Controversial role of toll-like receptors in acute pancreatitis

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Abstract

Acute pancreatitis (AP) is a common clinical condition with an incidence of about 300 or more patients per million annually. About 10%-15% of patients will develop severe acute pancreatitis (SAP) and of those, 10%-30% may die due to SAP-associated complications. Despite the improvements done in the diagnosis and management of AP, the mortality rate has not significantly declined during the last decades. Toll-like receptors (TLRs) are pattern-recognition receptors that seem to play a major role in the development of numerous diseases, which make these molecules attractive as potential therapeutic targets. TLRs are involved in the development of the systemic inflammatory response syndrome, a potentially lethal complication in SAP. In the present review, we explore the current knowledge about the role of different TLRs that have been described associated with AP. The main candidate for targeting seems to be TLR4, which recognizes numerous damage-associated molecular patterns related to AP. TLR2 has also been linked with AP, but there are only limited studies that exclusively studied its role in AP. There is also data suggesting that TLR9 may play a role in AP.

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INTRODUCTION

A central characteristic of the innate immune system is its capability to identify constitutive and conserved products of microbial metabolism. Several metabolic pathways are unique for invading microorganisms and absent in host cells. Being essential for survival, they are even highly conserved among a given class of microorganisms. A classical example is lipopolysaccharide (LPS), a molecule made by *Gram-negative bacteria*, but not by eukaryotic cells.

Molecules found in microorganisms, but not in host cells, can serve as molecular signatures that can be recognised by the innate immune system, starting an immunological response against the invasion and if necessary, assisting in the activation of the adaptive immune system[1]. As these molecules are conserved molecular patterns, they are called pathogen-associated molecular patterns (PAMPs) and interact with pattern-recognition receptors (PRRs), which are receptors of the innate immune system.

PAMPs are marvellous targets for innate immune recognition, as they are only produced by microbes, are invariant between microorganisms of a given class and are essential for microbial survival. Therefore, PRRs representes a vital component of the immune system, which probably developed early during evolution, as PRRs are found in all mammals, invertebrates and even in plants. PRRs are expressed on the cell surface or in intracellular

compartments, but they can be secreted into the blood stream and tissue fluids as well[2]. However, the role of PRRs in immune recognition is not solely associated to PAMPs, since PRRs in addition can recognize alarmins[3]. Alarmins are endogenous molecules released into the extracellular compartment by activated or necrotic cells in response to stress or tissue damage[4]. Even extracellular matrix molecules are alarmins when up-regulated upon injury or degraded following tissue damage[5]. Alarmins and PAMPs constitute damage-associated molecular patterns (DAMPs), which are the main targets of PRRs[3,4].

The relative recent discovery of a main PRR family, toll-like receptors (TLRs), has vastly increased our comprehension of the physiological and pathophysiological role of the innate immune system. While mice have twelve TLRs (TLR1 to TLR9 and TLR11 to TLR13), humans express only ten functional TLRs (TLR1 to TLR10) and ligands have been identified for all human TLRs, except for TLR10[3]. A selection of PAMPs and alarmins recognized by human TLRs are shown in Tables 1 and 2.

TLRs are type I transmembrane glycoproteins composed of an extracellular N-terminal that contains leucine-rich repeats, a single transmembrane domain and an intracellular C-terminal tail known as the Toll/IL-1 receptor (TIR)[6]. When TLRs form heterodimers or homodimers, an activating signal is started and TIR-TIR dimers are involved in the recruitment of five known signalling adaptor molecules: Myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adaptor protein (TIRAP), TIRAP inducing interferon β (TRIF), TRIF-related adaptor molecule (TRAM) and sterile-alpha and Armadillo containing motif protein[7-9].

Once the adaptor molecules are recruited, TLRs can activate two major intracellular signalling pathways. All TLRs except TLR3 can activate a MyD88-dependent pathway, in which IL-1R-associated kinases (IRAK), TNF receptor-associated factor 6 (TRAF-6) and mitogen-activated kinases are involved[5]. This pathway results in the transcription of pro-inflammatory genes through the activation of nuclear factor κβ (NFκB) and/or the activation of activating protein 1[9]. An alternative, non MyD88-dependent pathway can be activated by TLR3 and TLR4. In the TRIF pathway, the activation of interferon-regulated factors (IRF) *via* TRIF leads to the synthesis of interferon (IFN)[5].

As TLRs recognize several PAMPs and DAMPs, their involvement in the pathophysiology of several diseases has become a major research field[8,10]. Moreover, recent studies have reported the importance of NFκB pathways in the development of the systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS)[11,12]. However, NFκB pathways are not restricted to TLRs, which converts the elucidation of the role of TLRs in SIRS and MODS into a tremendous challenging task.

SIRS is a nonspecific condition that can be caused by infection, ischemia, trauma, and inflammation, or by the combination of several insults. Considered by many

Table 1 Human Toll-like receptors: Localization, known pathogen-associated molecular patterns and their producing microorganisms

