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Immunity to infection

Research Article

Kupffer cell activation by different microbial lysates: Toll-like receptor-2 plays pivotal role on thromboxane A_2 production in mice and humans

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Thromboxane (TX) A_2 has been identified as an important intrahepatic vasoconstrictor upon Kupffer cell (KC) activation during infections such as spontaneous bacterial peritonitis (SBP). The study aimed to investigate the role of TLRs in the TXA_2 increase in liver nonparenchymal cells and their related mechanisms. Here, we identified TLR-2 as a common pathway for different microbials: microbial lysates including Gram-positive bacteria, Gram-negative bacteria, and fungi all increased TXA_2 secretion via activation of TLR-2 in human KCs, accompanied by increased expression and phosphorylation of Myd88-related pathway. Of all TLR agonists, only TLR-1, -2, and -4 agonists increased TXA_2 in human KCs. These results were further confirmed by mouse liver nonparenchymal cells. Comparing the effects of TLR-1, -2, and -4 antagonists, only TLR-2 antagonist showed inhibitory effects with all tested microbial lysates. Pretreatment with TLR-2 antagonist in human KCs blocked the secretion of IL-10, CXCL-10, TNF-α, and IL-6 induced by Gram-positive and Gram-negative bacterial stimulation. IL-23 and IL-1β were only induced by Gram-negative bacteria. Thus, TLR-2 might be a potential marker and an attractive target for future treatment of patients with SBP. In addition, IL-23 and IL-1β might distinguish early between Gram-positive and Gram-negative SBP.

Keywords: bacterial peritonitis · kupffer cell · MAPK · thromboxane A_2 · TLR2

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Spontaneous bacterial peritonitis (SBP) is the most common infection associated with acute-on-chronic liver failure (ACLF), which is characterized by high morbidity and mortality due to a systemic

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inflammatory response syndrome and subsequent multiple-organ dysfunction [1]. Gram-negative aerobic organisms such as *Klebsiella pneumoniae (K. pneumoniae), Escherichia coli* (*E. coli*), and *Enterobacter cloacae* (*E. cloacae*) are the most common cause of SBP patients, other common organisms include *Enterococcus faecium* (*E. faecium*), *Streptococcus pneumoniae* (*S. pneumoniae*), and *Staphylococcus aureus* (*S. aureus*), while *Candida albicans* (*C. albicans*) is reported as the most common pathogen of spontaneous fungal peritonitis [2]. Nonparenchymal cells (NPCs), such as Kupffer cells (KCs), sinusoidal endothelial cells (SECs), and hepatic stellate cells (HSCs), are the first cells to meet bacteria or their products in the liver [3,4].

Recognition of microorganisms and activation of immune responses are mediated through pattern recognition receptors (PRRs), which mainly include toll like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and DNA sensors such as absent in melanoma 2 (AIM2). The expressions of NOD1, NOD2 and inflammasome receptors (NLRP1, NLRP3, AIM2) in non-stimulated liver NPCs are almost undetectable [5]. In contrast, TLR genes are widely expressed in NPCs: TLR-2 to -5 in HSCs; TLR-2 to -4 in KCs; and TLR-2 to -4 and TLR-9 in SECs. TLR-2, which can form complexes with TLR-1 or -6, is expressed in all NPCs but is highest in KCs [5,6]. TLR-2 receptors in monocytes are significantly overexpressed in patients with tuberculosis, systemic lupus erythematosus, and myelodysplastic syndromes [7,8]. The synergistic effects of the inflammasome (the heterogeneous group of multiprotein complexes) and TLR signals are the main causes for cytokine release. Activation of inflammasome can cause autocatalysis and kinase activation, leading to the maturation of proinflammatory cytokines [9]. Once activated by microbial antigens, inflammasome and TLR signal transduction become activated through Myd88 and MAPK resulting in the translocation of NF-κB with subsequent induction of inflammatory cytokines [10-12].

Besides inflammatory cytokines, thromboxane (TX) A_2 has been recently identified as an important factor of bacterial defense and the progression of ACLF [3]. TXA_2, mainly secreted by KCs in the liver, is an important vasoconstrictor and plays a crucial role in intrahepatic vascular constriction, platelet activation, and aggregation [13]. TXA_2 can be upregulated by non-alcoholic fatty liver disease, ischemia, and inflammation in in vitro and in vivo experiments [14]. In patients, increased TXA_2 synthesis was also linked to infections, acute myocardial ischemia, and heart failure [15]. The pathophysiology of KC activation and the function of TXA_2 to increase portal perfusion pressure following TLR agonist stimulations have been well investigated in our previous publications [3,4,16]. However, which TLRs on KCs are responsible for the TXA_2 secretion caused by bacterial infections remained unclear.

In this study, KCs isolated from human and mouse liver tissues were stimulated by microbial lysates derived from cultured isolates of SBP patients. By investigating the effects of TLR antagonists in combination with different microbial lysates, we aimed to identify the individual receptors and cascades of bacterial-induced increase in TXA_2. Besides, we compared the differences in inflammatory cytokine profiles induced by different types of microorganisms.

Results

Microbial lysates induced TXB_2 secretion in KCs

CD163, CD68, and CD11b are exclusively expressed on macrophages and are often used to identify macrophages in tissue sections such as KCs. Immunostaining results showed positive cells with typical morphological characteristics in both human and mouse KCs (Fig. 1A–C), verifying those isolated cells were our target cells.

According to our dose-response experiments (Supporting Information Fig. S1), 8 μg/mL microbial lysates were chosen to stimulate primary KCs. Lysates of Gram-negative bacteria (GNB, including *K. pneumoniae*, *E. coli*, and *E. cloacae*), Gram-positive bacteria (GPB, including *E. faecium*, *S. pneumoniae*, and *S. aureus*) and *C. albicans* all significantly increased Thromboxane B_2 (TXB_2, the stable degradation product of TXA_2) TXB_2 secretion in both human and mouse KCs (Fig. 1D and E).

