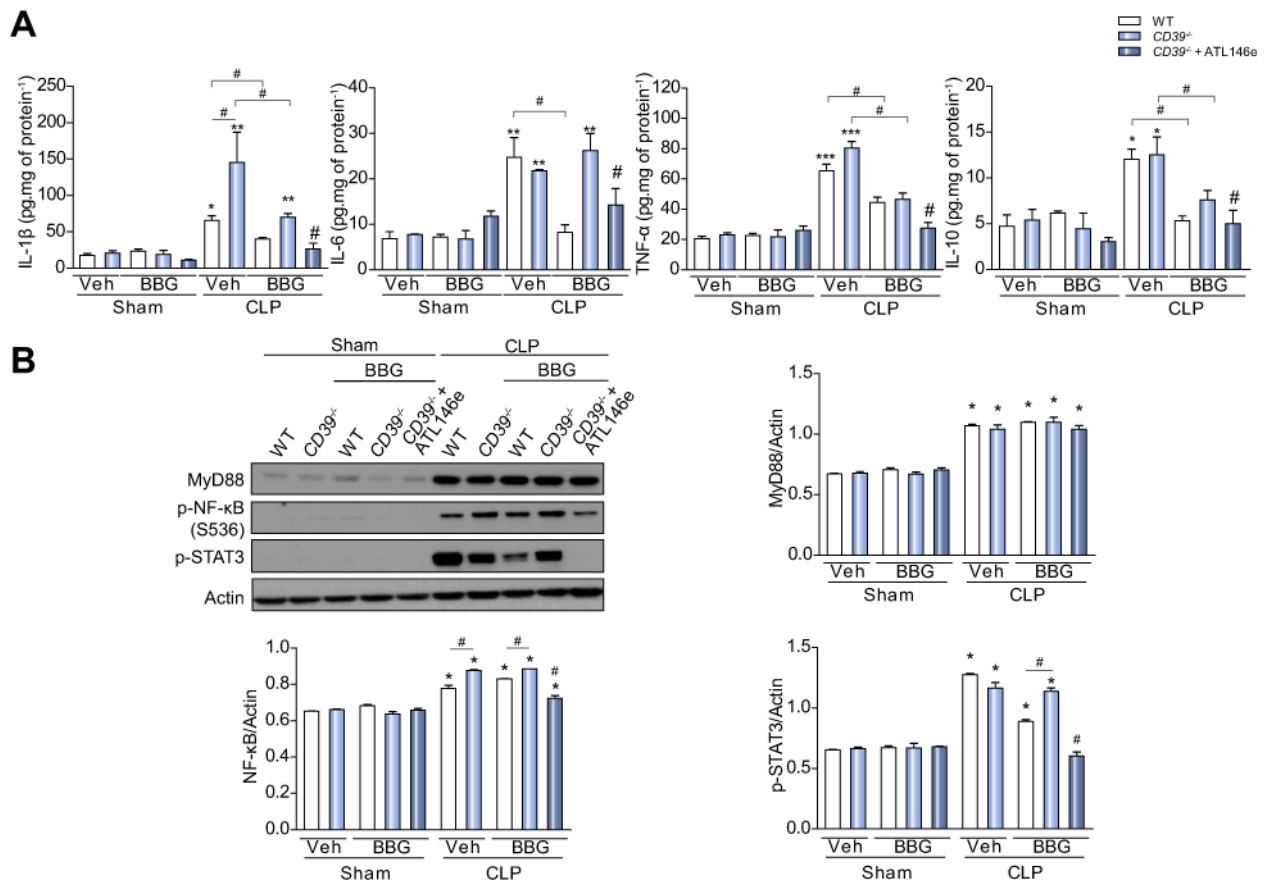
Fig. 6. P2X7 receptor blockade and concurrent adenosine A$_{2A}$ receptor activation inhibit inflammatory cytokine production and NF-κB/STAT3 activation in the liver from septic WT or CD39$^{-/-}$ mice

The liver was harvested 24 h after surgery and (A) cytokine (IL-1β, IL-6, TNF-α, and IL-10) production were determined by ELISA. (B) MyD88, p-NF-κB and p-STAT3 protein levels in the liver from septic mice treated or not treated with BBG or BBG + ATL146e. Data are presented as mean ± SEM from three independent experiments (n = 6) analyzed by one-way ANOVA, Turkey tests. Statistically significant differences between Sham and CLP, and between CLP groups (CLP vehicle *vs.* CLP BBG, CLP vehicle *vs.* CLP BBG + ATL146e) are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) and by the number sign (#, $p < 0.05$), respectively.
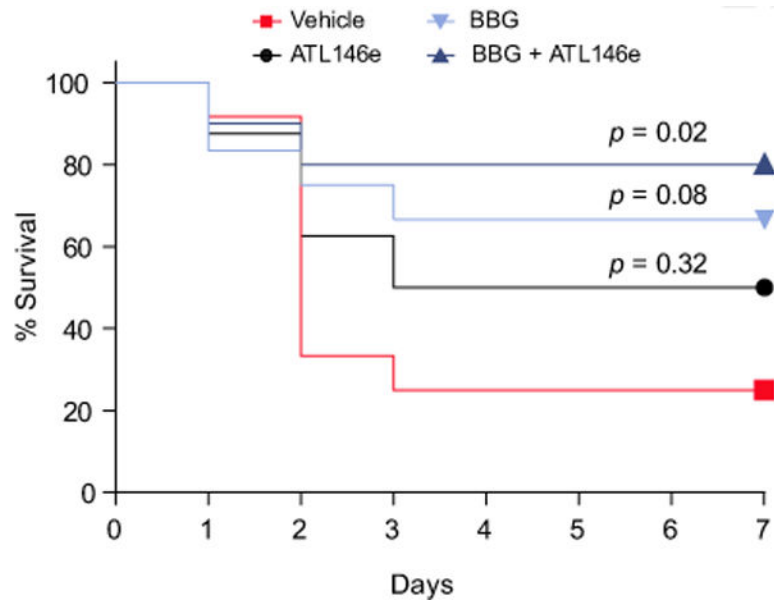
Fig. 7. P2X7 receptor blockade and adenosine A$_{2A}$ receptor activation prolong time to euthanasia and death of septic mice

