Gene Profiling of Toll-like Receptor Signaling Pathways in Neutrophils of Patients With Acute-on-chronic Liver Failure

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Research Article

Keywords: Toll-like receptors, mRNA, pathogens and organ/tissue injury, acute-on-chronic liver failure, neutrophil

Posted Date: June 9th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-541965/v1>

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Abstract

Objectives: Toll-like receptors (TLRs) of neutrophils play a crucial role in detecting pathogens and organ/tissue injury in acute-on-chronic liver failure (ACLF). However, little is known about the exact mechanisms and the potential signaling pathways. The aim of this study was to investigate alterations of TLR signaling pathways in neutrophils of ACLF patients.

Methods: Twenty-seven patients with compensated cirrhosis (n=9), de-compensated cirrhosis (n=9) and ACLF (n=9) were enrolled in the study. Neutrophils were isolated, and alterations in TLR signaling pathways were evaluated using an RT² Profiler™ PCR Array. The fold change for each gene ($2^{(-\Delta\Delta CT)}$) was compared among the groups. Genes with a fold change ratio of ≥ 2 or ≤ 0.5 along with a *p* value of < 0.05 were considered to be differentially expressed.

Results: A total of 17 genes were up-regulated in neutrophils from patients with compensated cirrhosis, which were mainly distributed in adaptors, TLR-interacting proteins and downstream pathways. Six genes were detected in patients with de-compensated cirrhosis. A trend of down-regulation of genes in the TLR signaling pathway was observed in neutrophils of patients with cirrhosis and ACLF. *TLR3*, *IFNG*, *IL1B*, *TBK1*, *CCL2* and *LTA* were downregulated in neutrophils. Moreover, CD14 and IL10 were up regulated in neutrophils of ACLF patients.

Conclusions: TLR signaling pathway genes were differentially regulated in neutrophils between cirrhosis and ACLF. In ACLF patients, there was defective expression of TLR3 and IFN, along with enhanced CD14 and IL10 expression, characterized by transcriptional alterations of neutrophils.

Introduction

Acute-on-chronic liver failure (ACLF) is a new clinical entity defined as acute exacerbation of underlying chronic liver disease, which usually triggers multiple organ failure and a high short-term mortality (Hernaez, Sola, Moreau, & Gines, 2017). As is known to all, the key driver of ACLF is the burst of systemic inflammation caused by bacterial infections, acute liver injury, variceal bleeding, or other acute injury (Bernardi, Moreau, Angeli, Schnabl, & Arroyo, 2015). To be specific, the severity, duration and type of systemic inflammation are reported to substantially affect the outcome of ACLF (Claria, Arroyo, & Moreau, 2016; Claria, Stauber, et al., 2016). To our best knowledge, function of neutrophils is critical in determining the nature of systemic inflammation (Wu et al., 2018). On this basis, it is reasonable to assume that functional modulation on the innate immune system may serve as a potential treatment option for ACLF.

Toll-like receptors (TLRs), probably the most important class of pattern-recognition receptors, are crucial in the regulation of innate immune system (Kawai & Akira, 2010). They were first identified as key receptors in the recognition of pathogen-associated molecular patterns (PAMPs). Currently, 11 types of TLRs have been identified in human beings, among which 10 are functional (Kawai & Akira, 2010). For example, in combination with lipopolysaccharide-binding protein and CD14, TLR4 on the surface of

innate immune cells can recognize lipopolysaccharide (LPS) that was a component presented in most gram-negative bacteria (Poltorak et al., 1998). In addition, TLR3 located on endosomes could recognize the retroviral double-stranded RNA (Alexopoulou, Holt, Medzhitov, & Flavell, 2001). Recently, TLRs have been shown to involve in sterile inflammation caused by tissue damages (Zitvogel, Kepp, & Kroemer, 2010). TLRs were capable of recognizing damage-associated pattern molecules (DAMPs), which subsequently activated the innate immune cells (Zitvogel et al., 2010). For instance, cellular HMGB1, a chromatin protein, can be recognized by TLR4 on neutrophils, leading to formation of neutrophil extracellular traps (NET) (Huang et al., 2015).

PAMPs and DAMPs had been detected in the serum or plasma samples of ACLF patients (Du et al., 2017; Michelena et al., 2015). Increased TLR expression and altered neutrophil responses were reported in ACLF patients (Bernsmeier, van der Merwe, & Périainin, 2020; Wasmuth et al., 2005). However, there is still a lack of a comprehensive understanding on TLR signaling cascades. In this study, RNA qRT-PCR array technology was utilized to evaluate the relevant expression of TLR-regulated genes in neutrophils from ACLF patients. We aimed to identify the key TLR pathways that may serve as therapeutic targets for treating ACLF.