TLR-1, -2, and -4 agonists increased TXB_2 secretion in KCs

TLR-1 to -9 agonists (names and related dosages were described below) were then used to investigate the effective TLRs increasing TXB_2 in KCs. Twenty-four hour stimulation with Pam3CSK4, heat-killed Listeria monocytogenes (HKLM) or LPS increased TXB_2 secretion in both human and mouse KCs (Fig. 2A and B). The same concentrations of TLR-1 to -9 agonists treating primary KCs were also tested in mouse SECs and HSCs. TLR agonists had no effects on TXB_2 secretion in SECs (Fig. 2C). Interestingly, an overall reduction was observed in HSCs after TLR agonists' stimulation and significant reduction of TXB_2 was found by applying TLR-3, -5, and -9 agonists (Fig. 2D).

TLR-2 antagonist showed the strongest effect in reducing bacterial-induced TXB_2 increase

According to the results above, TLR-1, -2, and -4 seemed to be most possibly related to bacterial-induced TXB_2 increase in KCs. CU-CPT22 (10 μM), 1 μg/mL Mab-mTLR2, and 1 μM TAK242 selected by dose-response experiments (Supporting Information Fig. S2) were used as antagonists for TLR-1, -2, and -4 in the following experiments.

Antagonists were added to human KCs 1 h before microbial stimulation. TXB_2 increase caused by *K. pneumoniae*, *E. coli*, *E. cloacae*, *E. faecium*, and *S. pneumoniae* were attenuated by TLR-1, -2, or -4 antagonists (Fig. 3A–E). TLR-1 and -2 antagonists also reduced TXB_2 secretion by *S. aureus* (Fig. 3F). Only Mab-mTLR2 decreased *C. albicans*-induced TXB_2 production (Fig. 3G). Comparing among TLR-1, -2, and -4 antagonists, Mab-mTLR2 seemed to be the most effective antagonist.

The effects of combined TLR antagonists were also compared with each single substance. Either single TLR-1, -2, and -4

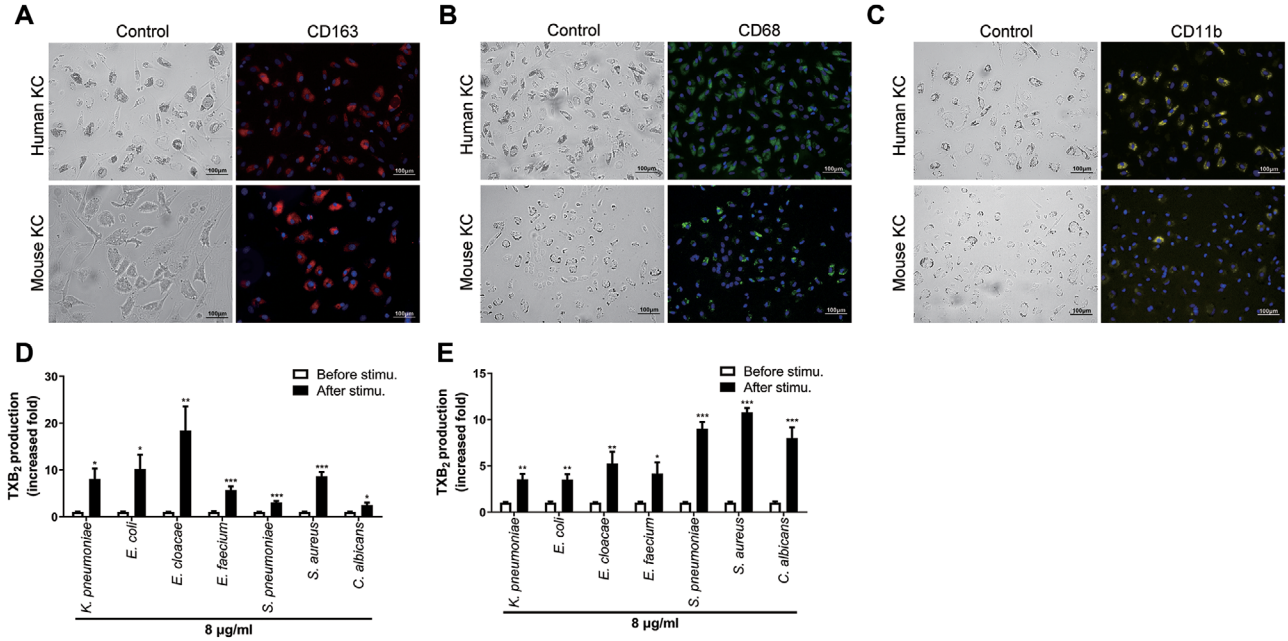

Figure 1. Stimulation with microbial lysates increased TXB_2 secretion in KCs. (A–C) Anti-CD163 (red), CD68 (green), and CD11b (yellow) antibodies were used to confirm the primary KCs isolated from human tissues and mice and the nuclei was stained with DAPI (blue), each picture was representative from three separate immunostaining results; (blue color represents DAPI and scale bar represents 100 μm; 200× magnification). (D and E) 8 μg/mL microbial lysates including *K. pneumoniae, E. coli, E. cloacae, E. faecium, S. pneumoniae, S. aureus*, and *C. albicans* were used to stimulate primary KCs isolated from human or mice tissues. 24 h stimulation with each microbial lysates significantly ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, comparing between values before and after stimulation, two-sided Student's *t*-test) increased TXB_2 secretion in human KCs (D) and mouse KCs (E). TXB_2 was measured by ELISA as detailed in the Materials and Methods section. Data are expressed as mean ± SD, $n = 6$ samples, and are from three independent experiments with a total of three human tissue or 12 mice.

antagonists or combined substances significantly decreased TXB_2 concentrations after GNB activation. In addition, combined substance achieved a better effect than any single substances (Supporting Information Fig. S3).

Myd88 related pathway is essential for bacterial-induced TXB_2 increase

Myd88-related pathways were reported to be involved in the TXB_2 secretion. Due to the limited number of primary KCs, THP-1 cells (the most commonly used macrophage-like cell line with similar TLR protein expression to human KCs [5]) were used to replace KCs. We treated THP-1 cells with the same dose and time of microbial lysate, and detected the expression of Myd88 related pathway proteins. Compared with the control group (cells without stimulants), Myd88 expression and phosphorylation levels of MAPK and NF-κB increased after stimulation with TLR-2 agonists or microbial lysates. In addition, the expression of phosphorus-MAPK and NF-kB caused by GNB were higher than those caused by GPB (Fig. 4).