	Localization	PAMP	Origin of PAMP
TLR1	Plasma membrane	Soluble factors	*N. meningitidis*
		Triacyl lipopeptides	*Bacteria, mycobacteria*
TLR2	Plasma membrane	Glycoinositolphospholipids	*Trypanosoma cruzi*
		Glycolipids	*Treponema maltophilum*
		Haemagglutinin	*Virus*
		Lipoarabinomannan	*Mycobacteria*
		Lipoprotein/lipopeptides	*Various pathogens*
		Lipoteichoic acid	*Gram-positive bacteria*
		Peptidoglycan	*Gram-positive bacteria*
		Phenol-soluble modulin	*S. epidermidis*
		Porins	*Neisseria*
		Zymosan	*Fungi*
TLR3	Endosome	Double-stranded RNA	*Virus*
TLR4	Plasma membrane	Envelope protein	*Mouse-mammary tumour virus*
		Fusion protein	*Respiratory syncytial virus*
		Heat-shock protein 60	*Chlamydia pneumoniae*
		Lipopolysaccharide	*Gram-negative bacteria*
		Taxol	*Plants*
TLR5	Plasma membrane	Flagellin	*Bacteria*
TLR6	Plasma membrane	Diacyl lipopeptides	*Mycoplasma*
		Lipoteichoic acid	*Gram-positive bacteria*
		Zymosan	*Fungi*
TLR7	Endosome	Single-stranded RNA	*Virus*
TLR8	Endosome	Single-stranded RNA	*Virus*
TLR9	Endosome	DNA (CpG)	*Bacteria, virus*
		Haemozoin	*Plasmodium spp.*
			Rhodnius spp.
			Schistosoma spp.
TLR10	Endosome	Not determined	Not determined

Modified after Akira *et al*[7]. TLR: Toll-like receptor; PAMP: Pathogen-associated molecular patterns.

as a self-defense mechanism, SIRS results in a complex inflammatory cascade that involve humoral and cellular responses, complement, and cytokines cascades. Severe complications depend on the underlying etiology and the magnitude of the inflammatory response, and may include *e.g.*, single or multiple organ failure[13].

Acute pancreatitis (AP) is one common cause of SIRS and MODS. AP occurs with an incidence of about 300 or more patients per million annually[14,15], reported to have increased during the last decades[16,17]. Since the principal risk factors for AP are gallstones and excessive alcohol intake[18,19], plausible the augmented incidence reflecting the overweight epidemic observed in the Western World[20-22].

Most cases of AP are mild and self-limiting, with only brief need of clinical support[14,15]. However, about 10%-15% of patients will develop severe acute pancreatitis (SAP) and of those, 10%-30% may die due to SAP-associated complications (Table 3)[23]. Complications may be local or extrapancreatic and in up to one third, the pancreatic injury leads to pancreatic necrosis, acute fluid collections and pseudocyst formation. SIRS may develop early during the course of SAP and cause *e.g.*, adult respiratory dysfunction syndrome,

Table 2 Human toll-like receptors and a selection of known alarmins

	Proteins, peptides	Fatty acids, lipoproteins	Proteoglycans, glycosaminoglycans
TLR1	β-defensin-3		
TLR2	Antiphospholipid antibodies β-defensin-3	Serum amyloid A	Biglycan
	Eosinophil-derived neurotoxin		Hyaluronic acid fragments
	Heat-shock protein 60, 70, Gp96		Versiglycan
	High-mobility group protein B1 (HMGB1)		
	HMGB1-nucleosome complexes		
	Surfactant protein A, D		
TLR4	Antiphospholipid antibodies	Oxidised low-density protein	Biglycan
	β-defensin-2	Saturated fatty acids	Heparan sulphate fragment
	Fibrinogen	Serum amyloid A	Hyaluronic acid fragment
	Fibronectin (extra domain-A)		
	High-mobility group protein B1		
	Heat-shock protein 60, 70, 72, 22, Gp96		
	Lactoferrin MRP8, MRP14		
	Neutrophil elastase		
	Surfactant protein A, D		
	Tenascin-C (fibrinogen-like globe)		

Modified after Paccinini *et al*[5]. TLR: Toll-like receptor.

Table 3 Complications of severe acute pancreatitis

Pancreatic	Systemic
Abscess	Acute kidney failure
Fat necrosis	Acute liver failure
Hemorrhages	Adult respiratory distress syndrome
Infected necrosis	Disseminated intravascular coagulation
Pseudocyst formation	Encephalopathy
Sterile necrosis	Gut ischemia
	Hypocalcemia
	Paralytic ileus
	Shock

Modified after Baddeley *et al*[24].

acute renal and liver failure[24]. SIRS in association with SAP may also result in MODS, which carries a 40% mortality rate[15]. A model for the course of AP is shown (Figure 1).

Despite the improvements done in the diagnosis and management of SAP, the rate of mortality has only marginally declined during the last decades[24]. This is of particular concern, considering that AP patient numbers has been persistently increasing. In order to decrease the mortality rates, it is imperative to find new therapeutic strategies that target the underlying pathophysiological mechanism of SAP.

TLRs seem to play a major role in the development of numerous diseases[5,10], which make these molecules attractive as potential therapeutic targets. Since SIRS is a potentially lethal complication in AP, and TLRs are involved in the development of SIRS[25]; it is plausible that TLRs play a major role in severe acute pancreatitis.

In the present paper, we aim to explore the current knowledge about the role of different TLRs that have been reported associated with AP. In addition, future therapeutic strategies will be discussed.

