Kupffer cell activation by different microbial isolates: toll-like receptor-2 plays pivotal role on thromboxane A_2 production

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Jiang Zhang

Für meine liebe Familie und Freunde

Contents

Zusammenfassung

Patienten mit komplizierte Leberzirrhose leiden häufig an spontaner bakterieller Peritonitis (SBP). Die Aktivierung der Kupffer-Zelle (KC) durch Toll-like-Rezeptoren (TLRs) und die endotheliale sinusoidale Dysfunktion sind wichtige Mechanismen für die portale Hypertonie und das Fortschreiten der SBP. Thromboxan A_2 (TXA_2, das stabile Abbauprodukt ist TXB_2) wurde bei der KC-Aktivierung als wichtiger Vasokonstriktor identifiziert.

Die Pathophysiologie der KC-Aktivierung und die Funktion von TXA_2 zur Erhöhung des Portalperfusionsdrucks nach Anwendung der TLR-Agonisten sind schon gut untersucht worden. Unklar ist jedoch welche TLRs auf KCs für die TXA_2-Sekretion verantwortlich sind. In diesem Experiment wurden aus humanen (HKCs), THP-1-Makrophagen (TMCs) und primären nichtparenchymalen Mauszellen [KCs, sinusoidale Endothelzellen (SECs) und hepatische Sternenzellen (HSCs)] isolierte primäre KCs untersucht. Zellen wurden durch mikrobielle Extrakte stimuliert, die aus kultivierten Isolaten von SBP-Patienten stammten. Mit speziellen TLR-Agonisten und -Antagonisten wollten wir die Funktion von TLRs in nicht-parenchymalen Leberzellen untersuchen.

Unsere Daten zeigten, dass TLR-Agonisten nur TXB_2 in Maus-KCs erhöhten, nicht jedoch in SECs oder HSCs. TLR-1, -2 und -4 spielten eine wichtige Rolle bei der TXB_2-Sekretion in KCs nach bakterieller Stimulation. TLR-2 erkannte alle Arten von

mikrobiellen Isolaten. Vorbehandlung mit TLR-1, -2 oder -4-Antagonist könnte den TXB_2-Anstieg durch gramnegative bakterielle Isolate (*Klebsiella pneumoniae, Escherichia coli und Enterobacter cloacae*) reduzieren. TLR-1 und -2-Antagonisten wirkten sich auch auf grampositive Bakterienisolate aus (*Enterococcus faecium, Streptococcus pneumoniae und Staphylococcus aureus*). Die Kombination von TLR-1, -2 und -4-Antagonisten erzielte eine bessere Wirkung als jedes einzelne Medikament.

Nach unseren Erfahrungen sind dies die ersten Versuche zur Behandlung humaner primärer KCs mit verschiedenen klinisch relevanten humanen mikrobiellen Isolaten. TLR-1, -2 und -4 spielen eine wichtige Rolle bei der bakterieninduzierten TXB_2-Sekretion in HKC. TLR-2 spielt wahrscheinlich eine zentrale Rolle, da es sich auf alle bakteriellen Produkte auswirkt. Daher könnte TLR-2 in der Zukunft ein potenzieller Marker und ein attraktives Ziel für die Behandlung von Patienten mit SBP sein.

Abstract

Background：

Spontaneous bacterial peritonitis (SBP) is a bacterial infection that often occurs in patients with cirrhosis and can be fatal. Sinusoidal endothelial cells (SECs), Kupffer cells (KCs), and hepatic stellate cells (HSC) are major components of liver non-parenchymal cells. They play an important role in combating pathogenic bacteria, usually the earliest cells that come into contact with antigens. Thromboxane A_2 (TXA_2) was proved to be the critical vasoconstrictor secreted by activated KCs.

Objection：

We aimed to investigate the role of toll-like receptors (TLRs) in bacterial-induced TXA_2 increase in liver nonparenchymal cells and to identify the most potent antagonists as potential therapeutic options in the future.

Method：

Primary nonparenchymal liver cells (including KCs, SECs, and HSCs) were isolated from mice, THP-1 macrophages (TMCs) differentiated by phorbol myristate acetate treatment and primary human KCs (HKCs) were investigated. Cells were characterized by immunostaining with special antibodies. Microbial isolates derived from patients with SBP and the special TLR-1 to -9 agonists were used to treat the related cells. TXB_2, the stable degradation of TXA_2, was measured in supernatants before and after stimulation. The special TLR-1/-2/-4 antagonists were additionally

added in some experiments 1 h before stimulation.

Result：

TLR agonists only increased TXB_2 in mouse KCs (MKCs) but not in SECs or HSCs. TLR-1, -2 and -4 rather than other agonists caused TXB_2 increase in HKCs and MKCs, TLR-1 and -2 agonists also worked on TMCs. Pretreatment with TLR-1, -2 or -4 antagonist in HKCs significantly reduced the TXB_2 increase caused by Gram-negative bacteria. TLR-1 and -2 antagonist also had effects on Gram-positive bacteria. Only Mab-mTLR2 worked on *Candida albicans*-induced TXB_2 increase. Combination of TLR-1, -2 and -4 antagonists achieved a better effect.

Conclusion：

This is the first study to investigate the effect of human microbial isolates on the vasoconstrictor TXA_2 after stimulation and activation of human KCs. TLR-1, -2 and -4 play an important role in bacterial-induced TXA_2 secretion, the TLR-2 antagonist showed the most effective reduction in bacterial-induced TXA_2 secretion. Therefore, TLR-2 might be a potential marker and an attractive target for the treatment of infections and liver cirrhosis.

Key words: toll-like receptor, spontaneous bacterial peritonitis, thromboxane A_2, Kupffer cell, nonparenchymal liver cells.

Abbreviations

Abbreviations	Full name
ACLF	acute-on-chronic liver failure
BSA	Bovine serum albumin
C. albicans	*Candida albicans*
Cys-LT	cysteinyl leukotrienes
DC	dendritic cells
E. coli	*Escherichia coli*
E. cloacae	*Enterobacter cloacae*
E. faecium	*Enterococcus faecium*
ELISA	The enzyme-linked immunosorbent assay
FCS	fetal calf serum
FcyR	Fc receptor
FSL-1	Pam2CGDPKHPKSF
GBSS	Gey's Balanced Salt Solution
Gram (+)	Gram-positive
Gram (-)	Gram-negative
HKC	human primary Kupffer cell
HKLM	heat-killed preparation of Listeria
HSC	hepatic stellate cells
IR	ischemia/reperfusion
KC	Kupffer cell
K. pneumoniae	*Klebsiella pneumoniae*
LPS-EK	Lipopolysaccharide E. coli K12
LTC4	leukotrienes C4
Mab-mTLR2	Monoclonal antibody to mouse TLR2

MKC	mouse primary Kupffer cell
NPC	mouse primary Kupffer cell
ODN	CpG oligonucleotides
Pam3CSK4	Pam3CysSerLys4
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PMA	phorbol myristate acetate
PFA	Paraformaldehyde
Poly(I:C)	Polyinosinic-polycytidylic acid
Poly(I:C)HMW	Polyinosinic-polycytidylic acid (high molecular weight)
Poly(I:C)LMW	Polyinosinic-polycytidylic acid (low molecular weight)
ssRNA	single-stranded ribonucleic acid oligonucleotide
SBP	spontaneous bacterial peritonitis
SD	standard deviation
SEC	sinusoidal endothelial cell
SFP	spontaneous fungal peritonitis
S. pneumoniae	*Streptococcus pneumoniae*
S. aureus	*Staphylococcus aureus*
ST-FLA	S. typhimurium Flagellin
TMC	THP-1 macrophages
TLR	toll-like receptor
TX	Thromboxane

1. Introduction

1.1 Role of infections in acute-on-chronic liver failure

Cirrhosis, the end stage of many kinds of chronic liver disease, is characterized by the presence of certain anatomic abnormalities and fibrosis of hepatic parenchymal architecture [1, 2]. The clinical manifestations are similar and dominated by consequences of diminished hepatic reserve and portal hypertension, regardless of origin (alcoholic liver disease, autoimmune hepatitis, hepatitis virus especially hepatitis B and metabolic disorders) [3-6]. Esophageal varices, ascites and splenomegaly with malfunction of platelets are common complications of liver cirrhosis [5, 6].

Patients with liver disease especially with cirrhosis often suffer from spontaneous bacterial peritonitis (SBP), a common and frequently fatal bacterial infection [7-10]. This is one of the most common and important complications of decompensated cirrhosis and is closely related to the patient's prognosis. Once SBP develops, the patient's mortality rate is greatly increased [7, 10, 11]. Therefore, the patient should be evaluated for liver transplantation once recovered from the first episode of SBP. The awareness and identification of early diagnosis and effective antibiotic strategy has reduced the mortality associated with first episode of SBP [7, 12].

However, the selection of resistant organisms leads to changes in the antibacterial spectrum and the occurrence of certain diseases (such as candida esophagitis) are

non-negligible side effects in antibiotic treatment. The increasing prevalence of antibiotic-resistant bacteria, causing several hundred thousand deaths yearly, is recognized as a major global threat and one of the greatest therapeutic challenges [7, 12, 13]. Misuse and overuse of antibiotics are considered to be the major driver for the progress of antibiotic resistance. As a result, alternative therapies of antibiotics are now being seeked [7, 9, 10, 12].

Gram (-) aerobic organisms such as *Escherichia coli* and *Klebsiella pneumoniae* are the most common cause of SBP patients, other common organisms include *Enterobacter cloacae* and *Staphylococcus aureus [14, 15]*. *Candida albicans* is reported as the most common pathogen of spontaneous fungal peritonitis [16, 17]. SBP is also the most common infection associated with acute-on-chronic liver failure (ACLF), which is characterized by a high morbidity and mortality due to systemic inflammatory response syndrome and multiple-organ dysfunction [18, 19]. ACLF is defined as the association of acute liver injury with established chronic liver disease leading to severe injury and can quickly lead to liver decompensation [20-22]. Alcohol and hepatitis viruses are the most important causes of disease in Europe and Asia respectively [22, 23]. Bacterial translocation and sepsis play important roles in the process of cirrhosis and can cause sudden decompensation [23-25]. (Fig.1-1)

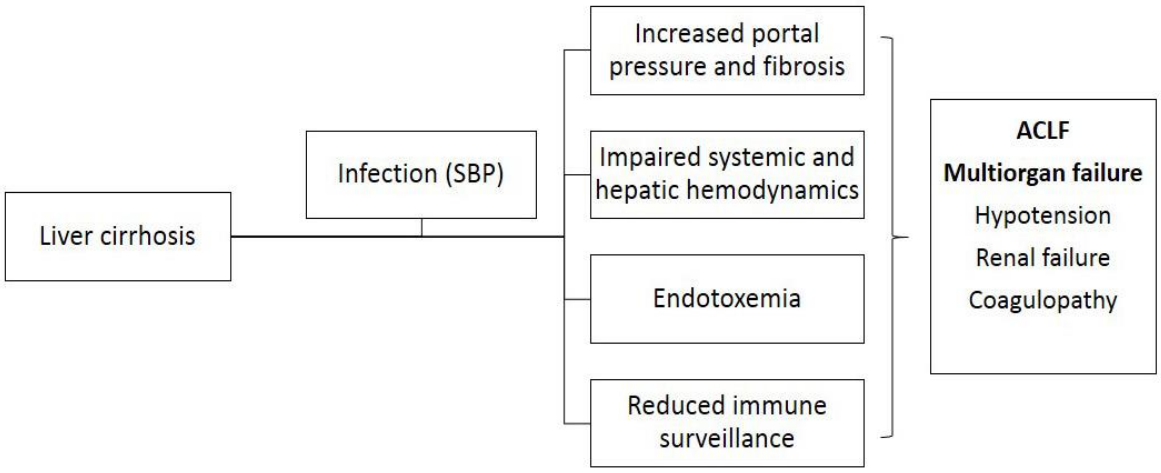

Fig.1-1 Role of infections in acute-on-chronic liver failure

1.2 Role of nonparenchymal liver cells and toll-like receptors

Nonparenchymal liver cells, mainly include hepatic stellate cells (HSCs), sinusoidal endothelial cells (SECs) and Kupffer cells (KCs), are the Important functional cells to meet bacteria (Fig.1-2) [26]. KCs are hepatic-resident macrophages that play a central role in the liver's inflammatory immune response. They are mainly involved in the recognition, presentation and phagocytosis of antigens.They can coordinate and regulate other nonparenchymal cells (NPCs) by secreting various cytokines. Shunting of blood away from KCs also allow a transient bacteremia to become more prolonged [14, 27]. KCs also express TLR-2, TLR-3, and TLR-9 and respond to their ligands [28-30]. KCs participate in immune activation and immunosuppression, both proinflammatory and anti-inflammatory cytokines such as interleukin (IL)-1b, IL-10 and tumor necrosis factor a (TNFa) can be secreted by KCs [29, 31-33].

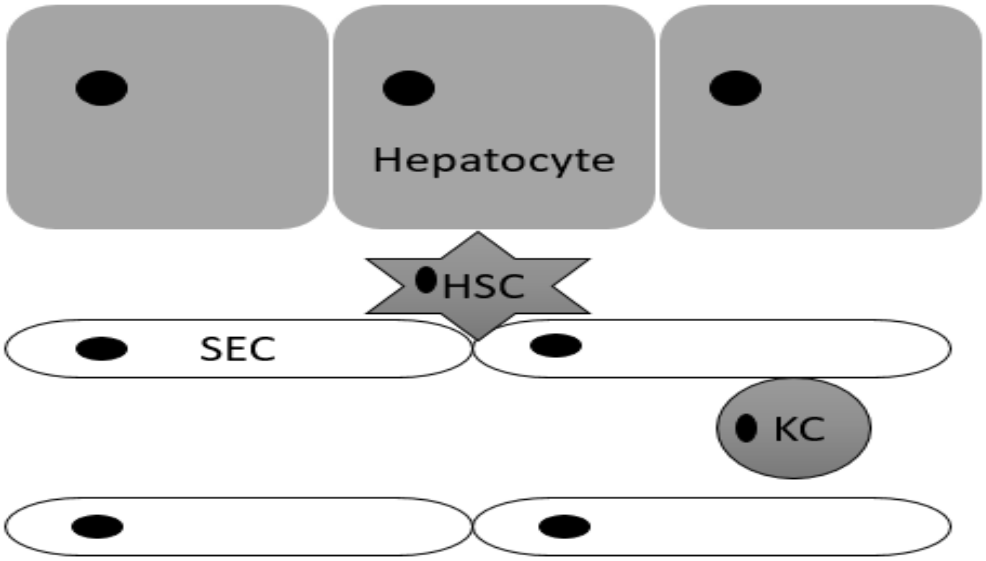

Fig.1-2 Architecture of the Liver

Toll-like receptors (TLRs) play vital roles in innate immune defenses against diverse set of pathogen-associated molecular patterns (PAMPs) by KCs. Examples of PAMPs include Gram (-) and Gram (+) bacterial components, fungi, and protozoa [34, 35]. The liver is continuously stimulated by antigens from the gastrointestinal tract and transmitted through the portal vein. With the arrival of foreign antigens in the liver, TLRs activate and maintain immune activation resulting in immediate release of cytokines and antimicrobial peptides by immune cells. In addition, TLR signaling also plays a key role in regulating immune tolerance [36-38]. Special agonists of TLR-1 to -9 have been widely used in different kinds of cell lines and primary macrophages [39]. TLR-2 can form complexes with other TLRs (TLR-6 or TLR-1…). TLR-3, -7, -8 and -9 are intracellular TLR sensors and mainly respond to nucleic acids such as double and single-stranded RNA from virus [40]. (Fig.1-3)

TLR-2 signaling activates the inflammasome primarily in KCs, but not in HSCs or hepatocytes [41, 42]. Expression of TLR-1, -2 and -4 also found as potential indicators for a variety of diseases. Expression of soluble TLR-2 were higher in patients with psoriasis [43]. TLR-2 and -4 expression in monocytes was found to be significantly overexpressed in patients with tuberculosis [44], systemic lupus erythematosus [45] and myelodysplastic syndromes [46]. TLR-2 and -4 were reported to be independently associated to poor outcome of patients with ischemia stroke and intracerebral hemorrhage [47, 48], higher methylation of TLR-2 may also confer susceptibility to adverse cardiac autonomic effects [49].