Effect of TLR-2 antagonist on the secretion of inflammatory cytokines

The inflammatory cytokine secretion by KCs via the Myd88 pathway is an important mechanism during bacterial infections. Twenty-four hour stimulation with GPB products significantly increased the secretion of IL-10, CXCL-10, TNF-α, and IL-6 in human KCs. Thereby, all the cytokine levels could be reduced by Mab-mTLR2 pretreatment (Fig. 5A–D). Besides the cytokines above, GNB also increased IL-1β and IL-23 secretion. The TLR-2 antagonist decreased the levels of IL-10, CXCL-10, IL-1β, and IL-23 among all six effective cytokines (Fig. 5A–F). *C. albicans* only induced TNF-α and IL-6 secretion and TNF-α increase can be blocked by TLR-2 antagonists (Fig. 5C and D). None of the microbial lysates had significant effects in IL-1RA or IL-12p70 (Fig. 5E and F).

Effects of purinergic signaling in the bacterial-induced TXB_2 increase

In addition to the TLR signal, purinergic signaling is also involved in the inflammatory immune response in the liver. Subtypes including type 2 P (P2Y) G protein-coupled receptors and P2X ATP-gated cation channels are widely expressed in hepatocytes and NPCs [17, 18]. P2 × 1R and P2 × 7R were reported to be expressed in KCs, the expression of P2 × 7R was increased during microbial stimulation while P2 × 1R showed no significant change (Supporting Information Fig. S4A and B). Furthermore, a P2 × 7R antagonist inhibited the effect of both GPB and GNB, confirming the role of P2 × 7R in bacterial-induced TXB_2 secretion in human

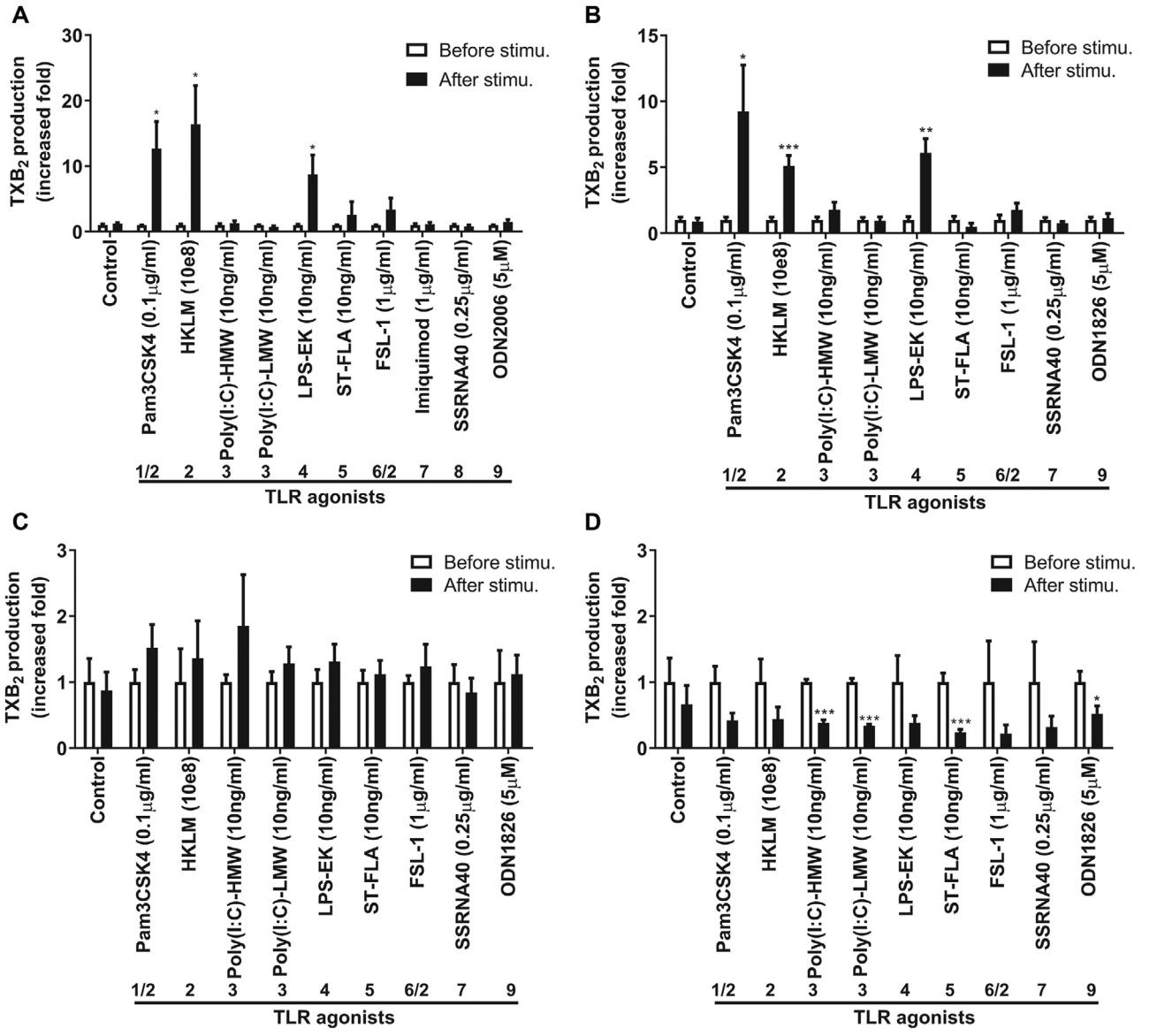

Figure 2. TLR-1, -2, and -4 agonists increased TXB_2 secretion in KCs. The same doses of special TLR-1 to -9 agonists were used in human KCs, mouse KCs, mouse SECs, and mouse HSCs for 24 h. (A and B) Among all ten kinds of agonists, only Pam3CSK4 (0.1 μg/mL), HKLM (10×10^8 cells/mL), and LPS-EK (10 ng/mL) significantly ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, comparing between values before and after stimulation, two-sided Student's *t*-test) increased TXB_2 secretion in human KCs (A) and mouse KCs (B). (C) None of TLR agonists significantly increased TXB_2 secretion in mouse SECs. (D) An overall reduction was observed in mouse HSCs after TLR agonists' stimulation. Furthermore, Poly(I:C)-HMW (10 ng/mL), Poly(I:C)-LMW (10 ng/mL), ST-FLA (10 ng/mL), and ODN1826 (5 μM) induced significant ($^*p < 0.05$, $^{***}p < 0.001$, comparing between values before and after stimulation, two-sided Student's *t*-test) effects. TXB_2 was measured by ELISA as detailed in the Materials and Methods section. Data are expressed as mean ± SD, $n = 6$ samples, and are from three independent experiments with a total of three human tissue or 12 mice.