TLR2-FAR AWAY FROM THE ANSWER

TLR2 is expressed on the plasma membrane of a large diversity of cells, including monocytes and macrophages, dendritic cells, polymorphonuclear leukocytes, B cells, T cells and microglia. This PRR recognises a wide range of DAMPs (Tables 1 and 2) and forms heterodimers with TLR1, TLR6 or TLR10[10]. Usually associated to the innate immune response against *Gram-positive bacteria* (several of its ligands origin in these microorganisms), TLR2 signals through a MyD88-dependent pathway (Figure 2A). CD14 is a protein found either in soluble form or anchored into the plasma membrane by a glycosylphosphatidylinositol tail. It has been reported that CD14 is required for the activation of TLR2-TLR6 dimers, since it facilitates the transport of specific ligands, such as peptidoglycan or lipoteichoic acid. Additionally, CD36, a membrane protein found in lipids rafts, seems to be important in the activation of TLR2-TLR6 pathways. However, it is unknown how CD36 enhances the formation of TL2-TLR6 dimers. CD44 is another membrane protein that has been associated to TLR2 and appears to enhance TLR2-mediated pro-inflammatory response[26]. However, the mechanisms are still unknown. There are also reports suggesting that CD44 may interact with TLR2, down-regulating TLR2-mediated inflammation[27,28].

Upon dimerization with TLR1, TLR6 or TLR10, TIR-TIR dimers recruit TIRAP, which is needed for the further recruitment of MyD88. MyD88 interacts with IRAK-4, which phosphorylates IRAK-1 and IRAK-2. IRAK-1 and IRAK-2 then activate TRAF-6, which in turn activates TGF-β activated kinase-1 (TAK-1). TAK-1 phosphorylates I-κ-β kinase β (IKKβ), which together with IKKα phosphorylate I-κ-β kinase α (IKKα). Upon phosphorylation IκBα becomes inactive, allowing the nuclear translocation of NFκB, with subsequent production of several cytokines and molecules involved in immune responses (Table 4)[29].

Besides its role in infectious diseases, TLR2 has also been reported involved in several non-infectious disorders, including atherosclerosis, asthma, renal disease, systemic lupus erythematosus and even sporadic colorectal cancer[30-34]. Though, the role of TLR2 in AP has only been evaluated in a limited number of studies.

Pancreatic damage
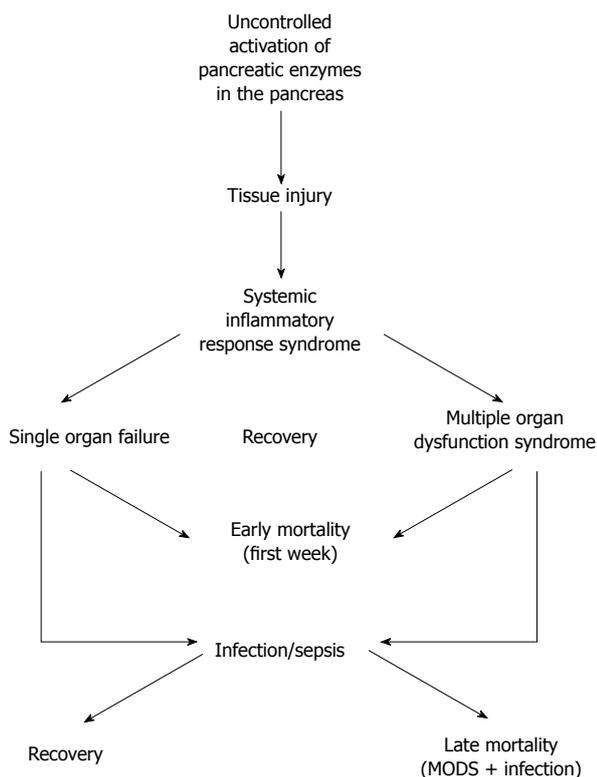The TLR2mRNA expression was found to be increased

Figure 1 Systemic inflammatory response syndrome in acute pancreatitis.

in the pancreas in cerulein-induced AP in rats[35]. Furthermore, TLR2 levels in the gland were also increased, as well as TNF-α, intracellular adhesion molecule 1 and IL-6. TLR2mRNA overexpression seemed to be caused by peroxisome proliferator-activated receptor-α (PPAR-α), since WY14643, a synthetic PPAR-α antagonist, decreased TLR2 levels in treated animals. Even if the observed TLR2mRNA overexpression was associated with PPAR-α, it is uncertain how important TLR2 was in the development of inflammation and tissue damage. Possibly, hyaluronic acid fragments found in the injured pancreas could be a major source of TLR2 upregulation[36]. Small molecular weight hyaluronic acid fragments seem to need TLR2 in order to stimulate the proinflammatory state in mouse macrophages[37]. Heat shock proteins may leak into the extracellular compartment after necrotic cell death in AP, interact with CD14/TLR2 and induce the production of inflammatory cytokines (especially TNF-α)[38].

Lung injury
Acute lung injury (ALI) is possibly the most serious complication associated to SAP, since it accounts for most deaths in untreated patients and in hospitalised patients that die during the first week after the onset of AP[39].

In 2005, Wu *et al*[40] described that TLR2mRNA overexpression in the lungs in a sodium taurocholate-based AP rat model was coupled to elevated TNF-α levels and lower nitric oxide (NO) levels, when compared to controls. Moreover, L-Arg administration decreased

TLR2mRNA expression and pulmonary TNF-α levels. Since L-Arg stimulates the production of NO, it is difficult to evaluate if the anti-inflammatory response was caused by NO itself or by a L-Arg-mediated TLR2 down-regulation. Chloroquine showed similar effects (both TLR2mRNA and TNF-α were decreased) but the lung injury was not significantly ameliorated. Chloroquine is a well-known anti-malaria drug that prevents against endosomal acidification[41] and is used to study the role of intracellular TLRs[42].

The authors concluded that SAP-associated ALI was coupled to higher TLR2 levels in the lungs and that reduced NO levels were in part responsible for the grade of inflammation, since NO has known anti-inflammatory properties. However, how NO levels are associated with TLR2 and its potential therapeutic use in AP, is still unknown.