Materials And Methods

Patients

A total of 27 patients with compensated cirrhosis (n = 9), stable cirrhosis (n = 9) and ACLF (n = 9) admitted to the First Affiliated Hospital of Zhejiang University were enrolled in this study. Meanwhile, 9 matched healthy volunteers received physical examination at the same period served as healthy control (HC). Those diagnosed with ACLF based on the EASL-ACLF criteria (Moreau et al., 2013) were included in this study. Patients with the following conditions were excluded from the study: those with human immunodeficiency virus infection; confirmed or suspected with malignancies; pregnant women; received organ transplantation previously; received administration of immunosuppressive drugs; or those with active bacterial infection. Written consent was obtained from each patient or their legal relations. The study protocols were approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University and complied with the Helsinki Declaration.

Isolation of neutrophils

Peripheral venous blood samples were collected within 24 h upon admission. Neutrophils were isolated from whole blood samples using a Polymorph prep kit (Axis-Shield PoC AS, Norway) according to the manufacturer's instructions. Cells were re-suspended in 1 ml TRIzol reagent (Invitrogen, CA, USA), followed by storing in liquid nitrogen.

RNA extraction, cDNA and PCR array

A RT² Profiler™ PCR Array for Human TLR Signaling Pathway (PAHS-018Z, QIAGEN) was used in this section. RNA extraction, cDNA synthesis and PCR array preparation were performed according to the manufacturer's instructions. Briefly, the experimental RNA samples were converted into first-strand cDNA using a RT² first-strand kit (QIAGEN). Afterwards, the cDNA was mixed with an appropriate RT² SYBR Green Mastermix (QIAGEN). The mixture was aliquoted into the wells of a RT² Profiler PCR Array. The amplification results were expressed as Ct and normalized with the housekeeping gene 18SrRNA (ΔCT). The fold change of gene expression was calculated as $2^{(-\Delta\Delta\text{CT})}$.

TLR signaling pathway mapping

Kyoto Encyclopedia of Genes and Genomes (KEGG) Mapper was used to analyze differentially expressed genes¹⁶. The ratio mean of gene expression was calculated in control and ACLF groups, which was visualized in color code in the TLR signaling pathway (KEGG hsa04620).

Statistical analysis

All data were analyzed using SPSS 16.0 software (SPSS, Chicago, IL). Student's t-test, Mann-Whitney U-test or Chi square test was utilized for the data comparison among groups. Two-sided P value of < 0.05 was considered to be statistically significant.

Results

Patient' characteristics

As shown in Table 1, age and sex were matched among the HC, compensated cirrhosis and stable cirrhosis groups, however, they were not matched among the ACLF patients. As expected, compared to other groups, ACLF patients had a severe hepatic dysfunction, jaundice and coagulopathy, as evidenced by laboratory measures and scores of disease severity.

Table 1
Demographic and clinical characteristics of study participants

Variables	Health controls (n = 9)	Compensated cirrhosis (n = 9)	De-compensated cirrhosis (n = 9)	ACLF (n = 9)
Male/Female	6/3	5/4	6/3	6/3
Age	53.4 ± 3.7	54.4 ± 6.7	58.3 ± 12.4	47.3 ± 10.9
Etiologies of cirrhosis				
HBV	0	7	6	8
Non-HBV	9	2	3	1
De-compensation events				
Ascites	-	0	6	2
UGH	-	0	3	1
HE	-	0	0	0
Jaundice	-	0	0	9
Organ failure	-			
Liver failure	-	0	0	9
Coagulation failure	-	0	0	6
Kidney failure	-	0	0	0
Cerebral failure	-	0	0	0
Circulation failure	-	0	0	0
Lung failure	-	0	0	0
WBC (10 ⁹ /L)	5.4 (3.5)	4.2 (2.8)	2.1 (2.8)	7.4 (7.1)
PLT (10 ⁹ /L)	123 (24)	89 (111)	60.5 (57)	109 (58)
TB (umol/L)	6 (3.4)	14 (15.5)	19.5 (19.5)	368 (135.3)
INR	1 (0.1)	1.1 (0.1)	1.4 (0.5)	2.6 (0.9)

HBV: hepatitis B virus; UGH: upper gastrointestinal hemorrhage; HE: hepatic encephalopathy; Jaundice: TBil ≥ 85umol/L; WBC: white blood cell; PLT: platelet; TB: total bilirubin; INR: international normalized ratio; MELD: model for the end-stage liver disease; CLIF-C ACLFs: Chronic liver failure Consortium Acute-on-chronic liver failure scores.