KCs (Supporting Information Fig. S4E). Although inhibitors of P2Y1R could block TXA_2 production by human platelets [10], the expression of P2Y1R and P2Y2R in human KCs was too low to be detected (Supporting Information Fig. S4C and D).

Discussion

Our study is the first to reveal the increase of the vasoconstrictor TXA_2 after the activation of human KCs with different human microbial lysates. TXA_2 has been identified as a highly relevant vasoconstrictor in the liver [3,4]. This present study investigated the role of TLRs in microbial-induced TXA_2 increase in liver nonparenchymal cells (NPCs), its related mechanisms, and the relationship of inflammatory cytokines to different kinds of microorganisms. Novel findings in the present study were: (1) GPB, GNB, and fungal lysates, all extracted from SBP patients increased TXB_2 secretion in human KCs, with the increased expression and phosphorylation of MAPK and NF-κB; (2) SECs and HSCs did not produce TXB_2 after TLR activation, while KCs did; (3) only TLR-1/2,

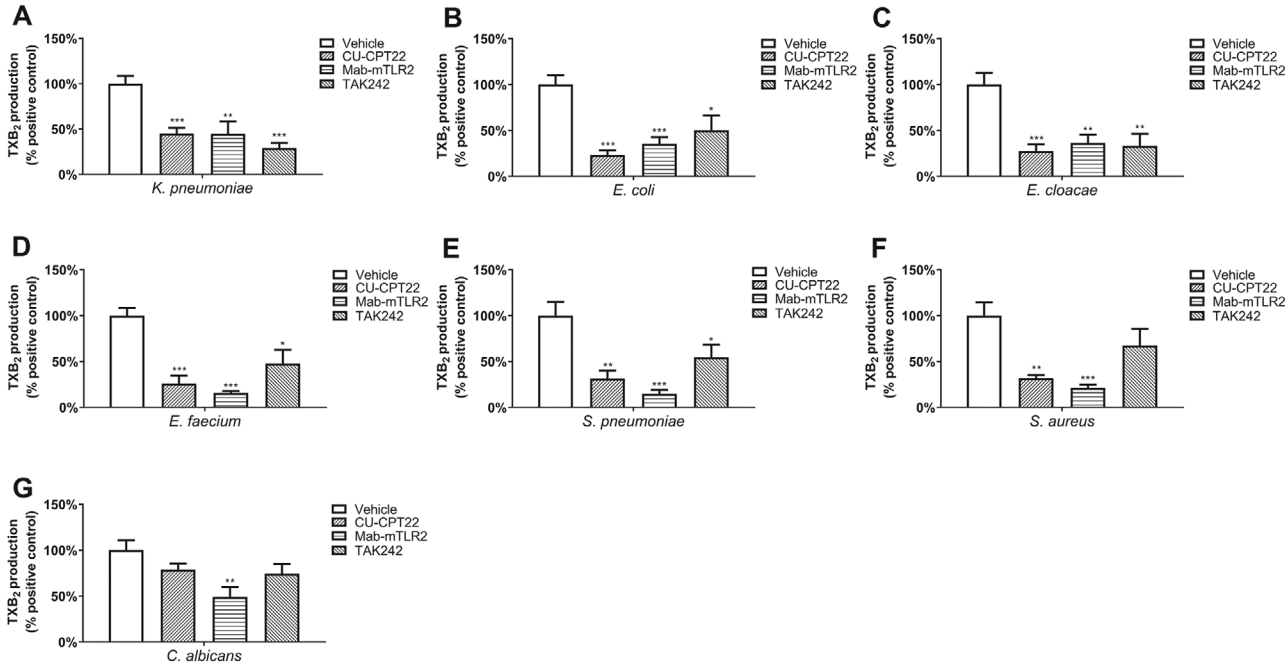

Figure 3. Effects of TLR-1, -2, and -4 antagonists differed in different kinds of microbial lysates. Human KCs were treated with the same concentration of TLR-1, -2, or -4 antagonist (CU-CPT22, Mab-mTLR2, or TAK242) for 1 h, and then stimulated with different microbial lysates for 24 h, including *K. pneumoniae*, *E. coli*, *E. cloacae*, *E. faecium*, *S. pneumoniae*, *S. aureus*, and *C. albicans* (8 μg/mL each). One hour pretreatment with CU-CPT22 (10 μM), Mab-mTLR2 (1 μg/mL), or TAK242 (1 μM) in human KCs respectively attenuated TXB_2 increase caused by *K. pneumoniae* (A), *E. coli* (B), *E. cloacae* (C), *E. faecium* (D), and *S. pneumoniae* (E). Cu-CPT22 and Mab-mTLR2 also decreased TXB_2 induced by *S. aureus* (F). Only Mab-mTLR2 had effects on *C. albicans*-induced TXB_2 increase (G). The solvent treatment group (Vehicle group) used as a positive control. Data are expressed as mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-sided Student's *t*-test) compared to the positive control. TXB_2 was measured by ELISA as detailed in the Materials and Methods section. Data are expressed as mean ± SD, $n = 6$ samples, and are from three independent experiments with a total of three human tissue or 12 mice.