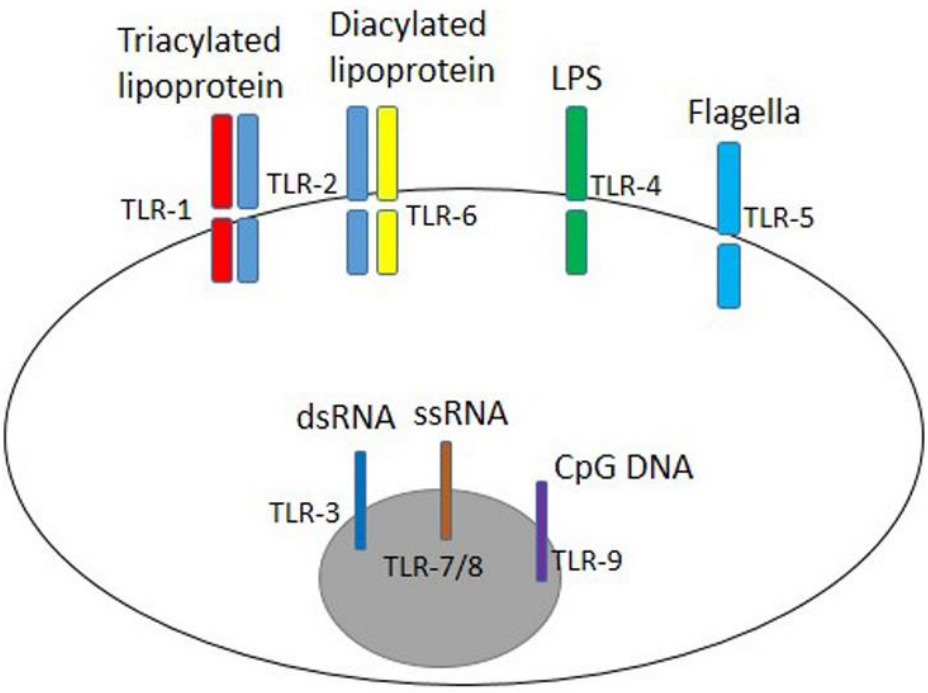

Fig.1-3 The role of TLRs

1.3 Development and reversibility of liver cirrhosis

Liver cirrhosis, the fourth cause of death due to non-communicable diseases, is characterized by patterns of evolution and complex pathophysiological changes. Traditionally, cirrhosis is a progressive and irreversible disease, and wound healing is an important starting event [50]. A marked reduction in mortality gained over the last decades because of the improved management and new knowledge on the mechanism, pathophysiology and therapy of cirrhosis [51, 52]. More and more researchers now approve that the description of liver cirrhosis as a static disease has now become inappropriate. At present, the process of cirrhosis is not only considered to be the result of physical effects of portal hypertension (such as scar and liver formation of cirrhosis), but also related to the production of a dynamic compound, namely the production of intrahepatic vasoconstrictors [50, 51, 53].

In this new way of understanding, pathological changes of vessels of the liver aggregating portal hypertension play vital roles on cirrhosis, resulting in a series of complications. Derangement of vascular networks, vascular thrombosis, formation of intrahepatic shunts, capillarization of sinusoids and perisinusoidal fibrosis are essential and important bases of the vascular pathological changes in cirrhosis [50, 51, 53]. One of the key target events for reversing fibrosis is the behavior of rapid closure that often occurs during chronic wound healing [50, 53]. Progressive reduction of necrotic inflammation is an important feature of the wound healing

process. Macrophages have been widely demonstrated to play an extremely important role in the study of fibrosis, not only in the progression of fibrosis but also in the regression of fibrosis [50, 54]. Soluble factors and cytokines such as IL-6. TNF-α, IL-8 and MCP-1 are important regulatory pathways that regulate macrophage's function, activity, and connection with other fibrosis-associated cells (such as HSC, SEC) [55]. Molecules associated with the development of fibrosis are widely expressed in these cells and interact with portal vein fibroblasts, which are usually mediated by pro-inflammatory and chemotactic cytokines [56]. Changes in inflammatory factors caused by bacterial translocation affect the progression of cirrhosis [57-59]. (Fig. 1-4)

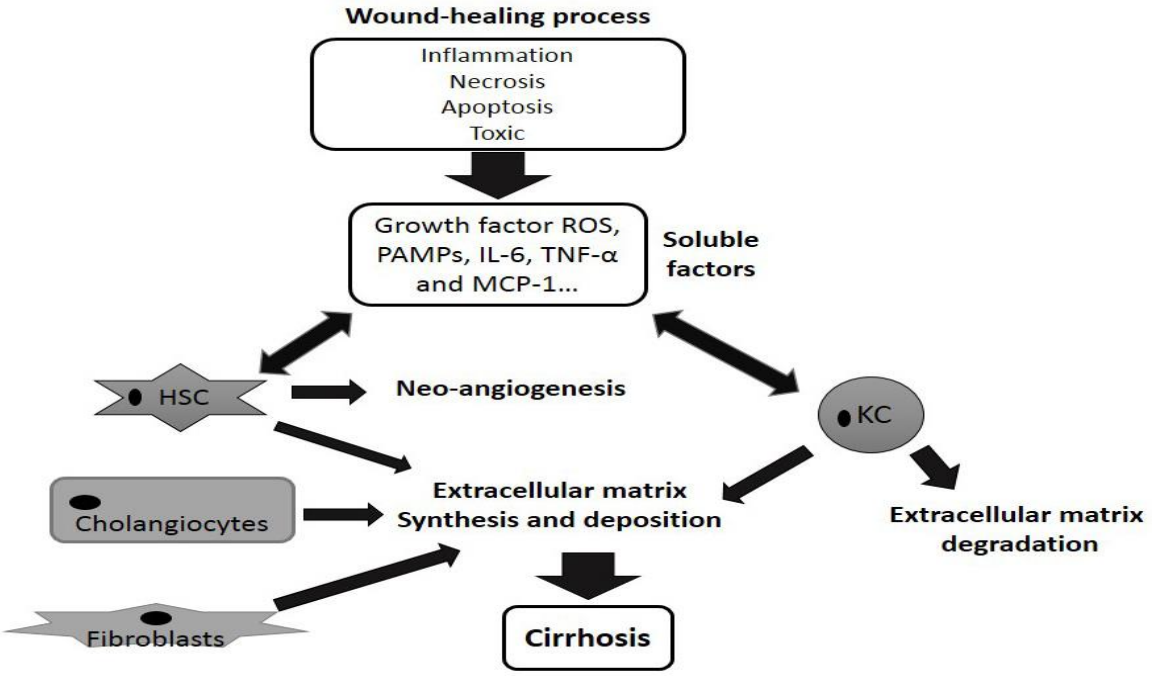

Fig.1-4 Mechanisms of liver cirrhosis

1.4 The pathophysiology of KC activation and function of TXA_2

KC activation by TLR agonists and endothelial sinusoidal dysfunction were important mechanisms for portal hypertension and progression of ACLF [14]. Thromboxane (TX) A_2 has been identified as an important vasoconstrictor in this process and plays important roles on vascular constriction, platelet activation and aggregation [60, 61]. TXA_2 exerts its specific biological activity by binding to thromboxane prostanoid (TP) receptor and activating the associated downstream proteins. During acute inflammation, hepatic microcirculatory dysfunction mediated by TNF-α can be regulated by TXA_2/TP receptor signaling pathway through enhancement of proinflammatory mediators [61-63].

TXA_2 and can also be induced by non-alcoholic fatty liver disease and activated by the interaction with blood components. In patients, increased TXA_2 synthesis was linked to infections, renal diseases, acute myocardial ischemia and heart failure [64, 65]. Aspirin has been proved to abolish TXA_2 generation and block platelet activation by responses [66, 67]. Significant changes in microcirculation effects occur when TXA_2 synthesis is inhibited [68]. Previous publications also reported TXA_2 inhibitors attenuated liver injury induced by ischemia/reperfusion, alcoholic liver injury, inflammation, fibrotic changes and liver injury induced by acetaminophen overdose elements [62, 69].

The pathophysiology of KC activation and function of TXA_2 to increase portal

perfusion pressure following TLR agonists have been well investigated in previous publications [14, 27, 32, 70]. In isolated rat liver perfusion models, TXA_2 and cysteinyl leukotrienes (Cys-LT) are proved to be important players in the portal pressure increase. We have demonstrated the important role for TXA_2 during the portal perfusion pressure increase after TLR activation: activated KCs release TXA_2 and then stimulate HSCs to produce subsequent effects, ultimately leading to contractile cell contraction and increased portal perfusion pressure. Intrahepatic vascular hyperreactivity and intrahepatic resistance are also important elements aggravating portal hypertension. (Fig. 1-5)

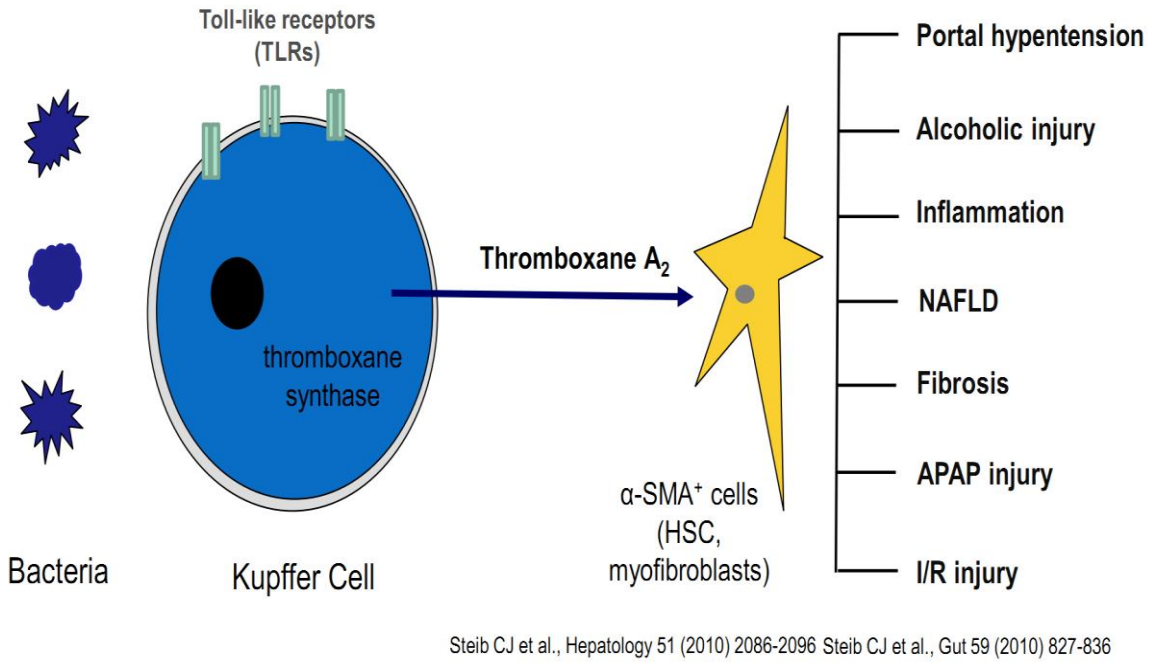

Fig.1-5 The pathophysiology of KC activation and function of TXA_2

1.5 Aims of the study

However, which TLRs on KCs are responsible for the TXA_2 secretion by bacterial infections are still unclear. In this experiment, primary nonparenchymal liver cells (including KCs, SECs, and HSCs) were isolated from mice, THP-1 macrophages (TMCs) differentiated by phorbol myristate acetate (PMA) treatment and primary human KCs (HKCs) were investigated. Cells were stimulated by microbial extracts derived from cultured isolates of SBP patients. Together with special TLR agonists and antagonists, we aimed to investigate the role of toll-like receptors (TLRs) in bacterial-induced TXA_2 increase in liver nonparenchymal cells and to identify the most potent antagonists as potential therapeutic options in the future.

2. Materials and methods

2.1 Animal and human tissue studies

All mice were adapted to the new environment for 1 week before experiment and were ethically treated according to rules and regulations. The Biobank of the Department of General, Visceral and Transplantation Surgery, Ludwig-Maximilians University provided human liver tissue under the administration of the Human Tissue and Cell Research (HTCR) Foundation.

2.2 Isolation of primary nonparenchymal liver cells

2.2.1 Separation of KCs and HSCs

2.2.1.1 Preparations

Material sterilization: 4 * empty beakers; 2 * beaker covered with 80 μm and 60 μm nylon mesh (Sigma, St. Louis, USA); 2 * Spoon; 2 * stirred fish; 2* aluminum foil; pipette tip kits: 1 * box for 1000 μl; 1 * box for 100 μl; 1 * box for 10 μl and several EP tubes.

Fresh Nycodenz solution: Nycodenz solution (0.75% NaCl with Tris –HCl, KCl and 0.3 mM CaNa2 EDTA) with stirring is heated to 50 °C, then slowly add Nycodenz powder (Axxis Shield, Rodelokka, Norway). After the Nycodenz powder is completely dissolved, the solution is naturally cooled to room temperature (RT). 0.75% NaCl solution and 60 ml Gey's Balanced Salt Solution (GBSS, Sigma, St.

Louis, USA) are finally added to achieve the required concentraction.

Pronase solution: 300 mg pronase (Sigma, St. Louis, USA) is dissolved into 20 ml GBSS and Closed with aluminum foil (light-shielded) and rolls until they are ready for use.

2.2.1.2 Obtain liver tissues from mice

Remove the animal from the cage and fix in the left hand. Intraperitoneal administration of 150 mg/kg Ketamine (Ketamidor, Chanelle, Galway, Ireland) + 10 mg/kg Xylazine (Xylavet, CP-Pharma Handelsges, Burgdorf, Germany) for anesthesia and return to the cage. After falling asleep, verify the depth of anesthesia based on its reflex. If necessary, reapplicate of Ketamine and Xylazine. The abdominal wall was then opened, exposure to the liver, portal vein and inferior vena cava. Flanking of the portal vein and perfusion with heparin-diluted PAA (RPMI 1640 from PAA, heparin 1,000 IU to 50 ml). The inferior vena cava is immediately cut off, causing the animal to bleed and die, and then remove the liver.

2.2.1.3 Liver slicing and digestion

Add the liver tissues from human or mouse without medium to a petri dish. Using 2 sterile scalpels, first superficially, then deeply cut. After the tissue is completely cut into 5–10 mm thick slices, the mass is added to a sterile beaker with a spoon and washed with sterile phosphate-buffered saline (PBS, Sigma, USA). Change PBS with 130 ml GBSS, DNase (Roche, Mannheim, Germany), the sterile pronase

(filtered with Minisart filter 0.2 µm pore size, Sigma, USA) and a sterile stirred fish in the beaker. Cover the beaker with sterile aluminum foil and pack it with an unsterile aluminum foil in a light-proof manner. Then the liver mass is incubated in the incubator (Incubator IF30, Memmert, Germany) at 37 °C for 20 min.

2.2.1.4 Filtration and centrifugation with Nycodenz solution

After 20 min of digestion, the solution is filtered through an 85 µm filter, which is stretched over a beaker. The rest tissues upon the filter are added into a Stomacher® 400 circular bags (Sigma, St. Louis, USA) with 100 ml GBSS + DNase. Close the Stormacher bag without bubbles and grind (Stomacher® 400 circulator, Seward, England). The solution from stomacher bag is then filtered through the 85 µm and 65 µm filter sequentially.

Distribute the solution under the 65 µm filter into approximately 6 * 50 ml falcon tubes. After distribution, centrifuge at 300 g for 10 min at 4 °C, with brake. Aspirate the supernatant, the pellet is resuspended with 30 ml GBSS in each tube. The Nycodenz solution is filtered in 6 * 50 ml Falcon tubes, 20 ml solution in each tube. The liver solutions are slowly added and layered on the sterile Nycodenz solutions (17.6% for KCs and 28.7% for HSCs). The mixtures are centrifuged at 600 g for 20 min at RT without brake.

Discard 20 ml of the upper layer, collect the interfaces in 2-3 orange tubes. The collections are centrifuged at 2000 g for 5 min with brake. The pellet is resuspended

in RPMI 1640 supplemented with penicillin (100 units/mL, Sigma, St. Louis, USA), and streptomycin (Sigma, St. Louis, USA) together with L-glutamine, fetal calf serum (FCS, PAA, Cölbe, Germany).

2.2.1.5 Cell counting and plating

After cell counting, KCs or HSCs are seeded in plates and cultured at 37 °C in 5% CO_2 (Incubator HeRA CELL 240, Heraeus, Germany). Before stimulation, the medium is changed by RPMI 1640 without FCS for 24 h.