Matsumura *et al*[43] reported that the expression of TLR2mRNA decreased in pulmonary macrophages in deoxycholate-induced AP in rats. Macrophages were obtained through bronchoalveolar lavage fluid (6 h after induction) and the cells were exposed to lipoteichoic acid. No change in TLR2mRNA was initially observed, but after six hours TLR2mRNA expression significantly decreased in macrophages. Additionally, the production of TNF-α after lipoteichoic acid stimulation was reduced. An important observation made was the increment of bacterial translocation (cultured from mesenteric lymph nodes) 18 h after AP-induction, indicating that impaired TLR2mRNA expression in pulmonary macrophages could partially be responsible for the development of ALI in SAP complicated by sepsis. It is widely accepted that TLR2-/- mice are vulnerable to infections[44,45]. Pulmonary macrophages have been pointed out as major players in ALI in SAP, as monocyte chemoattractant protein-1 and TNF-α are released by these cells[46]. Hence, diminished TLR2mRNA expression in pulmonary macrophages under AP is doubtfully important for the development of ALI when TLR2-related PAMPs are absent.

Liver involvement
Liver failure is a major complication in AP. Besides, there is increasing data suggesting that activated Kupffer cells mediates the inflammatory response and pulmonary damage seen in SAP[47].

Xiong *et al*[48] showed that hepatic TLR2mRNA expression was increased in AP-induced mice. The incremented TL2mRNA expression was observed 3 h after the induction, peaking at 12 h. Likewise, severe damage in the liver and SIRS ensued in the animals. However, in order to strengthen the immunological reaction, LPS was injected as well, leading to a TLR4mRNA expression increment in the liver. Thus, it is difficult to establish if SAP-related SIRS is associated with an increase in TLR2 or TLR4. Possibly, a combined increment of both TLRs account for the observed severity. Zhang *et al*[49] obtained similar results in a taurocholate-based rat model. Besides, decreased hepatic TLR2mRNA expression, chloroquine
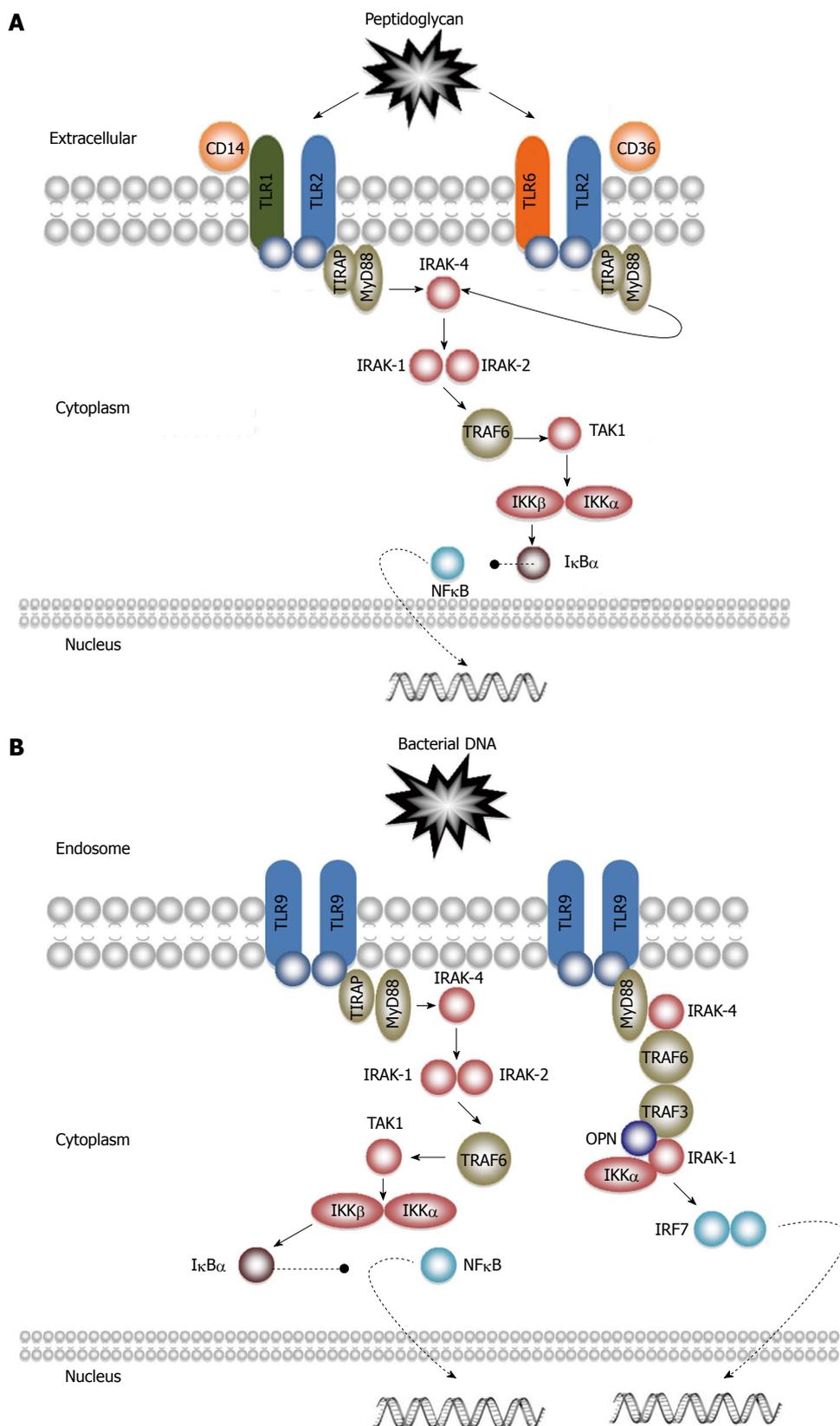
Figure 2 Toll-like receptor 2 and receptor 9 signalling pathways. A: Receptor 2; B: Receptor 9.

or L-Arg administration attenuated the liver injury and decreased TNF-α levels. Increased pro-inflammatory cytokines and chemokines are released following over-expression of TLR2mRNA in the liver of untreated ani-

mals. As previously shown in the lungs[40], NO levels also decreased in the liver. This is of special interest since reduced NO levels correlated with diminished secretion of anti-inflammatory mediators.