-2, and -4 agonists increased TXB_2 secretion in KCs among all TLR agonists; (4) TLR-2 antagonists showed the most effective reduction in microbial induced TXB_2 secretion compared with TLR-1 and -4 antagonists, combination of TLR-1, -2, and -4 antagonists attained even better effects; and (5) TLR-2 antagonists attenuated all inflammatory cytokines induced by GPB and partially cytokines induced by GNB. IL-1β and IL-23 might help to distinguish between GPB and GNB infections.

The seven most common pathogens extracted from SBP patients all increased TXB_2 secretion in human and mouse KCs (Fig. 1). The relation between TXB_2 production and TLRs especially TLR-2 and -4 via Myd88 related pathway has been proven in human alveolar macrophages, peritoneal macrophages, and various in vivo experiments [19]. According to results involving TLR -1 to -9 agonists in human and mouse KCs (Fig. 2A and B), TLR-1, -2, and -4 were identified as the most relevant TLRs in this process. The increased phosphorylation MAPK and NF-κB in human KCs following stimulation with microbial lysates also supported the role of TLRs in microbial-induced TXB_2 increase in human KCs (Fig. 4). Western Blot for THP-1 cells could give first support for the postulated NF-kB/MAPK pathway. In addition to THP-1 cells, the NF-κB and MAPK phosphorylation levels in LPS-stimulated peritoneal macrophages and RAW 264.7 macrophages infected with *S. aureus* also increased [20,21]. Alveolar macrophages isolated from LPS-treated mice also showed a higher ratio of phosphorus-NF-kB/ NF-kB that confirmed the relation between TLR-2/4 and MAPK/ NF-kB phosphorylation [22]. Previous studies have shown that TLR activation can regulate COX-1 and COX-2 through the MyD88-dependent NF-κB pathway, and cause changes in vasoactive factors such as Prostaglandin (PG) [23]. In our previous research, TXA_2 synthase inhibitors and COX-1 specific inhibitors can reduce TXB_2 secretion of KCs caused by Zymosan (an agonist of TLR-2 and -6), but COX-2 inhibitors do not have this effect [16]. Combined with the effects of TLR antagonists on KCs, we speculate that TLR-1, -2, and -4 activate MyD88-dependent pathways to regulate COX-1 is the main mechanism by which bacteria stimulate KCs to secrete TXA_2.

TLR-2 has a unique ability to heterodimerize with TLR-1 and -6. However, TLR2/1 agonists, but not TLR2/6 increased TXB_2 in human KCs consistent with previous results from studies involving platelets [10]. In unstimulated KCs, the ratio of expression levels of mRNA for TLR1, TLR2, and TLR6 was approximately 10:40:1 [24]. That may explain why both TLR-1/2 and TLR-2 agonists increased TXB_2 secretion while the TLR-6/2 agonist failed.

The effects of TLR agonists were also confirmed in mouse NPCs. Mouse KCs showed the same results as human cells after bacterial stimulation, confirming the results obtained within human KCs (Fig. 2B). TLR agonists induced no significant TXB_2

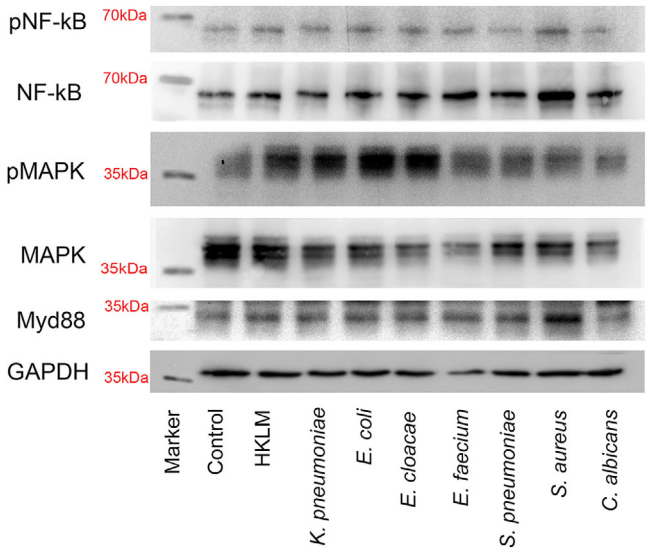

Figure 4. Myd88-related pathways were essential for bacterial-induced TXB_2 increase. TLR-2 agonist (HKLM, 10×10^8 cells/mL) and microbial lysates (*K. pneumoniae*, *E. coli*, *E. cloacae*, *E. faecium*, *S. pneumoniae*, *S. aureus*, and *C. albicans*, 8 μg/mL for each lysate) were used to stimulate THP-1 cells and the expression of Myd88-related pathway proteins were detected by Western blot: compared with the control group (cells without any stimulates), the Myd88, phosphorus-MAPK/MAPK, and phosphorus-NF-kB/NF-kB were significantly increased after stimulation of HKLM or microbial lysates. The Gram-negative bacterial lysates caused higher levels of phosphorus-MAPK and NF-kB than Gram-positive bacteria or *C. albicans*. GAPDH was detected as loading controls and each picture was representative from three separate Western blot results.

increase in SECs (Fig. 2C). The previous publication also showed that anaphylatoxin C5a significantly influenced the release of TXA_2 and PG in both KCs and HSCs, but not in SECs [25]. In contrast, TLR agonists induced an overall reduction in HSCs and TLR-3, -5, and -9 agonists even achieved significant decreases (Fig. 2D). HSC can present bacterial antigens to NK T cells, suppress the proliferation of T cells and activated HSC can also inhibit its activation through a negative feedback loop [26, 27]. These immunosuppressive characteristics may also explain why TLR agonists decreased TXB_2 secretion in HSCs.

PG and TXA_2 produced by NPCs in the liver play important roles in increasing portal pressure following TLR activation [3,28]. Yet, only a few publications reported the functions of TLRs on different NPCs. Our study was the first to investigate the comparison among mouse KCs, SECs, and HSCs after TLR agonists' stimulation, also taking into account complex stimulation patterns encountered with complex microbial extracts.

Cirrhosis can affect the function or phenotype of NPCs. In the fibrotic liver, KCs overexpressed platelet-derived growth factor (PDGF), which was the main factor that activates HSCs [29]. In normal livers, most HSCs were in a quiescent condition, while in cirrhotic livers, HSCs were activated by soluble factors (such as PDGF), differentiated into myofibroblasts, and then produced excess ECM [30]. Cirrhosis-induced SEC changes included a lack of capillarization, SEC fenestration, and the formation of organized basement membranes [31].