Variables	Health controls (n = 9)	Compensated cirrhosis (n = 9)	De-compensated cirrhosis (n = 9)	ACLF (n = 9)
Creatinine(umol/l)	78 (22)	64 (20)	68 (12)	66 (30.8)
Albumin(g/L)	43 (5.7)	40.3 (11.2)	33 (9.8)	31.7 (5.6)
MELD score	-	5.3 (7.4)	8.5 (7)	25 (5)
CLIF-C ACLFs	-	-	-	41.5 (8.3)
HBV: hepatitis B virus; UGH: upper gastrointestinal hemorrhage; HE: hepatic encephalopathy; Jaundice: TBil $\geq$ 85umol/L; WBC: white blood cell; PLT: platelet; TB: total bilirubin; INR: international normalized ratio; MELD: model for the end-stage liver disease; CLIF-C ACLFs: Chronic liver failure Consortium Acute-on-chronic liver failure scores.				

PCR array in polymorphonuclear neutrophils (PMNs)

Genes of the TLR signaling pathway were differentially expressed in PMNs from compensated cirrhosis to ACLF patients compared to those of HC. Patients with compensated cirrhosis represented a predominant up-regulation of TLR pathway-related genes, including *NFKBIA*, *NFKB*, *NFRKB*, *NFKBIL1*, *MAP4K4*, *TNFRSF1A*, *IRF1* and *ELK1*, which were distributed in downstream pathways and target genes (i.e. *MAPK8IP3*, *TIRAP*, *CD14*, *TOLLIP* and *HSPA1A*) among adaptors and TLR-interacting protein genes, *SIGIRR* and *TLR9* among TLR genes, and *FADD* and *IRAK1* among the effector genes. Only *TLR3* and *PTGS2* were downregulated. Patients with de-compensated cirrhosis represented a more limited alteration in TLR signaling genes, including 6 of the 17 up-regulated genes observed in compensated cirrhosis, *SIGIRR*, *IRAK1*, *HSPA1A*, *TOLLIP* and *ELK1*, as well as down-regulation of *IL-1B*. In contrast, ACLF patients had a different type of alteration in TLR signaling genes, which was featured by down-regulation of *TLR3*, *CXCL8*, *IFNG*, *IL1B*, *TBK1*, *CCL2* and *LTA*. In addition to *FADD*, *IRAK1*, *CD14*, *HSPA1A*, *NFKBIL1* and *ELK1* were commonly up-regulated in cirrhosis, however, *IL-10* was uniquely up-regulated in ACLF (Table 2). The gene expression profile in different groups was listed in Fig. 1 and Supplementary Table 1. Individual gene expression profile was listed in Fig. 2.

Table 2

List of differentially expressed genes of TLR signaling pathways between patients and health controls

Gene symbol	Gene	Neutrophils		
		C-LC	D-LC	ACLF
		Fold change	Fold change	Fold change
		P value	P value	P value
Toll like receptor	<i>TLR10</i>	-	-	-
	<i>TLR3</i>	0.4231	-	0.2398
		0.0478		0.0016
	<i>TLR9</i>	4.0564	2.5115	2.6805
		0.0009	0.03556	0.023
	<i>SIGIRR</i>	2.2314	2.1148	-
		0.0105	0.0447	
Effectors	<i>FADD</i>	2.022	-	2.2185
		0.0302		0.0133
	<i>IRAK1</i>	5.4967	3.3386	3.4884
		0.0003	0.0193	0.0089
	<i>IRAK2</i>	-	-	-
Interacting proteins & adaptors	<i>CD14</i>	2.1815	-	2.3584
		0.0116		0.0033
	<i>HSPA1A</i>	4.0346	2.3591	3.7515
		0.0017	0.0183	0.0039
	<i>MAPK8IP3</i>	2.0727	-	-
		0.0129		
	<i>TOLLIP</i>	2.4367	2.111	-
		0.0113	0.0039	
	<i>TIRAP</i>	2.4229	-	-
		0.0012		

C-LC: compensated liver cirrhosis; D-LC: de-compensated liver cirrhosis; ACLF: acute-on-chronic liver failure.