Table 4 A selection of genes regulated by nuclear factor κB	
Acute phase proteins	C-reactive protein
	Complement factor Bf
	Complement factor C3
	TNFβ
Adhesion molecules	E-selectin
	Intracellular cell adhesion molecule 1
	Vascular cell adhesion molecule 1
Chemokines	IL8
	Monocyte chemoattractant protein-1
	Chemokine C-C motif ligand 5 (also known as RANTES)
Cytokines	IL2, 6, 12
	ILβ
	IL1β
	TNFβ
	TNFα
Growth factors	Granulocyte colony stimulating factor
	Granulocyte-macrophage colony stimulating factor
	Macrophage colony stimulating factor

Modified after Christman *et al*[11]. TLR: Toll-like receptor; TNF: Tumour necrosis factor; IL: Interleukin.

Even if several studies associate TLR2 to AP, it is still debated if TLR2 plays a pathophysiological role. Awla *et al*[50] reported that the progression of SAP was not significantly different in TLR2$^{-/-}$ mice when compared with wild-type mice. No significant differences were observed concerning pancreatic tissue damage, chemokine formation and neutrophil recruitment in taurocholate-induced AP.

TLR2 and AP in humans

In humans, microsatellite polymorphism in intron 2 in the human *TLR2* gene was associated with an increased risk for AP in Japan[51]. For this experiment, DNA was harvested from 202 patients of which 80 were diagnosed with SAP. When compared to healthy Japanese controls, AP patients showed significantly increased polymorphism rates in *TLR2* genes. Since the same polymorphism has been associated with susceptibility to colorectal cancer, tuberculosis, rheumatoid arthritis and sarcoidosis[34,52-54]; it is conceivable that TLR2 signalling is altered if intron 2 is changed. However, it remains to be elucidated if the relationship between changed *TLR2* genes and the risk for AP is just limited to Japanese subjects, or if the results can be extrapolated to other populations. Other studies have shown that gene-related vulnerability for AP in a country could not be demonstrated in other populations[55,56].

A very interesting observation was made by Szabo *et al*[57]. Monocytes were harvested from healthy volunteers before alcohol consumption and 24 h thereafter. The cells showed a decreased pro-inflammatory profile. Furthermore, *in vitro* acute alcohol stimulation of monocytes activated the pro-inflammatory state and decreased IL-production when TLR2 and TLR4 ligands were added. However, if only TLR2 ligands were added, no significant changes were observed. Therefore, acute alcohol stimulation appears to inhibit TLR2 expression in monocytes, worsening by this way the innate immune response against microorganisms that produce TLR2 specific ligands. Acute alcohol stimulation seems to be a double edged sword, since it could induce both anti and pro-inflammatory responses depending on which TLRs are involved.

In summary, TLR2 seems to be up regulated in the pancreas, lungs and liver in experimental AP animals. Moreover, TLR2-deficiency or inhibition (chloroquine or L-Arg) seems to ameliorate ALI in SAP.

How the increased TLR2 levels in vital organs are associated with SAP is still unknown. It is plausible that the overexpression of TLR2 in the pancreas, lungs and liver, amplifies the inflammatory response observed in SIRS when DAMPs released in SAP are recognised.

Despite an association found in Japan, the relationship between modified human *TLR2* genes and AP has not been reported in other populations. Even if TLR2 seems to be relevant for the development of ALI in SAP, further research has to be done before this important PRR can be considered as a potential therapeutic target in AP.

TLR4-THE MAIN CANDIDATE?

TLR4 was the first TLR identified and is widely expressed on the plasma membrane of various immune cells, including macrophages and dendritic cells[58]. TLR4 recognises several DAMPs, including LPS, fibrinogen, and various heat shock proteins (Tables 1 and 2). Upon activation, TLR4 forms homodimers or heterodimers with TLR6. The TLR4 recognition of many DAMPs demands several accessory molecules. As for TLR2, CD14 is needed for binding LPS to TLR4 dimers. Another vital protein for LPS recognition is myeloid differentiation protein-2[3].

In the same matter as other TLRs, except TLR3, TLR4 signals through a MyD88-dependent pathway, leading to the activation of NFκB (see previous section). Besides, TLR4 can signal *via* a TRIF-dependent pathway. Once activated, TIR-TIR dimers recruit TRIF and TRAM. TRIF then activates I-κ-β kinase epsilon (IKKε), which binds to TANK-binding kinase 1 (TBK1). Finally, IKKε-TBK1 phosphorylates and activates IRF3, culminating in the transcription of IFN-α and IFN-β (Figure 3)[26].

TLR4 was initially mainly studied because of its involvement in Gram-negative bacterial infection, but has in recent years also been associated with an increasing number of diseases. Many reports suggest its involvement in atherosclerosis, liver disease, obesity, cardiac disease, and renal disease, among others[59-63].