Endotoxin level and TLR expression were positively correlated with the degree of liver fibrosis in patients, which may be important factors that cirrhosis affected in NPCs [32]. Human primary cells were rarely involved in the study of cirrhosis on NPCs. In this experiment, we obtained two human samples of liver cirrhosis, one of which was moderate and the other was severely cirrhotic. The number of KCs in the moderate cirrhotic samples was almost the same as that in normal tissues, while the number of KCs from the severe cirrhotic sample was very low (almost 1/4 or 1/3 of normal tissues). KCs extracted from normal or cirrhotic samples all responded to the stimulation of bacterial lysates, however, the increased TXA_2 level was lower in the severe cirrhotic sample than in normal or moderate cirrhotic samples (data not shown). Due to the limited number of NPCs from severe fibrosis samples, the impact of cirrhosis on SEC and HSC needs further investigations.

Antagonists of TLR-1, -2, and -4 were additionally added to human KCs to investigate potential treatment options to reduce the bacterial-induced TXB_2 secretion. GNB (including *K. pneumoniae*, *E. coli*, and *E. cloacae)* induced TXB_2 secretion could be attenuated by all three kinds of antagonists (Fig. 3A–C). TLR-1 and -2 antagonists decreased TXB_2 secretion following GPB (including *E. faecium*, *S. pneumonia*, and *S. aureus*) stimulation (Fig. 3D–F). Besides TLR-1 and -2 antagonists, *E. faecium* and *S. pneumonia* induced TXB_2 production was also reduced by TLR-4 antagonist. Previous studies have reported that GPB and LPS induce homology and cross-tolerance in the secretion of TXB_2 and TNF-α in human macrophages and this effect is achieved through the MAPK-related pathway [33]. The higher phosphorylation levels of MAPK and NF-kB caused by GNB than GPB supported the relation and our results further demonstrated that the cross-resistance of GPB may be caused by TLR-2 (Fig. 4).

Inflammatory cytokines produced by KCs via MAPK related pathway is an important mechanism defending microbial infections. Mab-mTLR2 attenuated the production of all cytokines induced by GPB stimulation, supporting the pivotal role of TLR-2 in this process (Fig. 5A–D). GNB increased more inflammatory cytokines than GPB, while TLR-2 antagonist failed to reduce all inflammatory cytokines significantly. LPS-induced TNF-α and IL-6 production were reported to be related to the $G\alpha_i$-coupled and Src tyrosine kinase-coupled signaling pathways, which may be independent of Myd88 signaling and therefore cannot be affected by TLR-2 antagonist [33] (Fig. 5C, D). IL-1β can induce cross-tolerance to LPS-induced mediators and does not alter MAPK or NF-κB activation, suggesting that LPS-induced IL-1β production is not solely dependent on MAPK-related pathways [23]. That may explain why only GNB increased IL-1β and IL-23 production (Fig. 5E and F). The GNB-induced increase was attenuated by the TLR-2 antagonist, suggesting a cross-talk between TLR-4 and TLR-2 in IL-1β and IL-23 production. Previous studies have shown that the effect of *C. albicans* stimulation is more sensitive to PI3K inhibitors than the MAPK-related pathway [33]. TLR-2 was more related to the MAPK pathways and that may be the reason why TLR-2 antagonist failed to protect against *C. albicans* induced IL-6 secretion (Fig. 5C, D).