Gene symbol	Gene	Neutrophils		
		C-LC	D-LC	ACLF
		Fold change P value	Fold change P value	Fold change P value
Regulation of adaptive immunity	<i>IFNG</i>	-	-	0.173 0.0006
	<i>IL10</i>	-	-	2.6697 0.0249
	<i>IL12A</i>	-	-	-
	<i>IL1B</i>	-	0.4534 0.0017	0.4067 0.0025
	<i>IL2</i>	-	-	-
<i>Downstream pathway of toll like receptors</i>				
NFKB signaling	<i>FADD</i>	2.022 0.0302	-	2.2185 0.0133
	<i>IL10</i>	-	-	2.6697 0.0249
	<i>IL1B</i>	-	0.4534 0.0017	0.4067 0.0025
	<i>IRAK1</i>	5.4967 0.0003	3.3386 0.0193	3.4884 0.0089
	<i>IRAK2</i>	-	-	-
	<i>MAP4K4</i>	2.0512 0.0018	-	-
	<i>NFKB2</i>	2.2645 0.0060	-	-
	<i>NFKBIA</i>	2.0441 0.0170	-	-
C-LC: compensated liver cirrhosis; D-LC: de-compensated liver cirrhosis; ACLF: acute-on-chronic liver failure.				

Gene symbol	Gene	Neutrophils		
		C-LC	D-LC	ACLF
		Fold change	Fold change	Fold change
		P value	P value	P value
	<i>NFRKB</i>	2.056 0.0292	-	-
	<i>TNFRSF1A</i>	2.5753 0.0015	-	-
	<i>CXCL8</i>	-	-	0.0959 0.0422
	<i>LTA</i>	-	-	0.489 0.0157
JNK/p38 signaling	<i>ELK1</i>	2.9532 0.0012	2.2857 0.0128	2.4822 0.0054
	<i>IL1B</i>	-	0.4534 0.0017	0.4067 0.0025
	<i>MAPK8IP3</i>	2.0727 0.0129	-	-
JAK/STAT signaling	<i>CCL2</i>	-	-	-
	<i>IFNG</i>	-	-	0.173 0.0006
	<i>IL2</i>	-	-	-
Interferon regulatory factor signaling	<i>CXCL10</i>	-	-	-
	<i>IFNB1</i>	-	-	-
	<i>IFNG</i>	-	-	0.173 0.0006
	<i>IRF1</i>	2.7619 0.0012	-	-
C-LC: compensated liver cirrhosis; D-LC: de-compensated liver cirrhosis; ACLF: acute-on-chronic liver failure.				

Gene symbol	Gene	Neutrophils		
		C-LC	D-LC	ACLF
		Fold change	Fold change	Fold change
		P value	P value	P value
	<i>TBK1</i>	-	-	0.4399 0.0013
Cytokine signaling	<i>CCL2</i>	-	-	-
	<i>IL1B</i>	-	0.4534 0.0016	0.4067 0.0025
	<i>IRAK1</i>	5.4967 0.0003	3.3386 0.0193	3.4884 0.0089
	<i>IRAK2</i>	-	-	-
	<i>SIGIRR</i>	2.2314 0.0105	2.1148 0.0045	-
	<i>TNFRSF1A</i>	2.5753 0.0015	-	-
NF/IL6 pathway	<i>PTGS2</i>	0.4895 0.0038	-	0.1811 0.0002
C-LC: compensated liver cirrhosis; D-LC: de-compensated liver cirrhosis; ACLF: acute-on-chronic liver failure.				

TLR signaling pathway mapping

In this section, we explored the specific pathway of TLR involved in the pathogenesis of ACLF. KEGG TLR signaling network (KEGG hsa04620) was utilized to contrast the expression levels of HC and ACLF groups (Fig. 3). The TLR signaling pathway mapping showed that TLR3 pathway was significantly downregulated in neutrophils of ACLF patients.

Discussion

TLRs are trans-membrane proteins expressed in innate immune cells especially the neutrophils, which play a central role in detecting invading microbes or endogenous danger signals and initiating corresponding immune responses (Kawai & Akira, 2010). Ten TLRs (i.e. TLR1-TLR10) have been