Bacteria are important microorganisms in AP. In acute haemorrhagic-necrotizing pancreatitis, intestinal bacterial translocation into the injured/necrotic pancreas or the systemic circulation, may lead to SIRS, ALI and MODS[16-19]. As TLR4 is related to LPS and a wide range of alarmins released during inflammation and tissue damage, its role in AP appears to be highly possible.
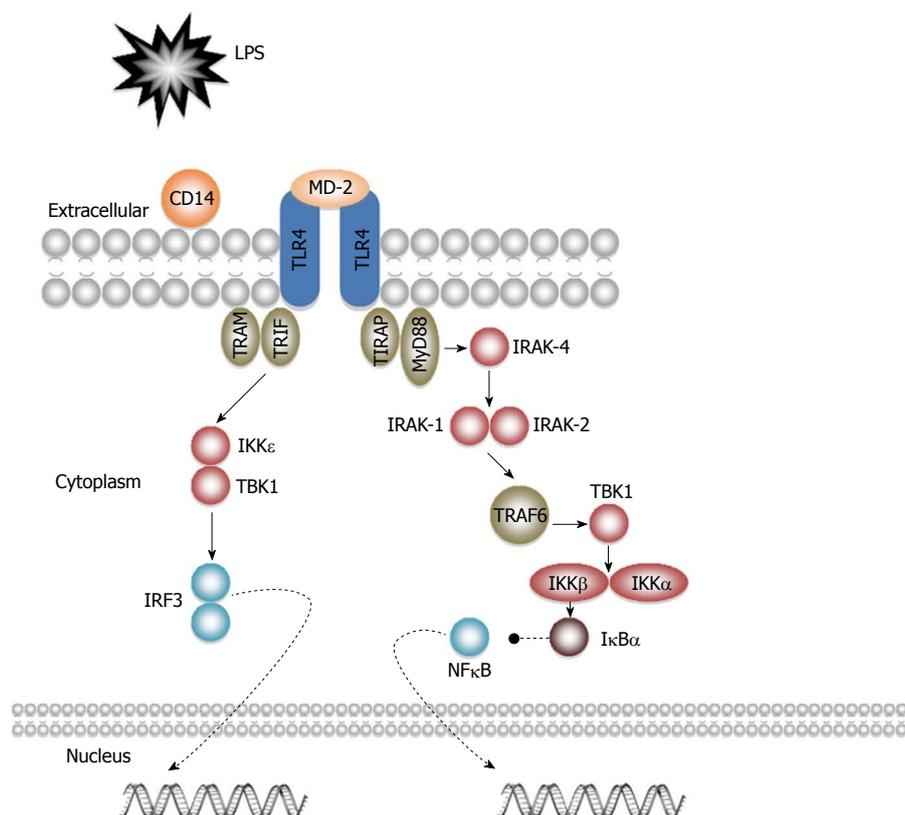
Figure 3 Toll-like receptor 4 signalling pathways.

Pancreatic damage

As for TLR2, overexpressed TLR4mRNA was also found in the pancreas in rats with cerulein-induced AP[35]. Li *et al*[64,65] reported (in the same animal model) that in both healthy and AP-induced animals, TLR4 was expressed mainly in the epithelium of the pancreatic duct and the pancreatic microcirculation. Even if some staining was observed in endocrine islets, no TLR4 protein was detected in the acinar cells. Likewise, TLR4 mRNA overexpression was observed, peaking after 1 h and returning to base levels after 4 h. In taurocholate-induced AP in TLR4^{-/-} mice[50], serum amylase and myeloperoxidase levels in the pancreas decreased as compared to wild-type induced mice. Additionally, acinar cell necrosis, oedema, and haemorrhage significantly reduced. Interestingly, pancreas and serum levels of CXCL2, a chemokine, released by monocytes and macrophages, recruiting neutrophils, were strongly diminished.

Opposite results were presented by Ding *et al*[66] using the same AP model. No significant changes in serum amylase levels and pancreatic histological scores were observed between TLR4^{-/-} and the controls. Curiously, TLR4^{-/-} mice showed decreased levels of pro-apoptotic proteins in the pancreas 2 h after induction, but not after 4 h. TLR4 can increase the apoptotic rate in different cells[67]. As some studies suggest that apoptosis is favourable in AP[68,69], it is feasible that TLR4 is involved both in the beginning and in the resolution of AP.

Wang *et al*[70] showed that the administration of anti-CD14 antibody prior to cerulein-induction and LPS chal-lenge in mice, reduced the severity of pancreatic injury, decreased pancreatic myeloperoxidase activity and down-regulated the secretion of pro-inflammatory cytokines.

TLR4 signalling pathways have been investigated in AP. Ding *et al*[71] showed in TLR4^{-/-} mice that IRAK-4 could play a role in AP, independently of TLR4. In this experiment, lacking TLR4-mediated response did not result in increased IRAK-4 levels as was expected. This suggest that TLRs other than TLR4 may also play a role in AP, as the activation of TLR2 and TLR9 also results in decreased IRAK-4 in macrophages[72]. The hypothesis is supported by a report in which TRAF6 mediated inflammation in AP in TLR4^{-/-} mice[73]. The author concluded that TLR4 may not be exclusively required for initiating AP, but its signal pathway may be of importance.