2.2.2 Separation of SECs

2.2.2.1 Preparation and liver tissue digestion are the same as KCs and HSCs.

2.2.2.2 Magenetic labeling and separation

Wash the pellet after centrifuge and then centrifuge again for 5 min at 300 g. Count and resuspend 1 ml of medium with 1×10^7 cells. Add 10µl Anti-SEC MicroBeads CD146 (Miltenyi Biotec, Germany) for immunolabeling. After incubation, seperate SECs with magnetically activated cell sorting system MACS (Miltenyi Biotec, Germany). Recount and then seed the SEC at the appropriate density and condition.

2.3 Culture and differentiation of THP-1 cell line

THP-1 monocytic cell (American Type Culture Collection, ATCC reference number TIB-202^{TM}) was a kind gift from prof. Peter Nelson. Cells were cultured in complete RPMI 1640 medium including 10% FCS. THP-1 monocytes were differentiated to macrophages by 24 h stimulation with medium containing 20 ng/ml phorbol

myristate acetate (PMA, Sigma, St. Louis, USA). Adherent cells were incubated with fresh RPMI 1640 medium without PMA after washing with PBS [72, 73]. Before stimulation, the medium was changed by RPMI 1640 without FCS for 24 h.

2.4 In vitro stimulation plan

The following microbes were isolated from patients with SBP: *Klebsiella pneumoniae (K. pneumoniae), Enterococcus faecium (E. faecium), Escherichia coli (E. coli), Staphylococcus aureus (S. aureus), Streptococcus pneumoniae (S. pneumoniae), Enterobacter cloacae (E. cloacae) and Candida albicans (C. albicans)* were cultured on special media (Becton Dickinson, Heidelberg, Germany) at 37°C under aeration. After growth, the isolates were gently taken off the media and resuspended in PBS. The isolates were washed three times to remove any residual media attaching to the isolates. Subsequently, the microbial pellet was collected by centrifugation (5000rpm, 4°C, 10min) and resuspended in a small volume of PBS and subsequently heat inactivated (98°C, 20min). The heat inactivated solution was vortex mixed and checked for sterility by plating it twice on Columbia 5% sheep blood media and inoculation in liquid broth. All extracts were found to be sterile and were measured for protein content using the Bradford technique. Extracts were diluted to achieve standardized protein concentration.

Preparation of human and mouse TLR agonist stock solutions (Invivogen, USA) was according to the instructions of the drugs (Table. 2-1). Administered

concentrations of TLR agonists were as following: TLR-1/2, Pam3CSK4, 0.1μg/ml; TLR-2, HKLM, 10^8cells/ml; TLR-3, Poly(I:C)HMW, 10ng/ml; TLR-3, Poly(I:C)LMW, 10ng/ml; TLR-4, LPS-EK, 10ng/ml; TLR-5, FLAST, 10ng/ml; TLR-6/2, FSL-1, 1μg/ml; Imiquimod, 1μg/ml (TLR-7 for human); ssRNA40, 0,25μg/ml (TLR-8 for human and TLR-7 for mouse, mouse lack TLR-8); ODN2006, 5μM (TLR-9 for human) and ODN1826, 5μM (TLR-9 for mouse).

In some experiments, the special TLR-1/-2/-4 antagonists (CU-CPT22, Merck, Germany; Mab-mTLR2, Invivogen, USA; TAK242, Merck, Germany) were additionally added 1 h before stimulation. Supernatants were stored at -80 °C before use.

Product	Application	Stock solution concentration	Volume of solvant
Pam3CSK4	Human, Mouse	100 ng/ml	100 μl H_2O
HKLM	Human, Mouse	10^{10} cells/ml	100 μl H_2O
Poly(I:C)HMW	Human, Mouse	1 mg/ml	500 μl H_2O
Poly(I:C)LMW	Human, Mouse	1 mg/ml	500 μl H_2O
LPS-EK	Human, Mouse	100 μg/ml	1 ml H_2O
FLAST	Human, Mouse	100 μg/ml	100 μl H_2O
FSL-1	Human, Mouse	100 μg/ml	100 μl H_2O
Imiquimod	Human	100 μg/ml	250 μl H_2O
ssRNA40	Human, Mouse	100 μg/ml	250 μl H_2O
ODN2006	Human	500 μM	26 μl H_2O
ODN1826	Mouse	500 μM	31 μl H_2O

Table. 2-1 Preparation of TLR agonist stock solutions

2.5 ELISA measurement

TXA_2 secretion was quantified by the release into supernatants of cells. The procedures were based on the instructions and protocols in our publications before [27, 32]. TXB_2 enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, USA) was used to measure the stable degradation product of TXA_2.

2.5.1 Sample and TXB_2 standard preparation

If necessary, dilute the sample to the appropriate concentration with ELISA buffer. Prepare the standard solution: Mix 100 µl of TXB_2 ELISA standard and 900 µl UltraPure water (Cayman Chemical, USA) into a tube. Use 8 clean test tubes, marking number from S1 to S8. S1 is filled with 900 µl and the rest tubes are filled with 600 µl ELISA buffer each. Transfer 100 µl standard solution to S1 and mix thoroughly. Then mix 400 µl from S1 with the solution in S2. Repeat this processs until S8. (Fig. 2-1)

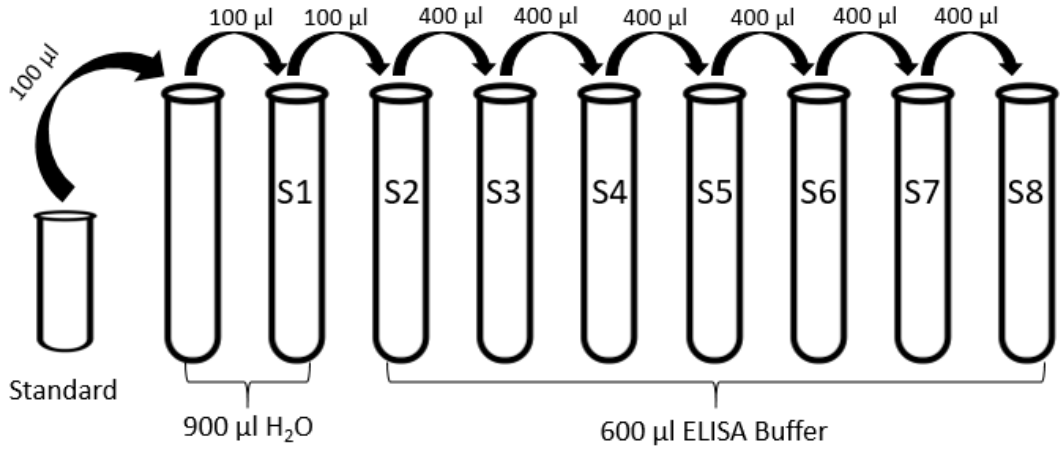

Fig. 2-1 Preparation of TXB_2 standard

2.5.2 Performing the assay

A clean 96-well plate was used to design and seperate samples into different groups (Fig. 2-2). Add the regents of ELISIA buffer, standard solutions, samples, TXB_2 AChE Tracer and TXB_2 ELISIA Antiserum into different kinds groups in the plate (Table. 2-2). After incubation overnight, empty the plate. Each well were filled with 200 µl fresh Ellman's Reagent after 5 times washing. Cover the plate with aluminum foil and develop. Develop the plate in 90-120 min with an orbital shaker (Orbital shaker SSL, Stuart, United Kingdom). Read plate at 405 nm wavelength light (Glomax Detection System, Promga, USA).

Blk: Blank
NSB: Non Specific Binding
B_0: Maximum Binding
S1-S8: Standards

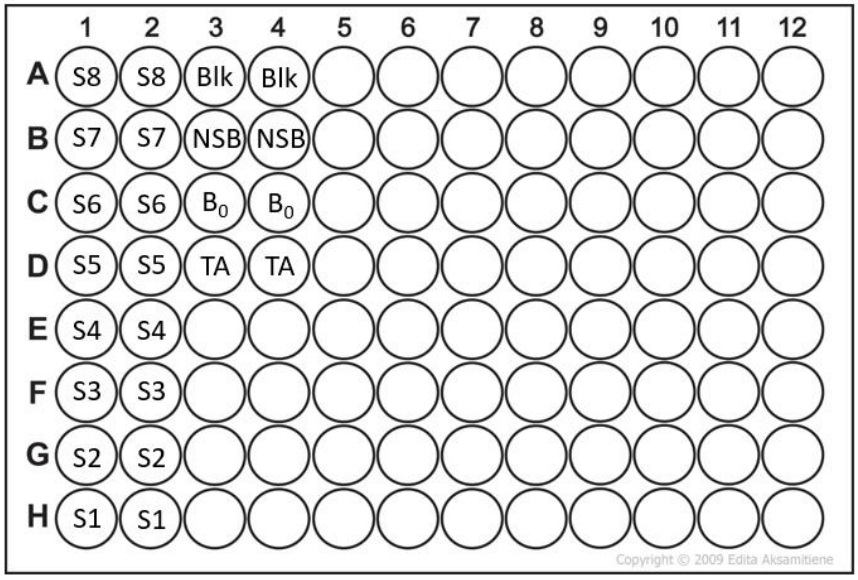

Fig. 2-2 Design of sample plate

Well	ELISA Buffer	Standard/Sample	Tracer	Antiserum
Blk	-	-	-	-
TA	-	-	5 µl (deve.step)	-
NSB	100 µl	-	50 µl	-
B_0	50 µl	-	50 µl	50 µl
Standard/Sample	-	50 µl	50 µl	50 µl

Table. 2-2 Addition of reagents

2.5.3 Calculation

Calculate and make the standard curve with the analysis temple from Cayman, then determine the sample concentration for each well.

2.6 Western Blot

2.6.1 Extraction and preparation of protein samples

Wash the cells with PBS on ice and then replace PBS with lysis buffer (1 ml containing 845µl RIPA-buffer (Sigma, St. Louis, USA), 40 µl cOmplete solution (cOmplete™ Protease Inhibitor Cocktail, Sigma, USA), 5µl Phenylmethylsulfonyl fluoride (PMSF, 200 mM, Sigma, USA), 5µl Sodium fluoride (NaF, 200 mM, Sigma, USA), 5µl Sodium orthovanadate (Na_3VO_4, Sigma, USA) and 100µl Triton-X-100 (Sigma, USA). Scrape cells and collect the suspension. After vortex oscillation, centrifuge at 12,000 rpm for 20 min (Biofugo, Heraeus, Germany). Transfer the supernatant to a new tube.

2.6.2 Protein concentration calculation and sample preparation

Prepare the standard solutions with Bovine serum albumin (BSA, 1 mg/ml, Sigma,

USA) as following

Standard density(μg/ml)	BSA volume(μl)	ddH_2O volume(μl)
0	0	200
10	2	198
100	20	180
200	40	160
400	80	120
600	120	80
800	160	40
1000	200	0

Fill the 96 well plates with 5 μl standard or sample in each well. Mix Bicinchoninic Acid (BCA) reagents A & B with the ratio 50:1, and then add 200 μl mixed regents in each well. After incubation, measure the absorbance at 562 nm wavelength light (Glomax Muti Detection System, Promga, USA). Add suitable volume 4X loading buffer (Sigma, USA).

2.6.3 Running the gel

Add samples and the marker (Sigma, USA) in the appropriate concentration of gel.

Protein Size	Gel pecentage
4-40 kDa	20%
12-45 kDa	15%
10-70 kDa	12.5%
15-100 kDa	10%
25-100 kDa	8%

Run the gel for proper time and voltage (Mini-PROTEAN® System, Bio-Rad, USA).

2.6.4 Transferring the protein to the membrane

Activate Polyvinylidene difluoride (PVDF) membrane with methanol and prepare the stack. Transfer for 60-90 min at 400 mA.

2.6.5 Antibody staining

Incubate the membrane with primary antibody after blocking for 1 h: anti-TLR2 antibody (1:1000, RabMab, England); anti-TLR1 antibody (1:1000, Arcis, Germany); ß-actin antibody (1:1000, Sigma, USA). Incubate with primary antibody overnight at 4 °C. Wash the membrane in Tris buffered saline with Tween 20 (TBST, Sigma, USA). Incubate the membrane with the secondary antibody. Then, wash the membrane.

2.6.6 Membrane development

Prepare Working Solution (SuperSignal™ West Femto, Thermo Fisher, USA). Add Working Solution on membrane. Remove the solution after incubation and then expose the blot to the imaging system (CHEMOCAM Imager 3.2, INTAS Science imaging, Germany).

2.7 Immunofluorescence and confocal microscopy

Primary antibodies against CD163 (1:100; Acris, Germany), CD68 (1:100; Arcis, Germany), CD11b (1:100; Santa Cruz Biotechnology, USA), F4/80 (1:100; RabMab, England) were used to confirm the isolated human and mouse primary KCs and differentiated THP-1 macrophages as following steps.

Culture the cells in the 8-well Culture Slides (Ibidi, Germany), about 1000 cells/well. Discard the medium, wash 3 times. Fix the cells with 4% paraformaldehyde (PFA) for 15 min. Add 10 mM Ethamlamine (Sigma, USA) for 5 min to remove remaining

PFA after washing with PBS. Wash again and add 0.01% Triton-X-100. Block with Albumin (1% BSA). Incubate with primary antibody overnight at 4 °C. The next day, incubate with secondary antibodies Alexa Fluor (Life Technologies GmbH, Darmstadt, Germany) for 1h in RT after washing with PBS. Wash again, 15min each. Add DAPI (10000x) 2min. Wash with PBS. Analyse cells (Zeiss LSM 510 META, Jena, Germany)

2.8 Statistical analysis

All samples were randomly distributed to different groups. Data were described as the mean ± standard deviation (SD). The Students t-test and One-way analysis of variance (ANOVA) were used when needed. Statistical significance was defined as A value of $p<0.05$; n denotes the number of samples used. SPSS and Graphpad were used for data analysis and figure drawing.

3. Results

3.1 Immunofluorescence results of different macrophages

Special antibodies were used to confirm the isolated primary KCs and differentiated THP-1 cells. Identification of macrophages such as KCs in tissue sections is usually stained by immunohistochemistry of CD68 or CD163 antibodies. The expression of CD68 in tissue macrophages is more extensive, while CD163 is more specific. In addition, CD163 is also commonly considered to be a M2 macrophages' marker [74, 75]. CD11b and F4/80 are specific markers for mature macrophages, of which F4/80 is mouse specific. Immunofluorescence with CD68, CD163 and CD11b positive confirmed the cells we isolated from human tissue were human KCs (Fig.3-1). CD68, CD163 and F4/80 positive immunofluorescent results in mouse primary cells also confirmed these cells were mouse KCs (Fig.3-2).

THP-1 cells, the most widely used monocytic cell lines to investigate macrophages, were suspension cells before PMA treatment. 24 h after PMA treatment, THP-1 cells became adherent cells with typical morphological characteristics. Immunofluorescent results with CD11b, CD68 positive and F4/80 negative in these cells also identified these THP-1 macrophages (TMCs) (Fig.3-3).