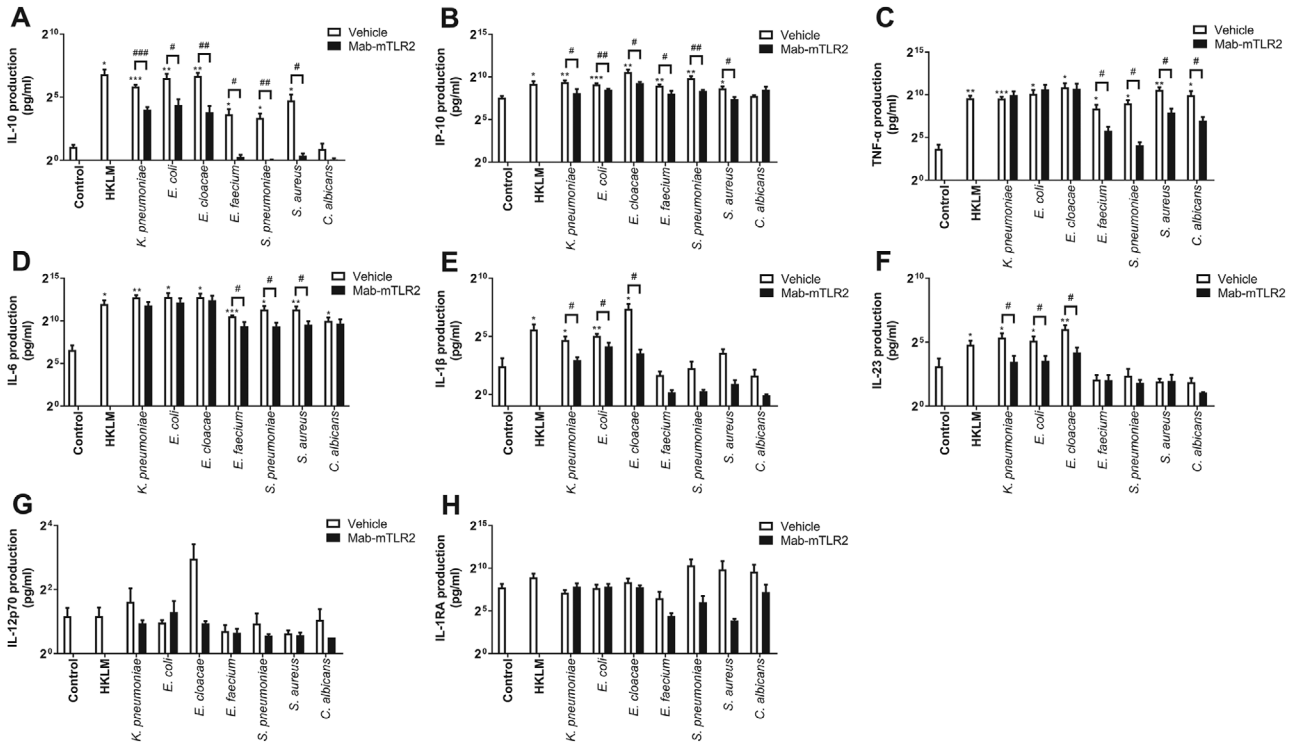

Figure 5. Effect of TLR-2 antagonist in the secretion of inflammatory cytokines. The inflammatory cytokines in the supernatants of human KCs were measured by cytometric bead array after 24 h stimulation with TLR-2 agonist (HKLM, 10×10^8 cells/mL) or microbial lysates (*K. pneumoniae*, *E. coli*, *E. cloacae*, *E. faecium*, *S. pneumoniae*, *S. aureus*, and *C. albicans*, 8 μg/mL for each lysates). (A–D) Both Gram-negative bacteria (*K. pneumoniae*, *E. coli*, and *E. cloacae*) and Gram-positive bacteria (*E. faecium*, *S. pneumoniae*, and *S. aureus*) significantly ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, comparing with the control group, two-sided Student's *t*-test) increased IL-10, IP-10, TNF-α, and IL-6 in human KCs after 24 h of stimulation. The TLR-2 antagonist attenuated all the cytokines increases caused by Gram-positive bacteria as well as the TNF-α and IL-6 secretion evoked by Gram-negative bacteria ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, comparing between vehicle and Mab-mTLR2 treatments, two-sided Student's *t*-test). *C. albicans* only induced TNF-α and IL-6 secretion ($^*p < 0.05$) and TNF-α' increase can be blocked by Mab-mTLR2 ($^{\#}p < 0.05$). (E and F) Gram-negative bacterial stimulation also increased IL-1β and IL-23 ($^*p < 0.05$, $^{**}p < 0.01$, comparing with the control group, two-sided Student's *t*-test), which can be reduced by TLR-2 antagonist ($^{\#}p < 0.05$ comparing between vehicle and Mab-mTLR2 treatments, two-sided Student's *t*-test). (G and H) None of the microbial lysates had significant effects on IL-1RA or IL-12p70. Data are expressed as mean ± SD, $n = 5$ samples, and are from three independent experiments with a total of three human tissues.

Comparing the effects of TLR-1, -2, -4, and P2 × 7R antagonists, only TLR-2 antagonist reduced *C. albicans*-induced TXB_2 increase (Fig. 3 and Supporting Information Fig. S4), this result was in accordance with previous investigations that TLR-2 but not TLR-4 was involved in the *Candida mannan* induced IL-17 production in macrophages [34]. Despite a rare condition, positive fungal cultures were found to be associated with significantly higher mortality in patients with SBP [2]. Lack of specific indicators and low culture success rates often lead to delayed diagnosis and treatment of concomitant fungal infection in SBP patients. The effects of TLR-2 antagonists in our experiments suggested TLR-2 as a potential marker and an effective treatment for fungal infections.

Resistant organisms are increasingly recognized as an emerging problem worldwide due to the widespread use of antibiotics in SBP [1,28]. Antibodies targeting TLRs may represent an attractive therapeutic option. OPN-305, the first humanized IgG4 monoclonal antibody against TLR-2 on monocytes, was tested in a randomized, double-blind, in-human phase I study [35]. In animal models, TAK242 pretreatment attenuated inflammation-mediated stress response and improved chronic pancreatitis and islet transplantation results [36]. Our results also support the combined use of TLR-2 and -4 antagonists in severely infected patients (Supporting Information Fig. S3).

There were some limitations in our experiments: the effect of cirrhosis on NPCs has not been well investigated due to the limited number of cells from severe cirrhosis samples; the results of in vitro experiments lack the validation of in vivo animal experiments. We have rat animal models for measuring portal vein pressure, but this technique does not apply to mice. Further improvements to this technique or new animal models may provide new evidence for our results.

Different microbial lysates induced KCs to secrete TXA_2 and inflammatory factors using the same route: activation of TLR-2 leading to factor secretion. Therefore, TLR-2 may be a potential marker and an attractive target for future treatment of patients with SBP and ACLF. In addition to the common pathway via TLR-2, GPB-products and GNB-products can be differentiated by the inflammatory cytokines IL-23 and IL-1β. This might serve as a novel basis to distinguish early between Gram-positive and Gram-negative infections.

Materials and methods

Animal and human tissue studies

Human liver tissue was provided by the Biobank of the Department of General, Visceral and Transplantation Surgery, Ludwig-Maximilians University (LMU), Munich, Germany under the administration of the Human Tissue and Cell Research (HTCR) Foundation. The framework of HTCR Foundation, which includes obtaining written informed consent from all donors, has been approved by the ethics committee of the Faculty of Medicine at the LMU (approval number 025-12) as well as the Bavarian State Medical Association (approval number 11142), Germany. A total of 22 normal liver tissue samples were involved in our experiments. The diagnosis of patient donors included liver metastasis from digestive tract tumor ($n = 16$), hepatocellular carcinoma ($n = 5$) and liver focal nodular hyperplasia ($n = 1$).

For animal experiments, only 6–8 weeks old male WT mice (C57BL/6 background, 16–22 g) were selected for the present study. All mice were adapted to the new environment for 1 week before the experiments. They were housed in a pathogen-free facility and handled in accordance with standard use protocols, animal welfare regulations, and the institutional guidelines. All animals and human tissues were ethically treated according to the criteria prepared by the National Academy of Sciences and published by the National Institutes of Health in addition to the legal requirements in Germany. All experiments were approved by the local government (Regierung von Oberbayern, Munich, Germany) and were reported to the responsible authorities annually.