However, the association between alarmins released and TLR4-mediated inflammation in AP appears to be robust. In mononuclear inflammatory cells, the enzyme pancreatic elastase seems to activate NFκB, inducing TNF-α secretion[74,75]. Hietaranta *et al*[76] found that when human myeloid cells were exposed to porcine elastase, increased expression of NFκB and AP1 was achieved. The effects of elastase seem to be mediated by TLR4, since the blocking of TLR4 with a specific neutralizing antibody strongly prevented the expression of pro-inflammatory factors in elastase-exposed cells. Premature elastase activation has been reported in AP, implying its role in the disease[77]. Hence, TLR4-mediated inflammation may be important in AP. Blocking elastase might attenuate the severity of AP.

The degradation of matrix components appears to be connected with the pathophysiology of AP. Reports indicate that low molecular weight polysaccharides from degraded hyaluronan activates dendritic cells through TLR4[78]. Besides, dendritic cells might protect the pancreas against cell stress in AP[79]. Blycans and oligosaccharides of hyaluronan seem to signal *via* TLR4 and induce the production and secretion of great quantities of TNF-α and macrophage inflammatory protein-2 in macrophages[80]. Elastase and trypsin, both involved in the initiation of AP, cleaves heparin sulphate (HS) from cell surfaces and extra cellular matrix *in vitro*[81]. This, probably may in turn lead to free endogenous HS in damaged tissues in AP. Johnson *et al*[25] demonstrated that the rapid degradation of HS in mice causes a TLR4-mediated SIRS-like reaction in mice. When soluble HS was injected intraperitoneally, almost all wild type, but no TLR4-deficient mice, perish. This reaction was HS specific since molecules structurally similar to HS did not stimulate TLR4 and HS was not contaminated with LPS. Moreover, wild type, but not TLR4-deficient, mice had increased TNF-α serum levels 1 h after administration. Continuing in this line, Axelsson *et al*[82] confirmed that HS is involved in the initiation of AP in the rat.

Akbarshahi *et al*[83] later showed that the HS-induced TLR4-dependent immune response in the murine pancreas is IRF3-mediated. When TLR4$^{-/-}$ or MyD88$^{-/-}$ mice were challenged with HS or LPS, myeloperoxidase activity was annulled in the pancreas. The same pattern with HS was observed in IRF3$^{-/-}$ mice. However, LPS administration initiated a strong immune response in these animals. This outcome could explain preceding reports about discrepancies between TLR4-deficiency and decreased TRAF6 and IRAK-4 levels in AP[71,73]. Additionally, in this experiment, pre-treatment with the TLR4 antagonist eritoran inhibited the otherwise occurring increase in myeloperoxidase production in HS-treated wild type mouse pancreas. Eritoran is a synthetic Lipid A analogue that bind to TLR4/myeloid differentiation protein-2, thereby competing with Lipid A, resulting in inhibited LPS-mediated immune response[84].

Possibly, the inhibition of TLR4, elastase or HS, alone or in combination, could ameliorate pancreatic damage in AP, thus considerably reducing the risk for developing SAP.

Lung injury

As the role of TLR4 in AP has been investigated previously together with TLR2, similar results have been reported (see TLR2 section). Briefly, TLR4mRNA expression was increased in the lungs of AP-induced rats; leading to increased TNF-α levels combined with decreased NO levels. Chloroquine and L-Arg decreased TLR4mRNA expression and attenuated the lung injury[40]. Moreover, pulmonary macrophages decreased the TLR4mRNA expression, which caused decreased LPS-induced TNF-α levels and predisposition for intestinal bacterial translocation[41].

Pastor *et al*[85] concluded that TLR4 may not play a role in AP-associated ALI, but it may participate in pulmonary injury mediated by endotoxemia. In a cerulein-induced pancreatitis model in TLR4$^{-/-}$ mice there were no significant changes in serum amylase levels, pancreatic myeloperoxidase activity, and pancreatic oedema and acinar necrosis when compared to wild type mice. Additionally, cerulein-related pulmonary damage did not decrease in TLR4$^{-/-}$. Although, when cerulein was combined with LPS, a significant decrease in pulmonary damage was reported. Thus, TLR4 would only be of importance in AP-related ALI when the disease is worsened by sepsis.

Sharif *et al*[86] obtained pole opposed results. TLR4$^{-/-}$ mice showed decreased serum amylase activity and pancreatic damage (oedema, myeloperoxidase activity, necrosis). Importantly, myeloperoxidase activity in the lungs also decreased, indicating reduced neutrophil sequestration. Furthermore, when AP was induced in CD14$^{-/-}$ mice, pancreatic and pulmonary damage were reduced as previously observed in TLR4$^{-/-}$ animals. Since TLR4/CD14 are very important for LPS-mediated TLR4 activation, TLR4 appears to be involved in the development of ALI in AP, even when endotoxemia is absent. Still, the subject is controversial, since ALI can ensure LPS-mediated TLR4/CD11b activation in CD14$^{-/-}$ mice[87].

Matsuda *et al*[88] proposed that the TLR4-associated inflammatory response in AP complicated by endotoxemia is mediated by macrophage migration inhibitory protein (MIF). Increased MIF expression in the lungs was observed after cerulein/LPS administration in mice. MIF levels were coupled to ALI and increased TLR4 expression in the lungs. Moreover, AP-induced MIF$^{-/-}$ mice showed lower TLR4 expression than in wild type mice; and anti-MIF antibody administration greatly suppressed the pulmonary expression of TLR4. It is important to stress that MIF can mediate inflammation independently of TLR4. For instance, the induction of cytosolic phospholipase A$_2$, an enzyme that has been linked to ALI, may be MIF-mediated[89,90].