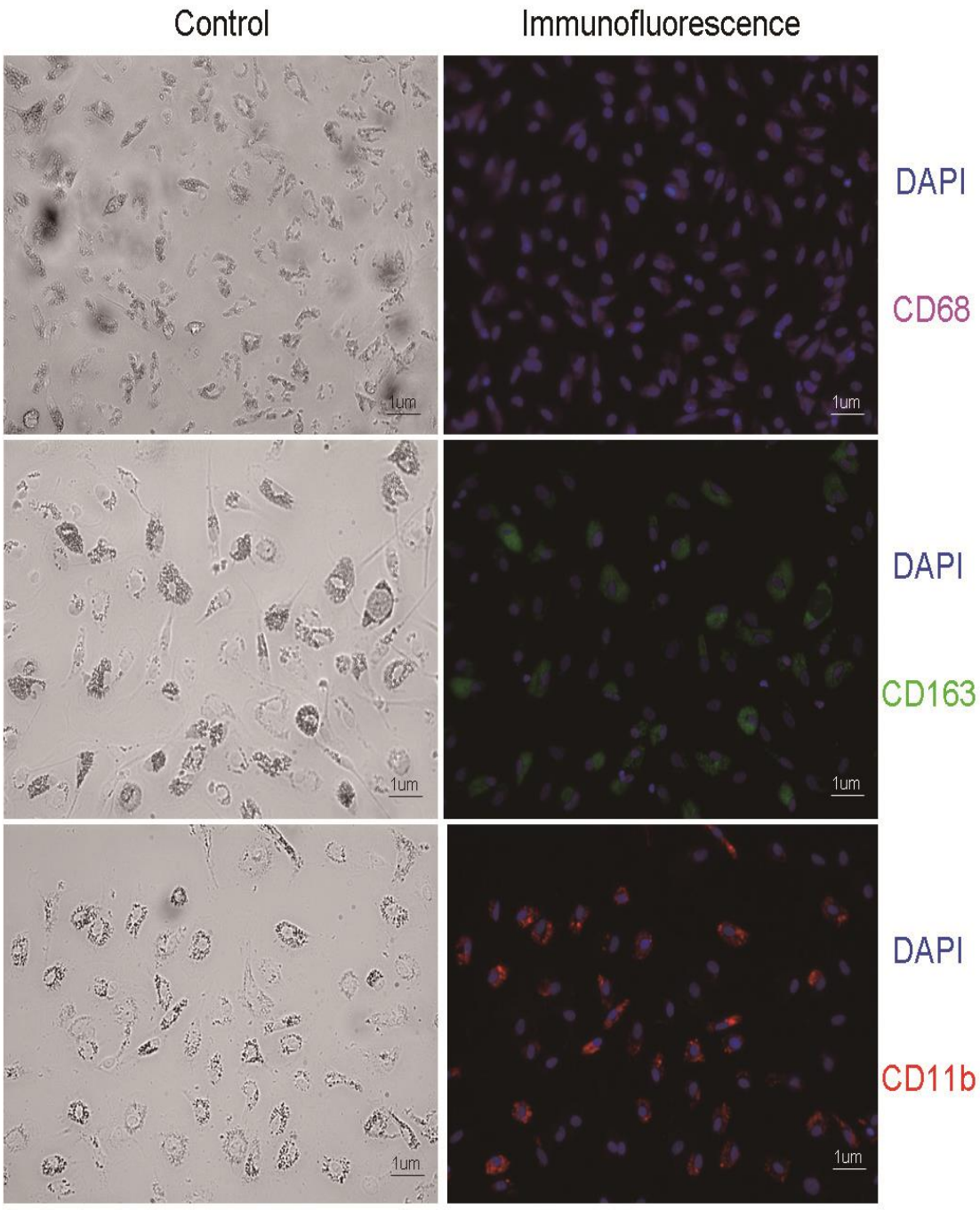

Fig.3-1 Immunofluorescence results of human KCs

(Each picture represents from three separate results, 200x magnification)

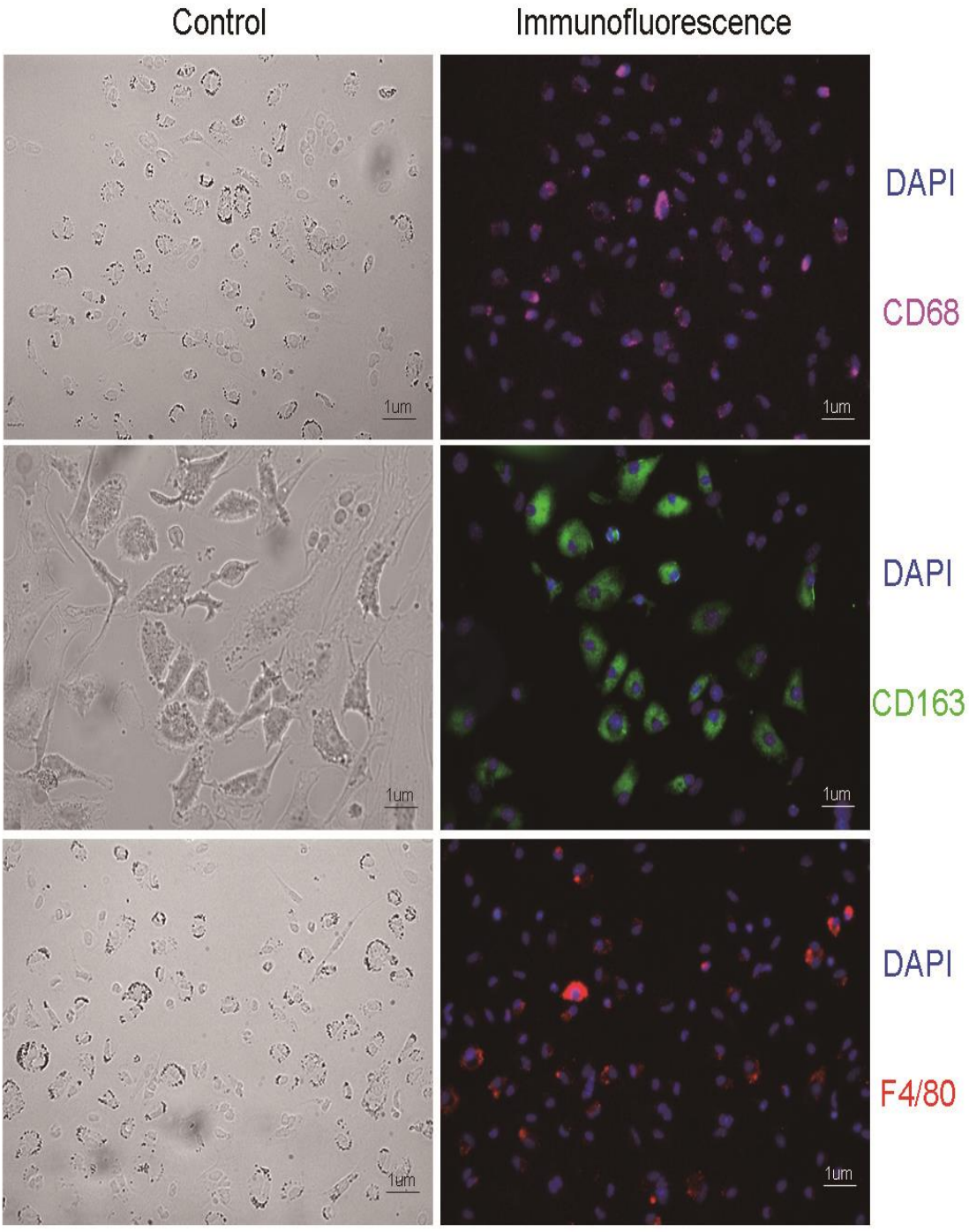

Fig.3-2 Immunofluorescence results of mouse KCs

(Each picture represents from three separate results, 200x magnification)

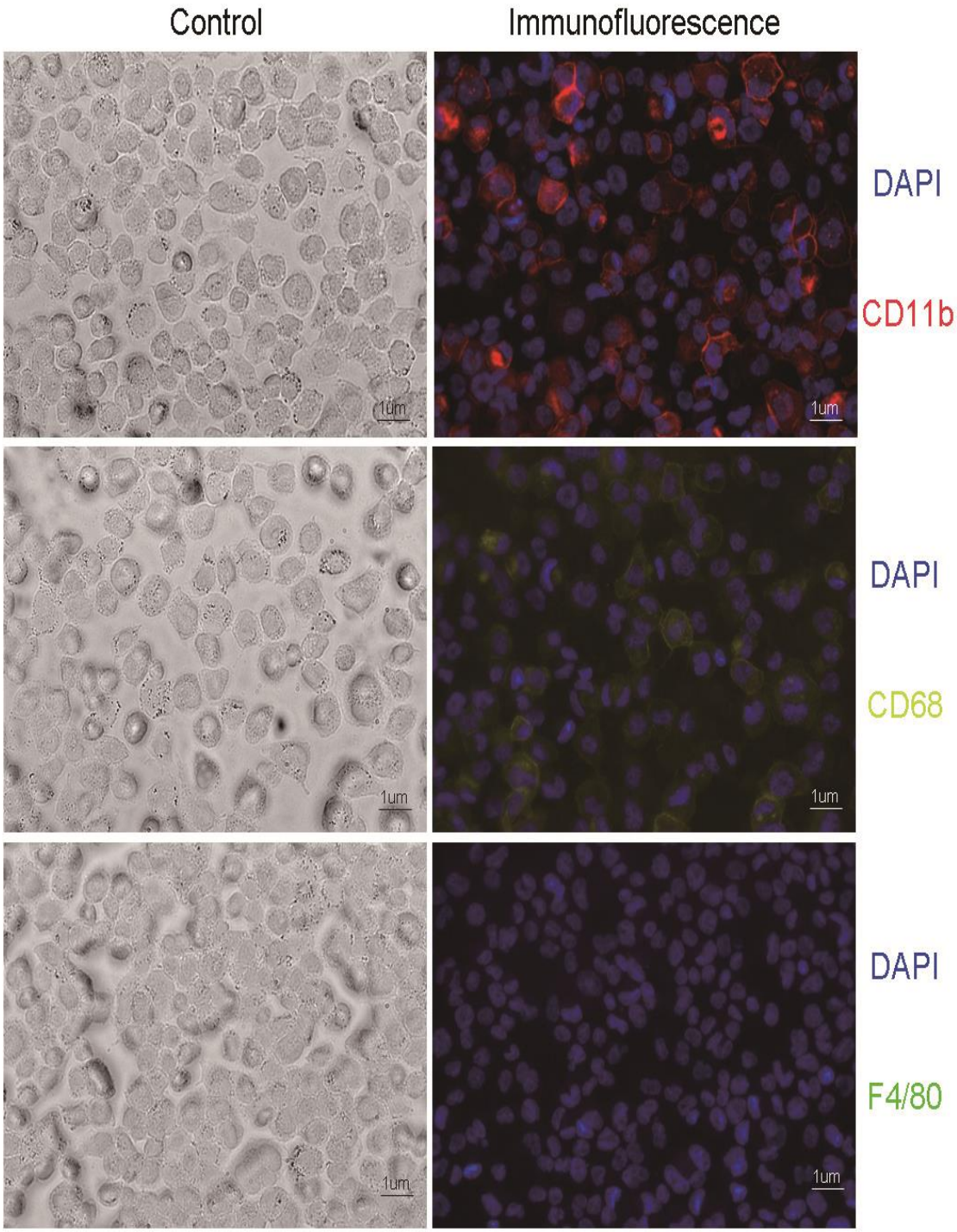

Fig.3-3 Immunofluorescence results of TMCs
(Each picture represents from three separate results, 200x magnification)

3.2 Influences of microbial isolates on TXB_2 secretion

According to published articles [76, 77] and our pretest results, we designed the concentration gradient experiments with low, middle or high concentrations (the standardized protein concentration) to confirm the suitable stimulating doses for different kinds of microbial isolates. The middle concentration showed the most effective increase in TXB_2 section on TMCs for all kinds of isolates (Fig. 3-4). The effects of 8μg/ml microbial isolates were also tested in primary KCs from mice and human. After 24 h stimulation, 7 kinds of microbial isolates all significantly increased TXB_2 secretion in HKCs (Fig. 3-5) and MKCs (Fig. 3-6). Due to individualized differences in the source of tissue specimens, primary KCs of human and mouse were compared with standardized results (multiple increases in TXB_2 levels before and after stimulation).

Microbial isolates	Human KCs (increased fold)	Mouse KCs (increased fold)	THP-1 macrophages (pg/ml)
K. pneumonia	1.00±0.13 vs. 8.02±5.51 *	1.00±0.20 vs. 3.52±1.48 *	117±58 vs. 5049±3490 *
E. coli	1.00±0.24 vs. 10.19±7.49 *	1.00±0.34 vs. 3.49±1.44 *	244±114 vs. 2435±1459 *
E. cloacae	1.00±0.11 vs. 18.44±12.62 *	1.00±0.18 vs. 5.23±3.17 *	275±268 vs. 7619±2032 *
E. faecium	1.00±0.45 vs. 5.71±1.83 *	1.00±0.29 vs. 4.14±3.01 *	104±68 vs. 4145±2613 *
S. pneumonia	1.00±0.09 vs. 3.07±0.78 *	1.00±0.20 vs. 8.99±1.83 *	192±192 vs. 3278±2163 *
S. aureus	1.00±0.15 vs. 8.65±2.19 *	1.00±0.29 vs. 10.77±1.19 *	257±291 vs. 4663±901 *
C. albicans	1.00±0.15 vs. 2.46±1.41 *	1.00±0.39 vs. 8.00±2.87 *	180±94 vs. 4724±2640 *

Table. 3-1 Influences of microbial isolates on TXB_2 secretion

Data are expressed as mean ± SD, *p < 0.05 comparing between values before and after stimulation, *p < 0.05, two-tailed t-test.

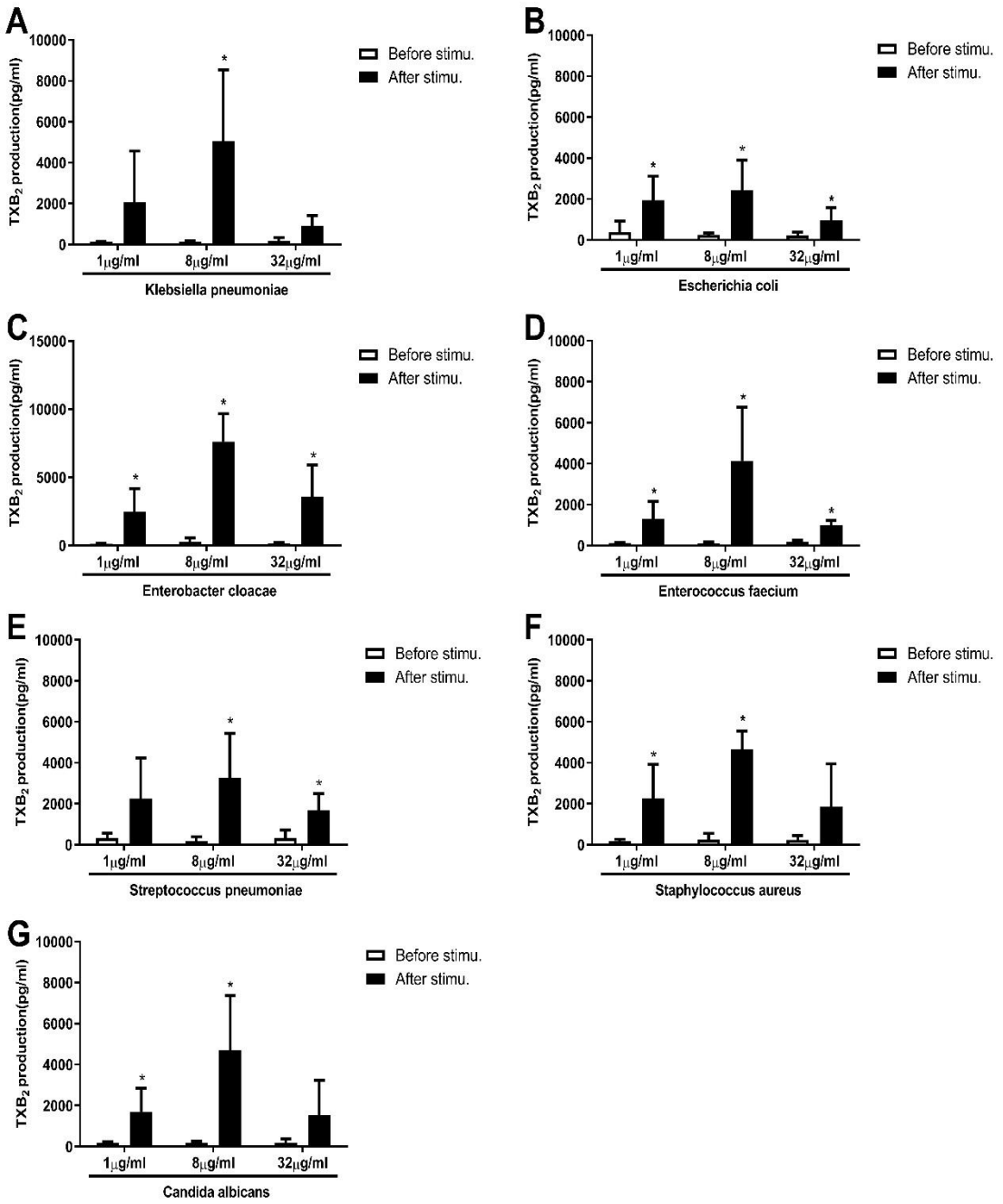

Fig.3-4 Concentration gradient experiments of microbial isolates

Low (1 μg/ml), middle (8 μg/ml) and high (32 μg/ml) concentrations of different kinds of bacteria were used to stimulate differentiated THP-1 macrophages, the middle concentration seemed to be the most effective for all kinds of bacteria. Data are expressed as mean ± SD, two-tailed t-test, *$p < 0.05$ comparing between values before and after stimulation, n=4 in each group.