Isolation of primary liver nonparenchymal cells

The isolation of NPCs was performed according to published protocols [4,37,38]. Briefly, liver tissues from mice and humans were cut into 5–10 mm thick slices and digested for 20 min at 37°C with pronase (Sigma Chemical Co, St. Louis, USA) and DNase (Roche, Mannheim, Germany). After filtration and centrifugation, the pellets were resuspended in GBSS and layered over fresh Nycodenz gradients (16.7% for KCs and 28.7% for HSCs, Axxis Shield, Rodelokka, Norway). The mixtures were centrifuged at room temperature without brake. The interfaces were collected after centrifugation and then centrifuged with brake. For isolation of SECs, Anti-SEC CD146 MicroBeads (Miltenyi Biotec, Germany) were added in the resuspended cells (10^7 cells/mL) for immunolabeling. After incubation, SECs were separated with magnetically activated cell sorting system MACS (Miltenyi Biotec, Germany). NPCs were collected and resuspended in RPMI 1640 supplemented with L-glutamine, FCS (PAA, Cölbe, Germany), and streptomycin/penicillin (Sigma, St. Louis, USA). The medium was changed after 6 h to remove non-adherent cells (such as erythrocyte). Cells were seeded in six-well plates and cultured at 37°C in 5% CO_2. Before stimulation, the medium was changed to RPMI 1640 without FCS or antibiotics for 24 h.

Cell culture line and treatment

THP-1 monocytic cells (American Type Culture Collection, ATCC reference number TIB-202™) were a kind gift from Prof. Peter Nelson. Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine and streptomycin/penicillin at 37°C in 5% CO_2. THP-1 monocytes were seeded in six-well plates and differentiated to macrophages by 24 h stimulation with 20 ng/mL PMA (Sigma, St. Louis, USA). After incubation, adherent cells were washed three times with PBS and incubated with fresh RPMI 1640 medium without PMA [39]. Before stimulation, the medium was changed to RPMI 1640 without FCS or antibiotics for 24 h.

In vitro stimulation plan

The following microbes were isolated from patients with SBP: *K. pneumoniae, E. coli, E. cloacae, E. faecium, S. pneumoniae, S. aureus*, and *C. albicans*. The isolates were cultured on Columbia 5% sheep blood media (Becton Dickinson, Heidelberg, Germany) at 37°C under aeration. After growth, the lysates were collected by centrifugation and resuspended in a small volume of PBS and subsequently heat-inactivated. All extracts were found to be sterile and were measured for protein content using the Bradford technique. Extracts were diluted to achieve standardized protein concentrations. After 24 h of stimulation, the supernatants of cells were collected and stored at -80°C before use. In some experiments, the special agonists or antagonists were used to treat cells (Supporting Information Table S1).

Western blot analysis

Cells were lysed in lysis buffer (RIPA-buffer supplemented with protease inhibitors) on ice, the proteins were collected by centrifugation. After measuring the protein concentration by BCA protein assay kit (Thermo Scientific). The cells were boiled with a suitable volume of loading buffer (Sigma). The samples and marker were loaded onto the appropriate concentration of SDS-PAGE gels and transferred onto polyvinylidene difluoride membrane with the Mini-PROTEAN® System (Bio-Rad, USA). The membranes were then incubated overnight at 4°C with primary antibody after blocking: anti-Myd88, MAPK, pMAPK, NF-kB, or p-NFkB antibodies (1:1000, Cell Signaling Technology); GAPDH antibody (1:1000, Sigma). Membranes were washed and then incubated with the secondary antibody (Sigma) for 1 h at room temperature. The activity was visualized by chemiluminescence: the working solution (SuperSignal™ West Femto, Thermo Fisher) was added on membranes and then the blot was exposed by the imaging system (CHEMOCAM Imager 3.2, INTAS, Germany).

ELISA measurement

TXA_2 is not a cyclic hormone and works within a short distance of its biosynthesis, it is rapidly hydrolyzed to TXB_2 after synthesis and

released into the circulation. As a result, TXB_2, the stable degradation product of TXA_2, was measured from the supernatants of the stimulated cells by TXB_2 ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). Supernatant samples were stored at -80°C until measurement. The procedures were well described in our publications before [4,16].

Cytometric bead array

Inflammatory-related factors, including TNF-α, IL-1β, IL-1RA, IL-6, IL-10, IL-12p70, IL-23, and C-X-C motif chemokine 10 (CXCL-10), were evaluated in the supernatant of human KCs using Cytometric bead array (CBA) method with LEGENDplex™ Human M1/M2 Macrophage Panel (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions. Methods were adhered to the guidelines of published articles [40]. A BD™ FACS Canto II flow cytometer was used to collect the data. The data were analyzed with the LEGENDplex software.

Confocal microscopy

Primary antibodies against CD163 (1:100; Acris, Sunnyvale, CA, USA), CD68 (1:100; Dako, Santa Clara, CA, USA), and CD11b (1:100; Santa Cruz, Dallas, Texas, USA) were used for immunofluorescence. The cells were fixed with 4% paraformaldehyde (Roth, Karlsruhe, Germany) in PBS for 15 min, then, washed with PBS three times and incubated with blocking buffer for 30 min at 37°C. Cells were generally stained with primary antibody overnight at 4°C. After additional washing steps with PBS, fluorescent-labeled secondary antibodies (Life Technologies GmbH, Darmstadt, Germany) were added. The cells were analyzed by confocal microscopy (Zeiss LSM 510 META, Jena, Germany).

Statistical analysis

All data were presented as the mean ± SD. All samples were randomly distributed to different groups. The two-sided Students *t*-test was used for paired or unpaired observations. One-way ANOVA was used when needed. A value of $p < 0.05$ was considered to be statistically significant; *n* denotes the number of samples used. SPSS and Graphpad were used for data analysis and figure drawing.

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Abbreviation: **ACLF**: acute-on-chronic liver failure · **GNB**: Gram-negative bacteria · **GPB**: Gram-positive bacteria · **HKLM**: heat-killed Listeria monocytogenes · **HSC**: hepatic stellate cells · **KC**: Kupffer cell · **MAPK**: mitogen-associated protein kinases · **Myd88**: myeloid differentiation factor 88 · **NPC**: nonparenchymal cell · **SBP**: spontaneous bacterial peritonitis · **SEC**: sinusoidal endothelial cells · **TX**: thromboxane

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