Survival curves of septic WT mice after pharmacological treatments (ATL146e, BBG or BBG + ATL146e) showing time to euthanasia or death. The statistical significances were analyzed using the Kaplan-Meier method and Log-rank test (n = 8–12).
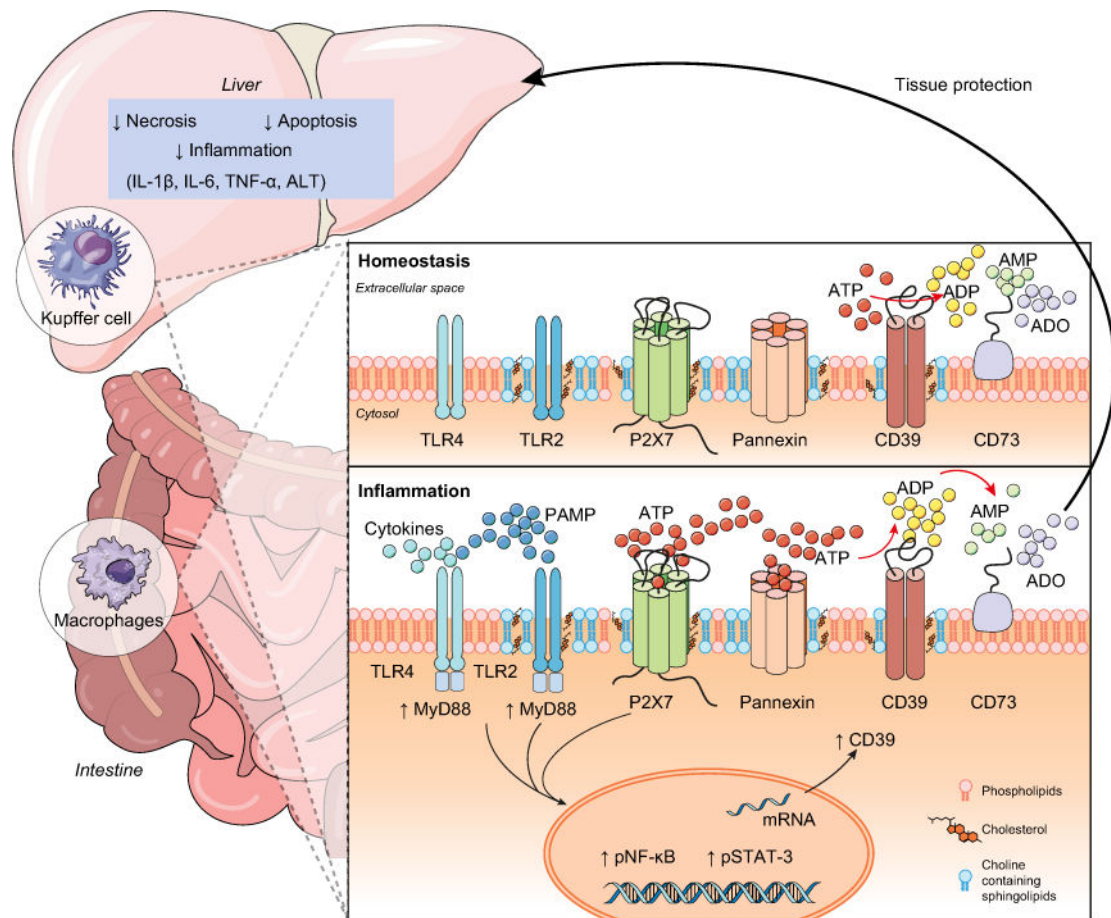
Fig. 8. Schematic representation of P2X7 receptor and CD39 interactions on monocyte-macrophages that modulate liver injury during inflammation

The recognition of PAMPs – such as bacterial LPS – by PRRs can induce ATP release activating the P2X7 receptor. P2X7 receptor activation induces large-scale ATP release that can occur mainly via pannexin hemichannels boosting purinergic signaling. CD39 functions to generate AMP from extracellular ATP to limit P2X7 activation and inhibit pro-inflammatory responses. Then, CD73 hydrolyzes AMP to adenosine (ADO) providing for anti-inflammatory effects and tissue protection. This purinergic regulatory mechanism is functional in limiting liver injury and restoring homeostasis during sepsis.