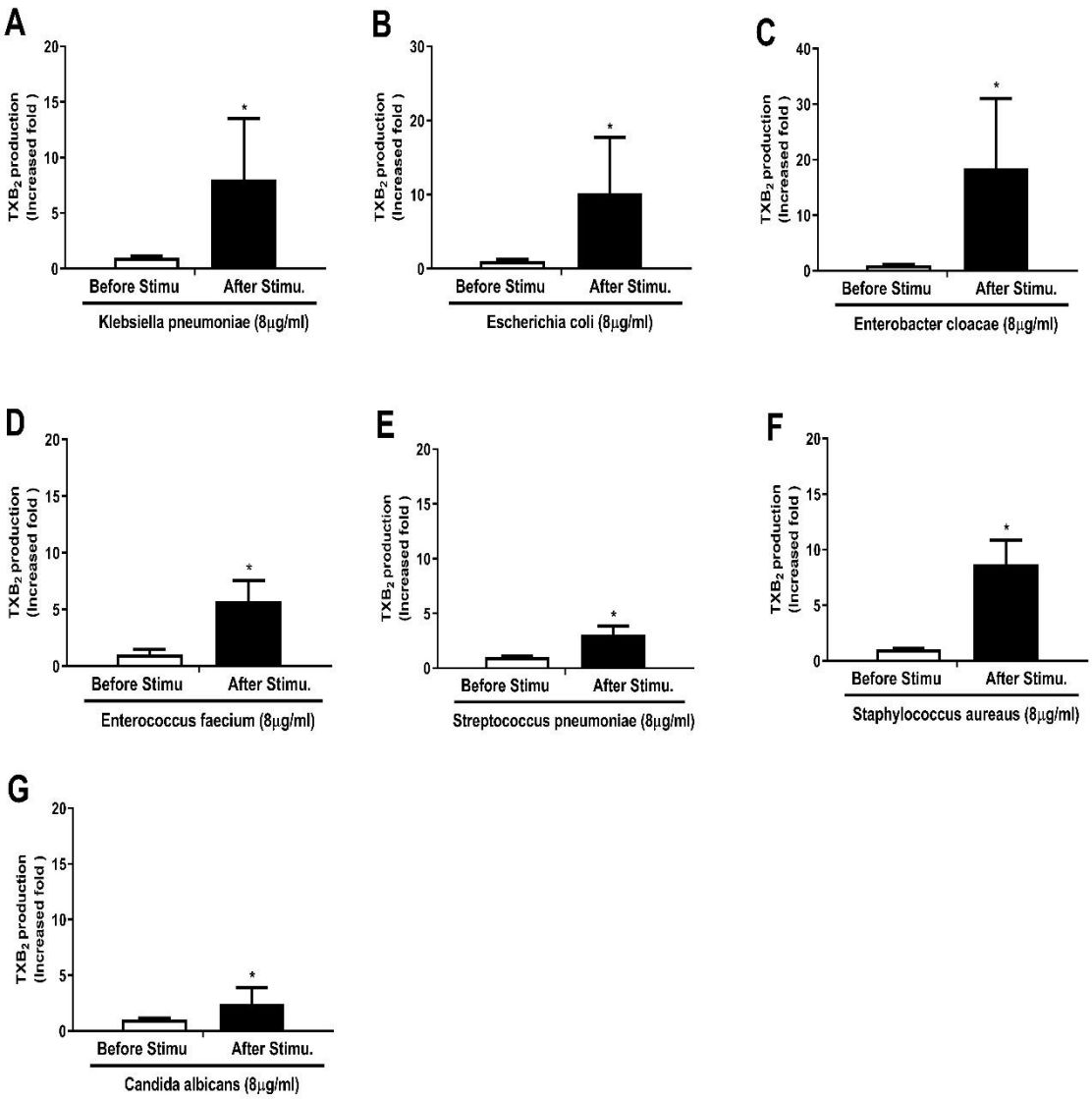

Fig.3-5 Microbial isolates increased TXB_2 secretion in human KCs

24 h stimulation with microbial isolates including *A-Klebsiella pneumoniae*, *B-Escherichia coli, C-Enterobacter cloacae, D-Enterococcus faecium, E-Streptococcus pneumoniae, F-Staphylococcus aureus and G-Candida albicans* significantly increased TXB_2 secretion in human KCs. Data are expressed as mean ± SD, two-tailed t-test, *$p < 0.05$ comparing between values before and after stimulation, n=6 in each group.

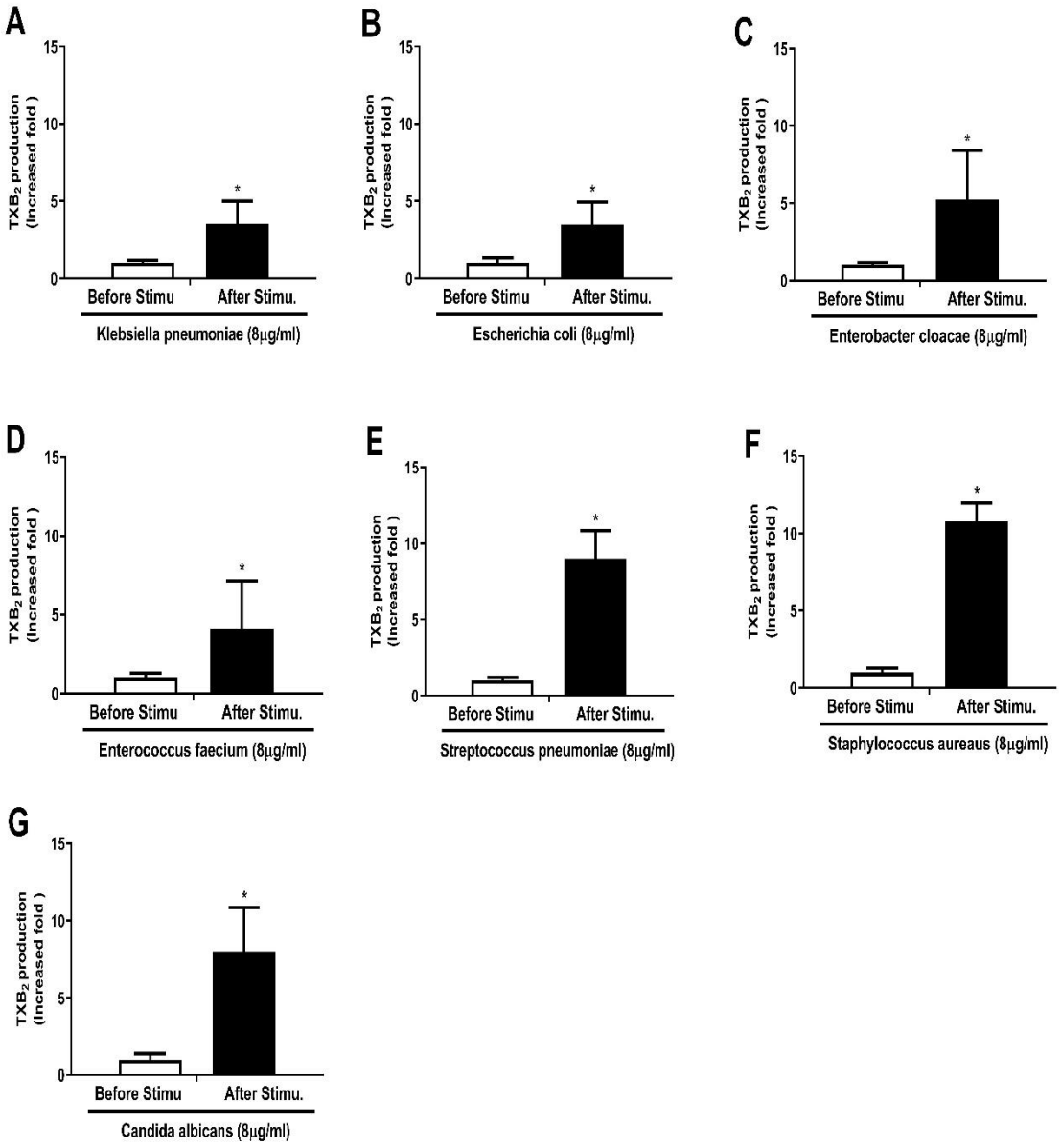

Fig.3-6 Microbial isolates increased TXB_2 secretion in mouse KCs

24 h stimulation with microbial isolates including A-*Klebsiella pneumoniae*, B-*Escherichia coli*, C-*Enterobacter cloacae*, D-*Enterococcus faecium*, E-*Streptococcus pneumoniae*, F-*Staphylococcus aureus* and G-*Candida albicans* significantly increased TXB_2 secretion in mouse KCs. Data are expressed as mean ± SD, two-tailed t-test, *$p < 0.05$ comparing between values before and after stimulation, n=6 in each group.

3.3 Influence of TLR-1 to -9 agonists in KCs and TMCs

TLR-1 to -9 agonists (names and stimulating dosages were described above) were used to investigate the effective TLRs increasing TXB_2 in KCs. 24 h stimulation with Pam3CSK4, HKLM or LPS significantly increased TXB_2 secretion in HKCs (Fig. 3-7). The same treatment with TLR-1/2, -2 and -4 agonists in MKCs also resulted in the increased TXB_2 production (Fig. 3-8). Besides Pam3CSK4 and HKLM, Poly(I:C)-LMW and ODN2006 also induced significant TXB_2 increase in TMCs (Fig. 3-9). Interestingly, LPS had no significant effect on TMCs (Fig. 3-9). Details are shown in Table. 3-2.

Comparing among the effects of TLR1-9 agonists on HKCs, MKCs and TMCs, TLR-1, -2 and -4 were most possiblily related to the microbial-induced TXB_2 increase in KCs. Western blot with anti-TLR-1 and anti-TLR-2 antibodies were then used to confirm the activated TLRs on TMCs following stimulation with different kinds of microbial isolates. TLR-1 and -2 expression on TMCs were elevated by all tested isolates, However, Gram (-) isolates (*K. pneumonia, E. coli* and *E. cloacae*) were more effective than Gram (+) isolates (*E. faecium*, *S. pneumoniae* and *S. aureus*) and *C. albicans* showed the least effect (Fig. 3-10).

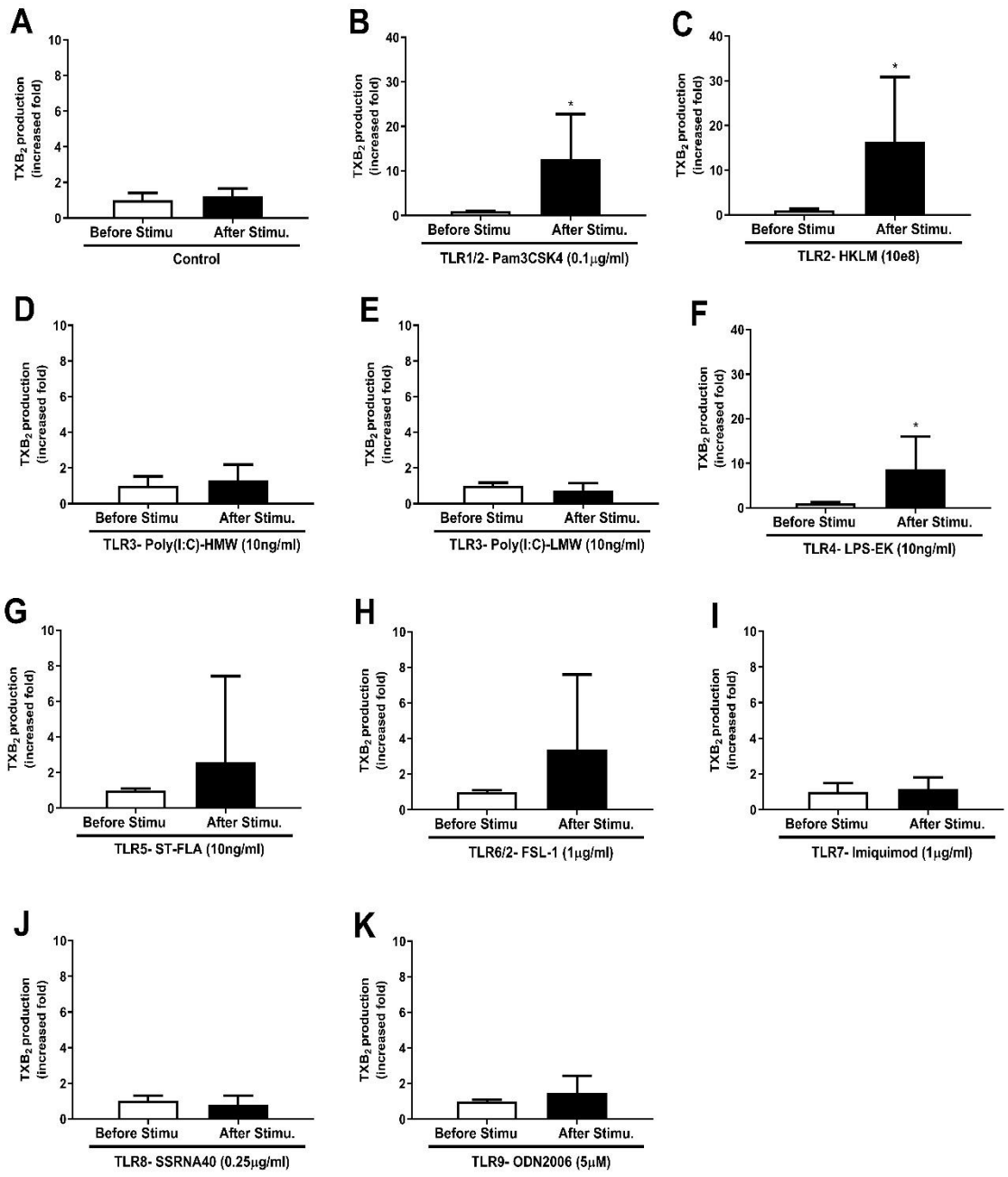

Fig.3-7 TLR-1/2, -2 and -4 agonists increased TXB_2 secretion in human KCs

Among all TLR-1 to -9 agonists, only Pam3CSK4 (B-0.1 μg/ml, 24 h), HKLM (C-10e8 cells/ml, 24 h) and LPS-EK (F-10ng/ml, 24 h) significantly increased TXB_2 secretion in human KCs. Data are expressed as mean ± SD, two-tailed t-test, *p < 0.05 comparing between values before and after stimulation, n=6 in each group.