identified in humans, while different TLRs could recognize various ligands, including bacterial cell wall components and viral double-stranded RNA(Xing, Li, Cao, & Huang, 2007). There were five housekeeping genes and 84 genes related to TLR-mediated signal transduction. These genes were divided into different groups according to their functions: TLR, adaptor and interacting proteins, effectors, regulation of the adaptive immunity group, downstream pathways and target genes. In this study, we found a stepwise down-regulation of TLR3 in neutrophils from patients with cirrhosis to those with ACLF. These results were consistent with a recent study showing defective TLR3 expression in ACLF patients(Bernsmeier et al., 2017). As previously described, TLR3 could recognize retroviral dsRNA, which then triggered the activation of transcription factor interferon regulatory factor-3 (IRF3) in a MyD88-independent manner. This allowed the induction of IFNs (Doyle et al., 2002). Thus, defective TLR3 signaling may lead to down-regulation of type I and II interferons, interferon regulatory factor TBK1 and interferon-stimulated gene (ISG) *CXCL 10* expression in immune cells of ACLF patients. Although TLR4 engagement also induced IFNs in immune cells (Fairfax et al., 2014), TLR4 signaling was not suppressed in ACLF patients. Therefore, defective TLR3 signaling may be a major contributor to the downregulation of interferons and their ISGs in ACLF patients. Along with the IFNs, genes regulating adaptive immunity, including IL-2 and IL-12A, were significantly down-regulated. It had been shown that enhanced activity of type I IFN signaling pathways was associated with excessive inflammation and tissue damages in mouse models of endotoxin shock (Karaghiosoff et al., 2003) and autoimmune diseases (Ronnblom, 2016). Thus, down-regulation of type I interferon regulatory signaling may be protective against excessive inflammation in ACLF patients. In parallel, the anti-inflammatory cytokine IL-10 was significantly up-regulated in neutrophils, and pro-inflammatory IL-1B was down-regulated specifically in neutrophils of patients with ACLF. Taken together, these findings suggested that peripheral neutrophils in ACLF patients showed defective expression of TLR3 and IFN, together with up-regulation of the anti-inflammatory cytokine IL-10 at the transcriptional level.

The TLR signaling transcriptional landscape of immune cells in ACLF was almost completely distinct from that in cirrhosis patients, which supported the clinical and experimental data that ACLF patients showed different immune dysfunctions (Albillos, Lario, & Alvarez-Mon, 2014). The transcriptional alterations in immune cells may have significant clinical implications. The prevailing theory hypothesized a shift from systemic inflammatory response syndrome (SIRS) to compensatory anti-inflammatory response syndrome (CARS) during the natural history of ACLF, which led to immune paralysis and increased susceptibility to infections (Jalan et al., 2012). Indeed, several clinical studies had confirmed that ACLF patients showed a higher incidence of bacterial infection than the non-ACLF counterparts (Fernandez et al., 2018; Shi et al., 2015). In human and animal studies, there was a link between impaired cellular immune function and an increased risk of bacterial infection, loss of infection control and infection-associated mortality (Bernsmeier et al., 2017; Hackstein et al., 2017; Vergis et al., 2017). Our findings revealed that a reduction in TLR signaling may impair TLR-driven neutrophils responses, which therefore represented a potential therapeutic target. A recent study demonstrated that the TLR-3 agonist poly(I:C) effectively reconstituted innate immune function in ACLF, which may serve as a promising TLR-based immunotherapy for ACLF (Bernsmeier et al., 2017).

In fact, our study has some limitations. First, further validation was required for the PCR array results. Second, the expression of genes could not represent the entire biological response. Thus, *in vitro* and animal studies are required to investigate the feasibility of crucial roles in the TLR3 signaling pathway that may serve as a target for the treatment of ACLF.

In summary, genes in the TLR signaling pathway were differentially expressed in neutrophils through different stages of cirrhosis. In addition, we identified a dysregulated key TLR pathway in the neutrophils of ACLF patients. In future, further investigation is required to test its potential as a therapeutic target.

Abbreviations

TLR: Toll-like receptor; ACLF: acute-on-chronic liver failure; PMN: polymorphonuclear neutrophil; DAMPs: damage-associated pattern molecules; HC: healthy control; C-LC: compensated liver cirrhosis; D-LC: de-compensated liver cirrhosis.

Declarations

Acknowledgement

Thanks to Dr. Lingtong Huang and Dr. Chunyun Ren for their help in data analysis.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

ZY wrote the manuscript; WW and WYJ revised the manuscript; XQ and TLJ did the data analysis; CZ and HM did the data collection. All authors reviewed the manuscript.

Funding

This research was funded by National Natural Science Foundation of China (grant number: 81671949).

Ethical approval

This study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of each participating hospital.

Informed consent

Informed consent was obtained from each patient included in the study

Data Availability Statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Supplementary Table

Supplementary Table 1 is not available with this version.

Figures

Figure 2

Individual TLR signaling pathway-related gene expression of PMNs among HC, compensated cirrhosis, de-compensated cirrhosis and ACLF groups. The relative expression level was indicated by color code. Genes with significant up-regulation were colored in red and those with significant down-regulation were marked in green. The ubiquitous expression level was shown in black.