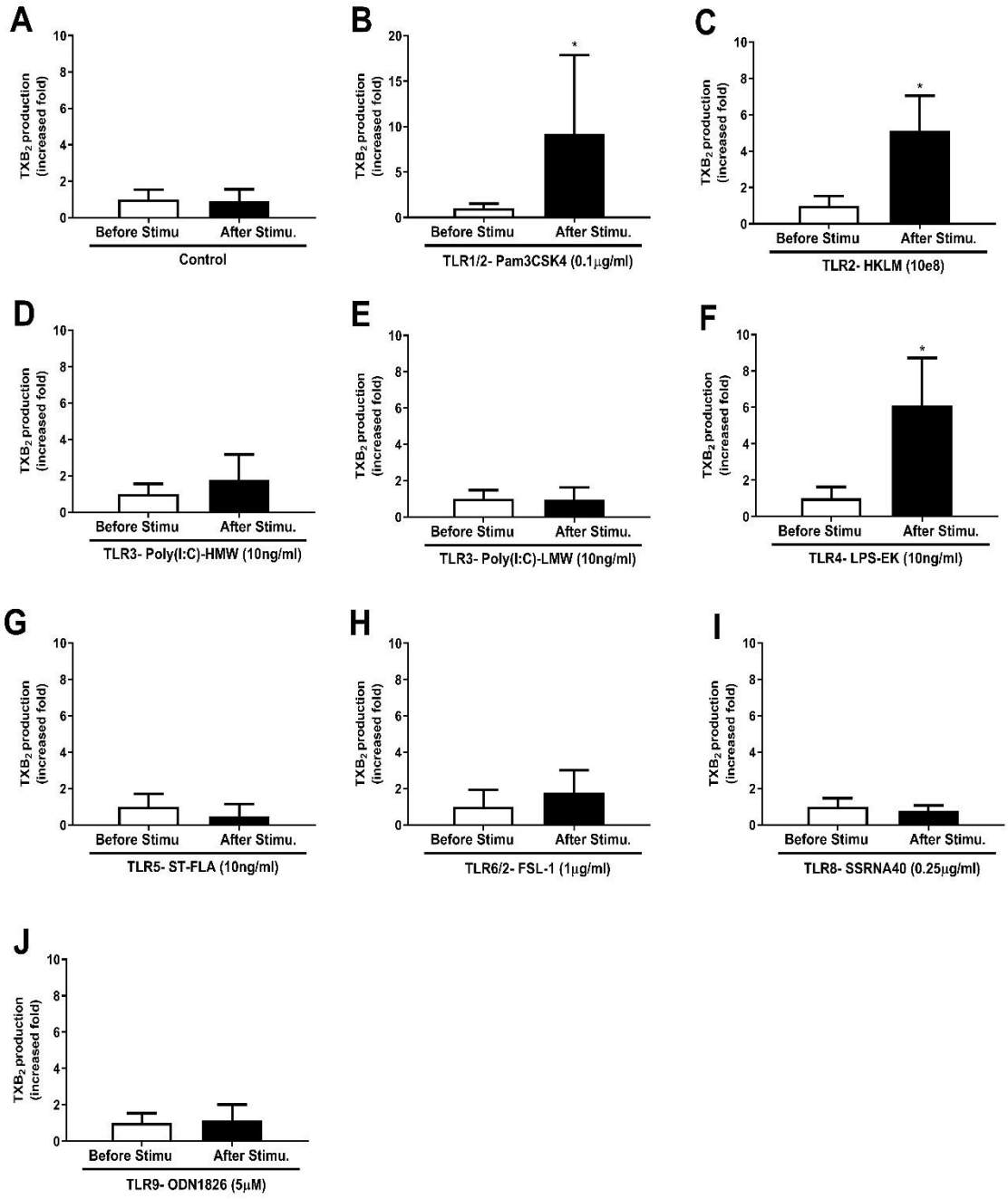

Fig.3-8 TLR-1/2, -2 and -4 agonists increased TXB_2 secretion in mouse KCs

Among all TLR-1 to -9 agonists, only Pam3CSK4 (B-0.1 μg/ml, 24 h), HKLM (C-10e8 cells/ml, 24 h) and LPS-EK (F-10ng/ml, 24 h) significantly increased TXB_2 secretion in mouse KCs. Data are expressed as mean ± SD, two-tailed t-test, *$p < 0.05$ comparing between values before and after stimulation, n=6 in each group.

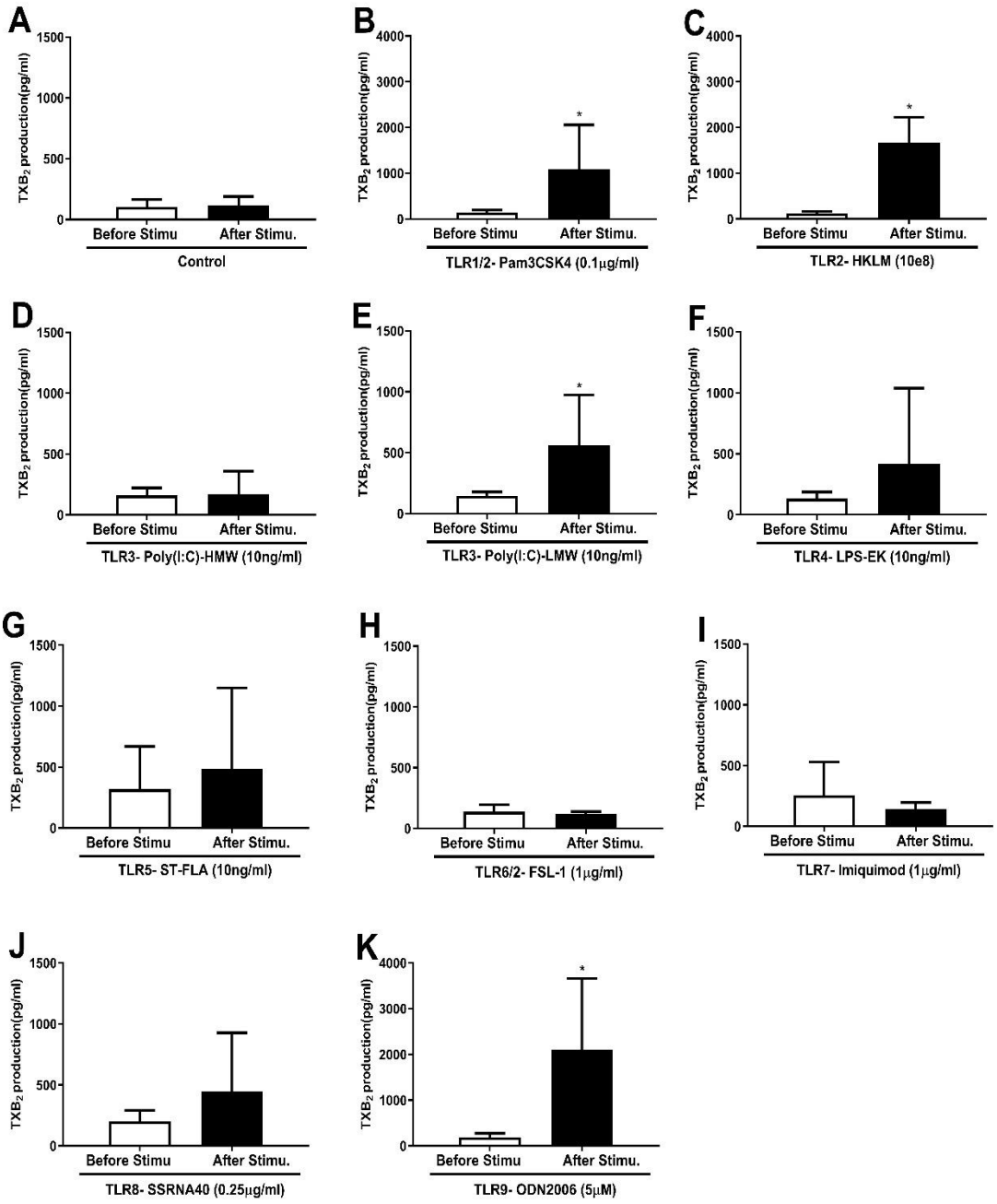

Fig.3-9 TLR-1/2, -2, -3 and -9 agonists increased TXB_2 secretion in THP-1 macrophages

24 h stimulation with Pam3CSK4 (B-0.1 μg/ml), HKLM (C-10e8 cells/ml), Poly(I:C)-LMW (E-10ng/ml) and ODN2006 (K-5μM) had significant effects in differentiated THP-1 macrophages. Data are expressed as mean ± SD, two-tailed t-test, *$p < 0.05$ comparing between values before and after stimulation, n=6 in each group.

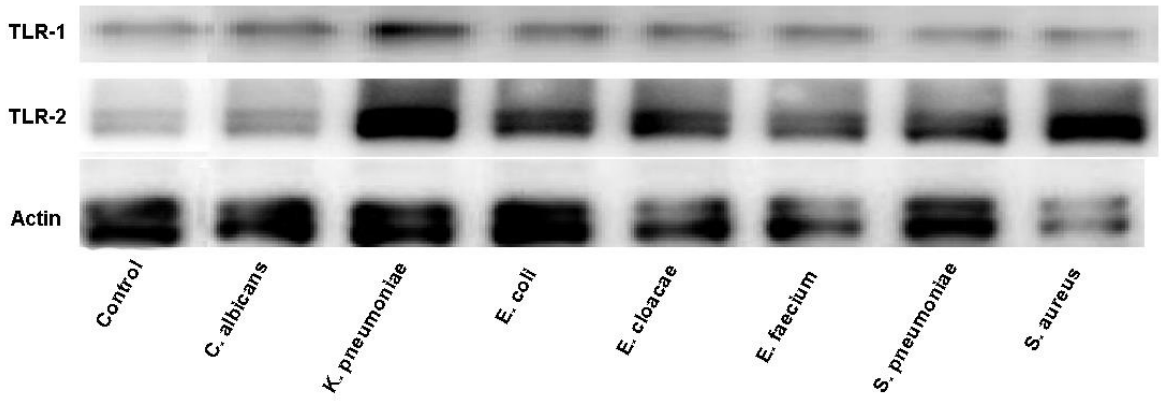

Fig.3-10 TLR-1 and -2 expression on TMCs following microbial isolates stimulation

24 h stimulation with microbial isolates including *K. pneumoniae, E. coli, E. cloacae, E. faecium, S. pneumoniae, S. aureus and C. albicans* (8μg/ml each, 24h stimulation) increased TLR-1 and -2 on TMCs. Expression by Gram (-) isolates were more effective than Gram (+) isolates and C.albicans showed the least effect.

TLR agonists	Human KCs (increased fold)	Mouse KCs (increased fold)	THP-1 macrophages (pg/ml)
Control	1.00±0.41 vs. 1.21±0.45	1.00±0.53 vs. 0.89±0.66	103±61 vs. 118±71
Pam3CSK4	1.00±0.05 vs. 12.69±10.10 *	1.00±0.53 vs. 9.23±8.63 *	145±47 vs. 1082±975 *
HKLM	1.00±0.39 vs. 16.41±14.43 *	1.00±0.54 vs. 5.10±1.95 *	123±41 vs. 1661±561 *
Poly(I:C)-HMW	1.00±0.53 vs. 1.30±0.89	1.00±0.56 vs. 1.78±1.40	160±60 vs. 172±187
Poly(I:C)-LMW	1.00±0.17 vs. 0.71±0.44	1.00±0.49 vs. 0.95±0.68	148±30 vs. 561±411 *
LPS-EK	1.00±0.34 vs. 8.72±7.34 *	1.00±0.62 vs. 6.09±2.63 *	132±54 vs. 416±621
ST-FLA	1.00±0.21 vs. 8.84±14.03	1.00±0.71 vs. 0.49±0.67	317±351 vs. 487±663
FSL-1	1.00±0.11 vs. 3.39±4.21	1.00±0.94 vs. 1.77±1.24	137±58 vs. 117±21
Imiquimod	1.00±0.49 vs. 1.15±0.66		254±276 vs. 139±57
SSRNA40	1.00±0.28 vs. 0.80±0.52	1.00±0.47 vs. 0.77±0.30	200±90 vs. 447±479
ODN2006/1826	1.00±0.09 vs. 1.46±0.98	1.00±0.54 vs. 1.14±0.87	189±86 vs. 2097±1564 *

Table. 3-2 Influences of TLR agonists on TXB_2 secretion

Data are expressed as mean ± SD, before vs. after stimulation. Two-tailed t-test, *p < 0.05, comparing between values before and after stimulation.

3.4 TLR agonists decreased TXB_2 secretion in mouse HSCs and had no effects in SECs

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. 3-11). Interestingly, an overall downward tendency was observed in HSCs after TLR agonist stimulation. Furthermore, significant reduction of TXB_2 was found by applying TLR-3, -5 and -9 agonists (Fig. 3-12). Complete data are shown in Table. 3-3.

TLR agonists	Mouse SECs (increased fold)	Mouse HSCs (increased fold)
Control	1.00±0.87 vs. 0.87±0.69	1.00±0.89 vs. 0.66±0.71
Pam3CSK4	1.00±0.47 vs. 1.52±0.86	1.00±0.59 vs. 0.42±0.27
HKLM	1.00±1.24 vs. 1.36±1.39	1.00±0.86 vs. 0.44±0.45 *
Poly(I:C)-HMW	1.00±0.28 vs. 1.85±1.91	1.00±0.11 vs. 0.38±0.12 *
Poly(I:C)-LMW	1.00±0.39 vs. 1.28±0.62	1.00±0.14 vs. 0.34±0.05 *
LPS-EK	1.00±0.47 vs. 1.31±0.65	1.00±0.99 vs. 0.38±0.27
ST-FLA	1.00±0.44 vs. 1.12±0.51	1.00±0.34 vs. 0.24±0.11 *
FSL-1	1.00±0.24 vs. 1.24±0.82	1.00±1.53 vs. 0.22±0.32
SSRNA40	1.00±0.65 vs. 0.84±0.54	1.00±1.50 vs. 0.32±0.40
ODN1826	1.00±1.18 vs. 1.12±0.71	1.00±0.41 vs. 0.52±0.29 *

Table. 3-3 Influences of TLR agonists on mouse HSC and SEC

Data are expressed as mean ± SD, before vs. after stimulation. Two-tailed t-test, *p < 0.05, comparing between values before and after stimulation.

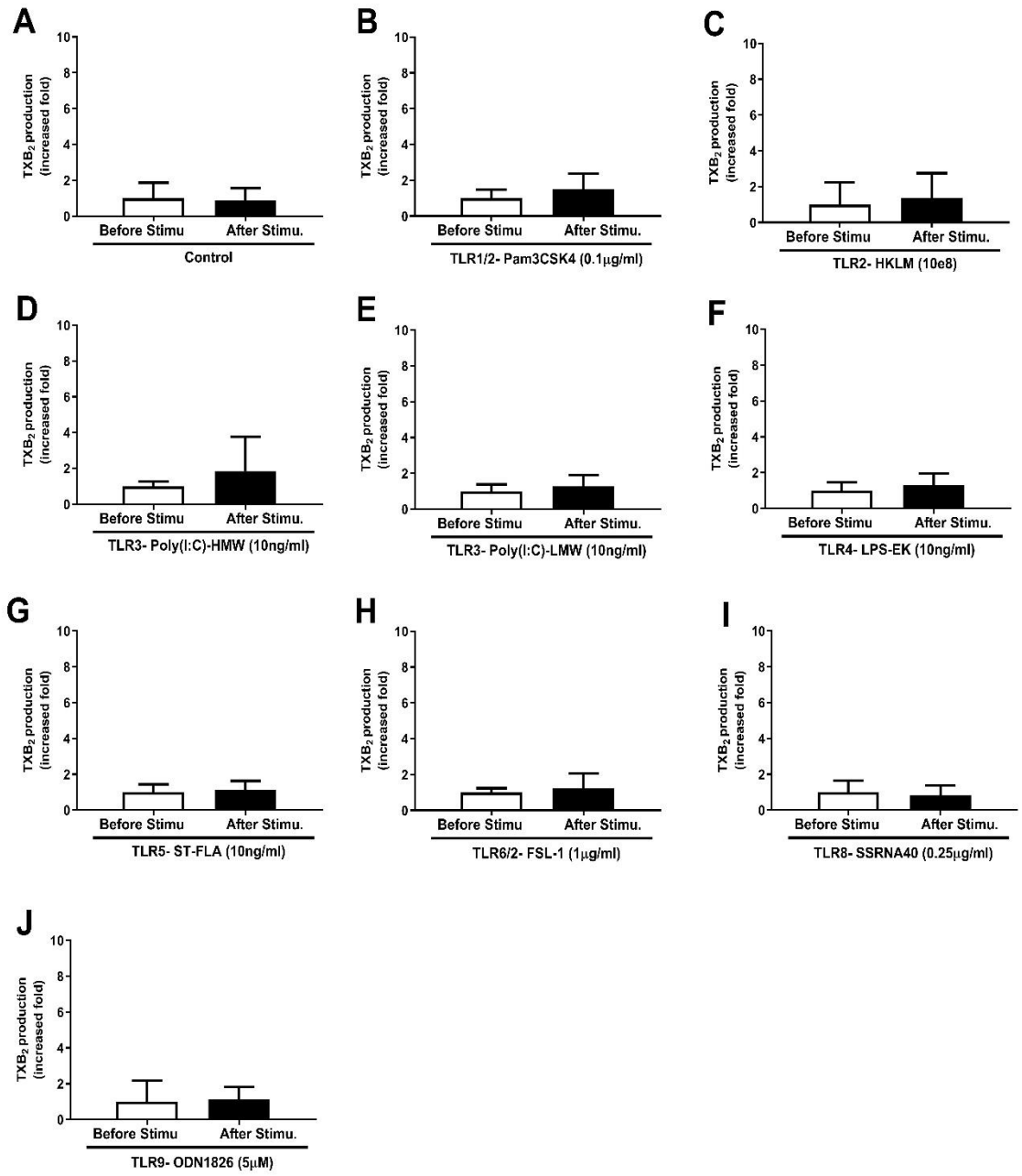

Fig.3-11 TLR agonists showed no effects in mouse SECs

Among all TLR-1 to -9 agonists, none of TLR agonists significantly increased TXB_2 secretion in SECs. Data are expressed as mean ± SD, n=6 in each group.

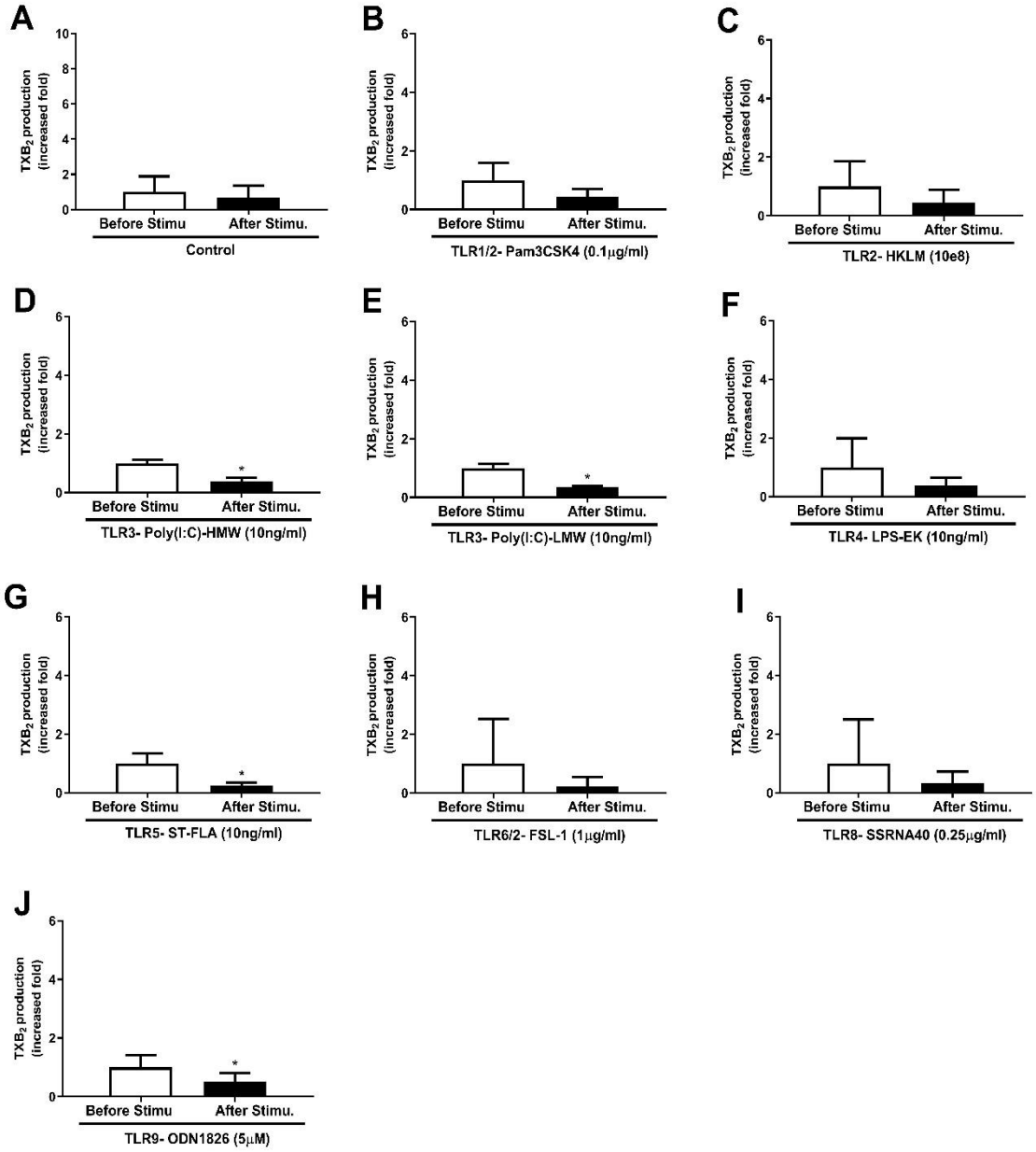

Fig.3-12 TLR-3, -5, -6 and -9 agonists decreased TXB_2 secretion in mouse HSCs

An overall downward tendency was observed in HSCs after TLR agonist stimulation. Furthermore, Poly(I:C)-HMW (10ng/ml, 24 h), Poly(I:C)-LMW (10ng/ml, 24 h), ST-FLA (10ng/ml, 24 h) and ODN1826 (10ng/ml, 24 h) induced significant effects. Data are expressed as mean ± SD, two-tailed t-test, *$p < 0.05$ comparing between values before and after stimulation, n=6 in each group.

3.5 Specific TLR-1, -2 or -4 antagonists reduced the TXB_2 increase caused by related TLR agonists

According to the results above, TLR-1, -2 and -4 seemed to be most possibly related to bacterial-induced TXB_2 increase. Before treating the HKCs with TLR antagonists, the gradient concentration experiments to select the effective concentration of these antagonists were tested in TMCs and MKCs (LPS failed to increase TXB_2 in TMCs, so TLR-4 antagonist was tedted in MKCs). 10μM CU-CPT22, 1μg/ml Mab-mTLR2 and higher concentrations of these drugs adding in TMCs significantly attenuated the increase of TXB_2 caused by Pam3CSK4 or HKLM (Fig. 3-13 A, B). 1 μM TAK242 or higher concentrations in MKCs also had significant effects in reducing LPS-induced TXB_2 secretion (Fig. 3-13 C).

In order to minimize the adverse effects of TLR antagonists on cells, we chose to treat cells with their lowest effective dose. 10μM CU-CPT22, 1μg/ml Mab-mTLR2 and 1 μM TAK242 were selected in the following experiments. The effects of the selected concentrations of TLR antagonists were identified in human and mouse KCs. 1 h pretreatment with CU-CPT22 (10μM), Mab-mTLR2 (1μg/ml) or TAK242 (1μM) in human Kupffer cells and mouse Kupffer cells siginificantly reduced the TXB_2 increase caused by Pam3CSK4, HKLM or LPS-EK (Fig. 3-13 D,E)

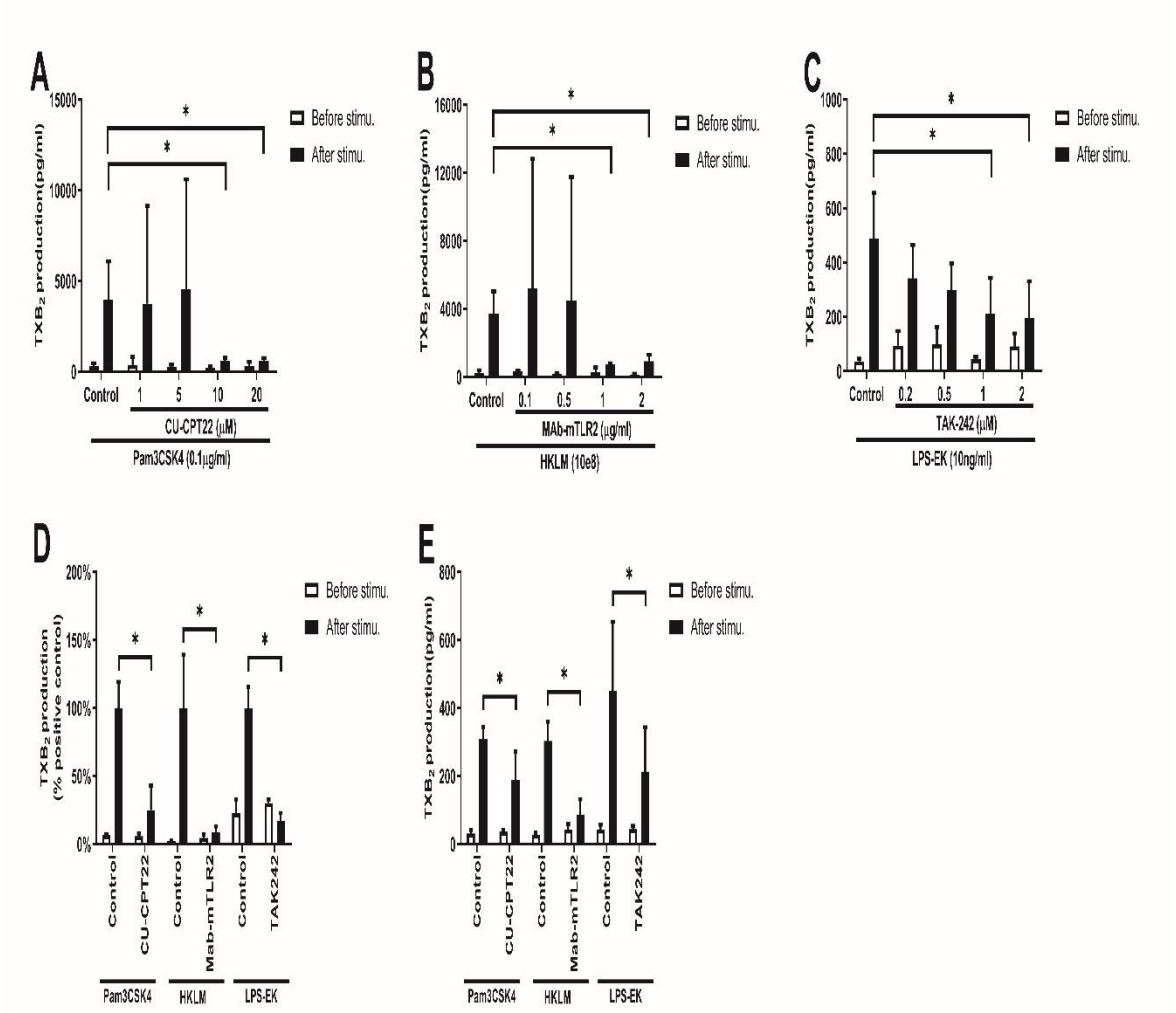

Fig.3-13 Concentration gradient experiments of TLR-1/-2/-4 antagonists

A-C: Different concentrations of CU-CPT22, MAb-mTLR2 or TAK242 were added 1 h before stimulation of TLR-1, -2 or -4 agonists. Pretreatment with 10μM CU-CPT22, 1μg/ml MAb-mTLR2, 1μM TAK242 and higher concentrations of these 3 drugs significantly attenuated the increase of TXB_2 by Pam3CSK4, HKLM or LPS-EK. Mean values of control group after stimulation used as positive control. 1 h pretreatment with CU-CPT22 (10μM), Mab-mTLR2 (1μg/ml) or TAK242 (1μM) in human Kupffer cells (D) and mouse Kupffer cells (E) reduced TXB_2 increase caused by Pam3CSK4 (0.1 μg/ml, 24 h), HKLM (10e8 cells/ml, 24 h) or LPS-EK (10ng/ml, 24 h). Data are expressed as mean ± SD, control after stimulation used as positive control in human KCs. Two-tailed t-test, *$p < 0.05$, n=6 in each group.

3.6 Effects of TLR-1, -2 and -4 antagonists on bacterial-induced TXB_2 increase

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. TXB_2 increase caused by Gram (-) bacterial products were attenuated by 1 h pretreatment with TLR-1, -2 or -4 antagonists before stimulation (Fig. 3-14 A-C). CU-CPT22 and Mab-mTLR2 also had effects on TXB_2 secretion following Gram (+) bacterial stimulation (Fig. 3-14 D-F). Only Mab-mTLR2 significantly decreased *C. albicans*-induced TXB_2 secretion (Fig. 3-14 G).

Microbial isolates	Control (%positive control)	CU-CPT22 (%positive control)	Mab-mTLR2 (%positive control)	TAK242 (%positive control)
K. pneumoniae	7.64±8.13 vs. 100.00±8.22	6.72±7.77 vs. 44.04±19.20 *	8.04±8.53 vs. 64.53±16.50 *	6.87±7.28 vs. 34.15±14.05 *
E. coli	15.48±13.64 vs. 100.00±24.79	13.02±10.76 vs. 23.20±12.22 *	15.25±12.68 vs. 35.16±18.15 *	15.33±15.01 vs. 50.07±39.66 *
E. cloacae	6.16±7.13% vs. 100.00±38.02	5.75±6.18 vs. 30.24±22.65 *	5.67±6.17 vs. 27.00±21.21 *	4.18±4.46 vs. 41.48±38.36 *
E. faecium	13.95±8.84 vs. 100.00±26.56	8.75±4.71 vs. 25.34±26.38 *	10.96±6.03 vs. 15.81±3.50 *	11.96±5.03 vs. 53.10±45.87
S. pneumoniae	25.97±29.51 vs. 100.00±26.66	6.19±6.38 vs. 37.23±25.18 *	9.96±10.57 vs. 16.35±13.66 *	28.13±37.54 vs. 52.42±41.85
S. aureus	2.59±0.61 vs. 100.00±45.45	4.17±1.07 vs. 27.95±8.12 *	4.72±4.45 vs. 25.90±6.38 *	6.47±5.79 vs. 61.16±42.37
C. albicans	8.94±2.17 vs. 100.00±33.23	11.57±5.30 vs. 80.16±15.63	16.83±10.69 vs. 45.00±29.99 *	7.66±1.34 vs. 74.14±32.76

Table. 3-4 Influences of TLR-1, -2 and -4 antagonists on bacterial-induced TXB_2 increase

Data are expressed as mean ± SD (mean values of control group after stimulation used as positive control), before vs. after stimulation. Two-tailed t-test, *$p < 0.05$, p value was calculated by comparing values after stimulation in each antagonist groups with control group.

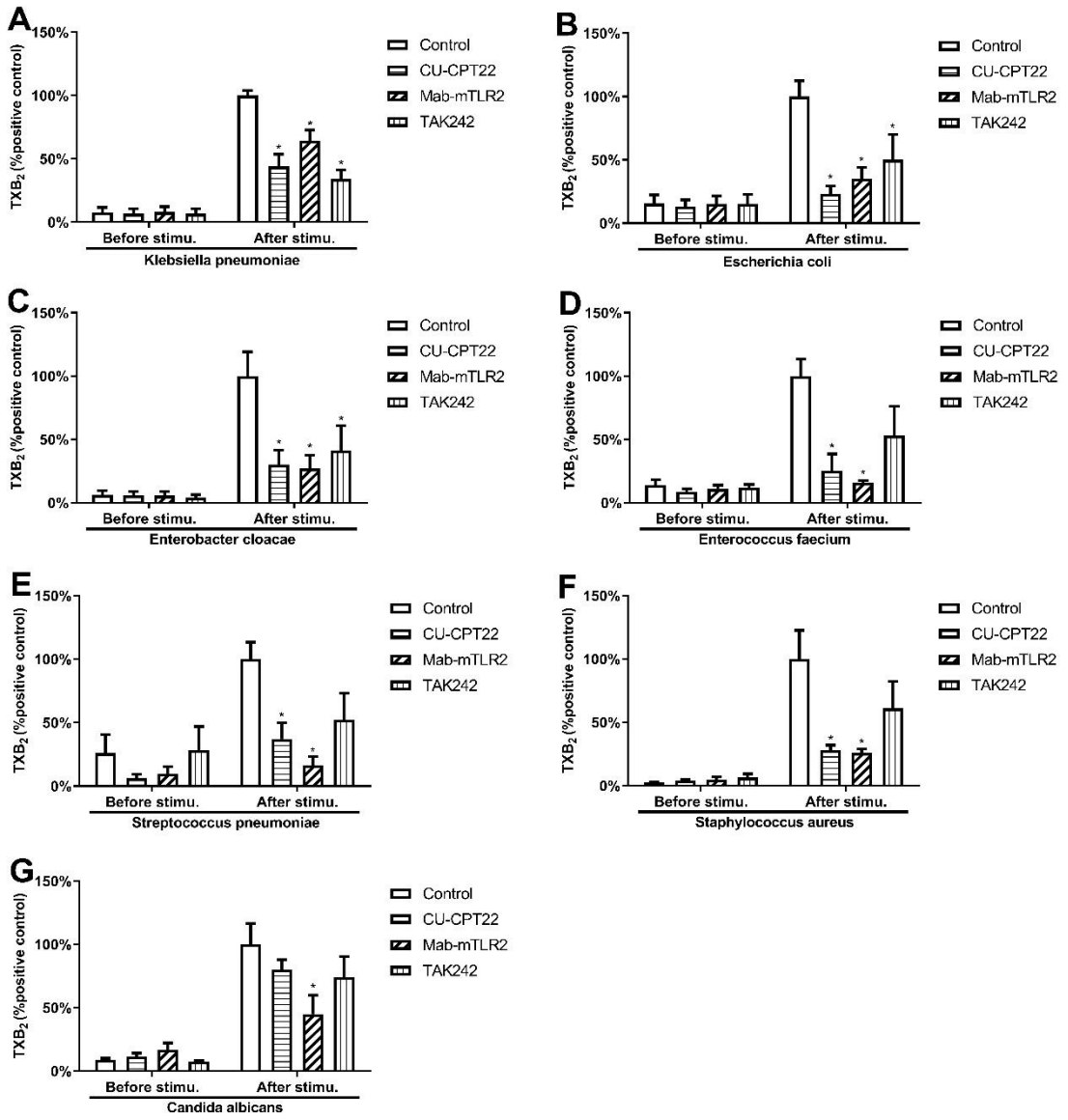

Fig.3-14 Effects of TLR antagonists in different kinds of microbial isolates

1 h pretreatment with CU-CPT22 (10μM), Mab-mTLR2 (1μg/ml) and TAK242 (1μM) in human Kupffer cells respectively attenuated TXB_2 increase caused by Gram (-) bacteria (A-*Klebsiella pneumoniae*, B-*Escherichia coli* and C-*Enterobacter cloacae*, 8μg/ml 24 h stimulation). Cu-CPT22 and Mab-mTLR2 also decreased TXB_2 induced by Gram (+) bacteria (D-*Enterococcus faecium*, E-*Streptococcus pneumoniae* and F-*Staphylococcus aureus*, 8μg/ml 24 h stimulation). Only Mab-mTLR2 had effects on fungus-induced TXB_2 increase (G-*Candida albicans*, 8μg/ml 24 h stimulation). Mean values of control group after stimulation used as positive control. *$p < 0.05$ compared to the positive control, n=4 in each group.

3.7 Effects of combined TLR antagonists on bacterial-induced TXB_2 increase

The effects of combined TLR antagonists were also compared with each single drug. Either single TLR-1, -2 and -4 antagonists or combined drugs (mix group, including Mab-mTLR2, CU-CPT22 and TAK242, 1 h pretreatment before stimulation) significantly decreased TXB_2 increase by Gram (-) bacterial stimulation (*K. pneumoniae*, *E. coli* and *E. cloacae*, 8μg/ml, 24 h stimulation), In addition, combined substance achieved a better effect than any single substances. Details are shown in Table. 3-5.

Microbial isolates	Control (Positive %)	CU-CPT22 (Positive %)	Mab-mTLR2 (Positive %)	TAK242 (Positive %)	Mix (Positive %)
K. pneumoniae	100.00±24.28	50.37±17.38 *#	36.25±18.87 *#	42.18±22.55 *#	15.62±10.32 *
E. coli	100.00±12.18	39.31±13.33 *#	64.83±17.14 *#	53.19±14.12 *#	20.84±4.48 *
E. cloacae	100.00±17.97	37.61±19.88 *#	43.38±13.46 *#	34.89±11.52 *#	14.97±2.70 *

Table. 3-5 Comparision between combination and single usages of TLR-1, -2 and -4 antagonists

Data are expressed as mean ± SD, the control group used as a positive control. P values were calculated by comparing values after stimulation in each antagonist groups with control group (*$p < 0.05$) or Mix group (#$p < 0.05$), two-tailed t-test.

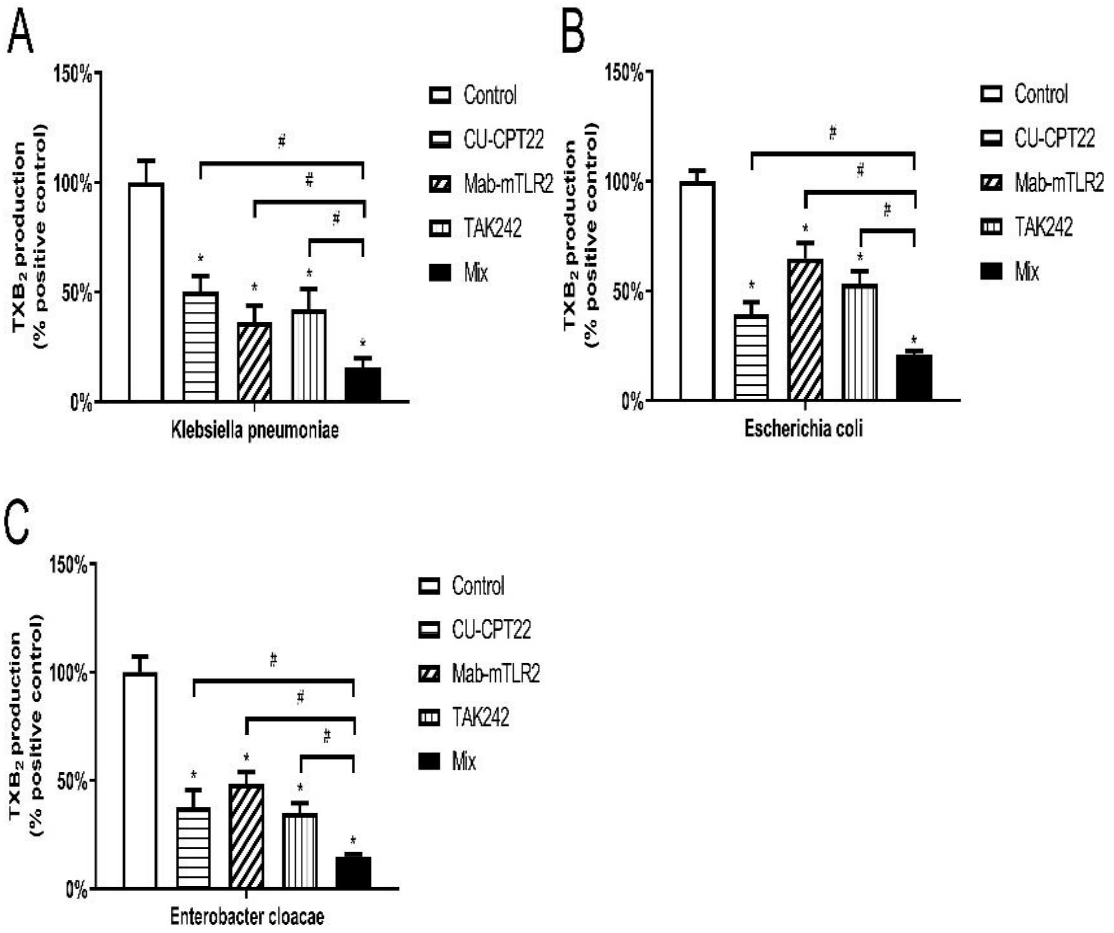

Fig.3-15 Combined use of TLR-1, -2 and -4 antagonists achieved a better effect on decreasing Gram-negative bacterial induced TXB_2 increase

1 h pretreatment in human KCs with CU-CPT22 (10μM), Mab-mTLR2 (1μg/ml), TAK242 (1μM) or mix drugs (combined use of these three substances) reduced TXB_2 increase following Gram-negative bacterial stimulations (A-*K. pneumoniae*, B-*E. coli* and C-*E. cloacae*, 8μg/ml 24 h stimulation). Effects of the combination were better than individual TLR-1, -2 or -4 antagonists. Data are expressed as mean ± SD, the control group used as a positive control. Two-tailed t-test, *$p < 0.05$ comparing with positive control, #$p < 0.05$ comparing with mix group, n=6 in each group.

4. Discussion

This study investigated the function of TLRs in bacterial-induced TXA_2 increase in nonparenchymal liver cells. Novel findings in our research were the following: (1) Gram-positive, Gram-negative and fungal microbial isolates extracted from SBP patients increased TXB_2 secretion in HKCs, MKCs and TMCs; (2) SECs and HSCs did not secrete TXB_2 by TLR activation, while KCs did. Only TLR-1/2, -2 and -4 agonists increased TXB_2 secretion in HKCs and MKCs among all TLR agonists; and (3) TLR-2 antagonist showed the most effective effect in reducing bacterial-induced TXA_2 secretion in comparison with TLR-1 and -4 antagonists. Combination of these 3 agents even attained a better effect. These results were achieved by using primary KCs from human and mouse, primary SECs and HSCs from mice and THP-1 cell line.

4.1 Responses to microbial isolates in different kinds of macrophages

The initial set of experiments showed the effective microbial isolates and TLR agonists increasing TXB_2 secretion after 24 h stimulation. 7 most common isolates extracted from SBP patients all increased TXB_2 secretion in 3 kinds of macrophages. Macrophages derived from humans (HKCs and TMCs) seemed to be more sensitive to Gram (-) bacterial isolates while Gram (+) bacterial isolates induced more TXB_2 in MKCs (Fig. 3-4, 3-5, 3-6). This discrepancy may implied the difference between human and mouse in immune function about bacterial defense. Previous

publications demonstrated differences between human and mouse immunology: the TLR-2 expression on mouse peripheral blood leukocytes was much lower than on human [78, 79]; macrophages from mice lacked CD4 while CD4 exited on human macrophages [80] ; expression of the IgG Fc receptor (FcγR) system also differed between mouse and human [81, 82]. However, researches spurred in differences between mouse and human on functions of TLRs in present study are still young, our results suggest more attention in this field in further investigations.

4.2 Responses to TLR agonists in different kinds of macrophages

Special TLR-1 to -9 agonists were then tested in different kinds of macrophages to find the responsible TLRs increasing TXB_2. Only Pam3CSK4, HKLM and LPS had significant effects in both HKCs and MKCs, Pam3CSK4 and HKLM also worked on TMCs (Fig. 3-7, 3-8, 3-9). These common effects implied TLR-1, -2 and -4 were most possibly related to the bacterial-induced TXB_2 increase.

Previous publications found the expression of TLR-4 on undifferentiated THP-1 cells was lower than primary human monocytes and differentiated THP-1 cells were even unable to see reliable TLR-4 expression on the surface [83]. Our results also confirmed that conclusion since only TMCs failed to increase TXB_2 by LPS stimulation. Both Poly(I:C)-LMW and ODN2006 had significant effects on TMCs, however, ODN2006 caused the most significant TXB_2 secretion while Poly(I:C)-LMW induced the least among all effective TLR agonists (Fig. 3-9). It has reported

earlier that Poly(I:C) is a potent adjuvant, acting through the TLR-9/MyD88-dependent pathway [84, 85]. We doubted that the TXB_2 increase caused by TLR-3 agonist was due to its adjuvant effect with TLR-9 pathway, and the effect may only exited on Poly(I:C)-LMW. In humans, TLR-9 is expressed primarily by plasmacytoid dendritic cells and B cells rather than macrophages [86, 87]. THP-1 cells can be differentiated to both dendritic cells and macrophages, which may be the reason why TLR-9 agonist only worked on TMCs.

TLR-2 has a unique ability to heterodimerize with TLR-1 and -6. In unstimulated KCs, the mRNA expression for TLR-1, TLR-2, and TLR-6 was approximately 10:40:1 [88, 89]. That may explain why both TLR-1/2 and TLR-2 agonists increased TXB_2 secretion while TLR-6/2 agonist failed. Another possible explanation may be that FSL-1 is a kind of pure diacylated lipoproteins needing the direct association of TLR-6/2 and CD36, whereas, TLR-1/2 heterodimers are formed in response to Pam3CSK4 (one kind of triacylated lipoproteins) in absence of CD36 [34]. CD36 is expressed on KCs but the basal expression is low [90, 91], we therefore suspected the process by which KCs recognize dimeric antigens requires the assistance of other cells containing CD36.

Numerous antagonists targeting TLRs including antibodies, oligonucleotides and small molecular inhibitors are currently under preclinical studies, clinical trials, in vitro and in vivo experiments [45, 47, 92]. The discrepancy of TLR function among

HKCs, MKCs and TMCs may also lay basis for translating results from animal experiments and cell lines to the human situation in future investigations.

4.3 Responses to TLR agonists in different nonparenchymal liver cells

Prostaglandin (PG) and TXA_2 produced by NPCs in liver played important roles in increasing portal pressure after TLR activation [14, 32, 93, 94]. Yet, only a few publications reported the functions of TLRs in different NPCs. Our study was the first to investigate the differences among mouse KCs, SECs and HSCs after TLR agonist stimulation. TLR agonists induced no significant TXB_2 increase in SECs despite their 70% amount in NPCs. This result was in line with previous publication that anaphylatoxin C5a significantly increased the release of TXA_2 and PG in both KCs and HSCs, but not in SECs [95-97]. Compared with SECs and HSCs, KCs possessed the largest amount of Gs-linked prostaglandin E2 receptor (GPER). However, the functional evidence for this receptor has not proved so far [93]. We speculated that the strong expression of GPER in KCs may be responsible for the increased TXB_2 after stimulation of TLR agonists.

HSC can present bacterial antigens to natural killer T cells and suppress proliferation of T cells [98-100]. Activated HSCs can also simultaneously inhibit its activation through a negative feedback loop via IL-10 [101, 102]. Thus, HSCs could limit bacterial infection and are critical in liver's immune tolerance. These immunosuppressive characteristics may also explain why TLR agonists decreased

TXB_2 secretion in HSCs. Myofibroblast-like cells are usually transdifferentiated from activated HSCs, which are often triggered by infections or hepatotoxic drugs. Transdifferentiation accompanied by a change in receptor expression such as G-protein coupled receptors for PGF2α and TXA_2 [103, 104]. This apparent discrepancy is also related to vitamin A droplets loss and augmented contractile activity [105-107]. Most TLR 1-9 agonists are products or components of bacteria, and may differentiate HSCs with an apparent and functional change. This differentiaction may also explain the oppsite effects of TLR agonists and anaphylatoxin C5a in HSCs.

4.4 Clinical applications

TLR-1, -2 or -4 antagonists were additionally added in HKCs. The number of effective antagonists was consistent with the levels of TXB_2 secretion caused by different microbial isolates: Gram (-) bacteria induced the most relevant TXB_2 secretion in HKCs, the increase was reduced by all 3 kinds of antagonists; TLR-1 and -2 antagonist attenuated TXB_2 secretion following Gram (+) bacterial stimulations; in comparison, only Mab-mTLR2 reduced *C.albicans*-induced TXB_2 increase (Fig. 3-14). Mab-MTLR2 had significant effects on all 7 kinds of microbial isolates, supporting TLR-2 may recognize the most diverse set of PAMPs including lipoarabinomanan, peptidoglycan, lipoteichoic acid from Gram (+) and Gram (-) bacteria [108-110] as well as fungi [111, 112]. TLR-2 expression in monocytes were

significantly overexpressed in patients with tuberculosis [44], systemic lupus erythematosus [45] and myelodysplastic syndromes [46]. TLR-2 polymorphisms were also related to increased susceptibility toward SBP in cirrhotic patients with ascites [24]. Despite a rare condition, positive fungal cultures were found to be associated with a significantly higher mortality than patients with SBP alone [16, 17, 113]. Lack of specific indicators and low culture success rate often lead to delayed diagnosis and treatment of concomitant fungal infection in SBP patients. The effects of TLR-2 antagonist in our experiments suggested TLR-2 as a potential marker for SBP patients accompanied with fungal infections.

Resistant organisms are becoming an emerging problem due to the wide-spread use of oral antibiotics in SBP patients [18, 24, 114]. 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. A single infusion of OPN-305 provided 80% inhibition of interleukin 6 release for at least 14 days, with no significant adverse findings. This study proved the safety and tolerability of OPN-305, providing further evidence of application of TLR2 antagonists in infections [115]. Sepsis associated death can be decreased by anti-TLR-2 or anti-TLR-4 antibody alone [116]. The synergistic effect of TLR-1, -2 and -4 antagonists in our experiments (Fig. 3-15) also support the combined application in patients with complicate and severe infections.

4.5 Conclusions

These are the first experiments to treat human primary KCs with different, clinically relevant microbial isolates. TLR-1, -2 and -4 play vital roles in bacterial-induced TXB_2 secretion. TXA_2 has been shown to be an important intrahepatic vasoconstrictor which increases the portal pressure and participate in the progression of ACLF. In the present study we identified TLR-2 as novel and important target in the pathway of TXB_2 production by human Kupffer cells. Different microbial isolates induced KCs to secrete TXA_2 and inflammatory factors using the same route: activation of MAPK-associated pathway by TLR-2 leading to factor secretion. Therefore, inhibition of TLR-2 might be a potential marker and an attractive treatment option for liver cirrhosis and infections. Hereby the dynamic and in particular TXB_2-dependent component of portal pressure increase might be attenuated with great benefit for these patients.

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