The role of TLR4 in AP-associated ALI is debated. While it is generally accepted that TLR4 plays a role in the course of ALI when endotoxemia ensues in AP; most reports are divided between those that suggests TLR4 as an important player (even in the absence of endotoxemia) and those that minimize its role. Fortunately, this discrepancy may lead to new experiments that focuses not only in LPS but also in the wide range of alarmins related to AP, ALI and TLR4. For instance, neuropeptide substance P, which has pro-inflammatory properties that increase vascular permeability and is correlated to AP and ALI[91]; has also been associated to the up regulation of TLR4 in AP[92]. Moreover, extracellular heat shock protein 70 can induce SIRS-like immune responses in cerulein-challenged mice[93]. The action of this protein appears to be mediated by TLR4, as TLR4$^{-/-}$ mice did not showed the same outcome.

Liver, kidneys and intestine

Similarly to TLR2, TLR4mRNA expression is increased

in the liver during AP-associated SIRS[48,49]. Briefly, after AP-induction, higher levels of liver enzymes and TNF-α were observed. Hepatic NO levels decreased. All these changes appeared to be related to the TLR4mRNA overexpression in the liver. The administration of chloroquine and L-Arg decreased TLR4mRNA expression, thus reducing the liver damage.

Peng *et al*[94] showed that the deletion of TLR4 attenuated liver injury in AP. AP was induced in mice by choline-deficient ethionine diet. Besides the expected augmentation of TLR4 mRNA in mice liver, an increment in protein kinase C-zeta (PKCζ) was detected. However, induced TLR4^{-/-} mice showed less apoptosis in hepatic cells and the hepatic PKCζmRNA expression was clearly reduced. PKCζ activates NFκB, which is essential for the production of cytokines in Kupffer cells[95]. Consequently, TLR4-deficiency protects the liver in AP through down-regulation of PKCζ, resulting in less inflammation and hepatic cell apoptosis.

Sawa *et al*[96] showed that in SAP, TLR4 expression is not only increased in the liver, but also in the kidneys and small intestine. Closed duodenal loop operation was performed in mice. The increased expression of TLR4 occurred 4 h after the ligation and returned to baseline after 12 h. Curiously, TLR4-deficient mice showed the same tendency and histological analyses of the liver and kidney did not show any differences between the different groups. However, apoptosis was seen in the liver and kidney of ligated TLR4-deficient, but not in wild type, mice. Additionally, Gram-negative bacterial translocation into the pancreas occurred in higher rates in TLR4-deficient animals. The translocation ensued 12 h after induction, but not before. Even Gram-positive bacterial translocation was registered, but the difference between the groups was not significant. Bacterial translocation highly correlated to fluctuations in TLR4 expression. According to the authors, as MODS develops in the early or the late phase of SAP, the early inhibition and late stimulation of TLR4 could result in a milder clinical course, since it could prevent MODS and bacterial translocation. Yet, results from another experiment in mice challenges this hypothesis[97]. van Westerloo *et al* showed that the immune response against Escherichia coli in AP-induced TLR4^{-/-} mice was not different from that observed in controls. Nevertheless, there is a chance that mice developed the compensatory anti-inflammatory response syndrome (CARS), which is generally (but not exclusively) related to SIRS and sepsis[98]. CARS is a systemic deactivation of the immune system, that can ensue after SIRS and sepsis in order to restore homeostasis[99]. Thus, CARS could explain why LPS-mediated TLR4-activation was not observed in TLR4^{+/+} mice after bacterial challenge in AP.

TLR4 and AP in humans
Despite controversy, there is an increasing amount of data suggesting that TLR4 plays a role in experimental AP in rodents. However, in humans, the role of TLR4 in AP patients has been investigated mainly indirectly *via*

DNA analysis or immune cells taken from blood.

In a Chinese study 310 patients were diagnosed with AP according to the Atlanta severity classification[100,101]. Pancreatic necrosis was recognized in 115 patients and in 37 of those, TLR4 mutation (*896G allele*) was identified. When compared to AP patients without TLR4 mutation (*896A allele*), patients with mutated TLR4 had an increased morbidity following Gram-negative infection. When compared to healthy volunteers (*n* = 80), TLR4 mutation frequency was significantly higher in patients with pancreatic necrotic infection. Thus, according to this study, TLR4 *Asp299Gly* polymorphism appears to be associated with an increased risk for pancreatic necrotic infection in AP. However, this study may have limited value as all patients were recruited from one hospital located in southwestern China, thus questioning its representative value for the whole Chinese population. In a later study, Zhang *et al*[102] could not find any significant difference in the occurrence of *Asp299Gly* polymorphism between 238 Chinese AP patients and 121 healthy volunteers.

DNA samples were harvested from 92 AP patients in a Hungarian medical centre[103]. Not only TLR4 *Asp299Gly* polymorphism was considered, but also as *Thr399Ile* polymorphism was taken into account. No significant differences were detected between AP (*n* = 42) and SAP patients (*n* = 50) in any polymorphism analysed. Likewise, no differences were found between the 92 patients and healthy controls (*n* = 200). Perhaps, the strongest data against the involvement of TLR4 mutations in AP comes from an intercontinental study reported by Guenther *et al*[104]. No significant differences could be found between 521 AP patients (343 from Germany and 178 from United States) and healthy controls (128 Germans and. 265 Americans, respectively) in the incidence of *Asp299Gly* and/or *Thr399Ile* polymorphism. Thus, current knowledge indicates that the investigated TLR4 polymorphisms are not important for the development and clinical course of AP, even if these mutation have been linked to impairment of LPS-induced TLR4-mediated immune response in humans[105].

In vitro acute alcohol stimulation seems to inhibit TLR4 expression in human monocytes[57]. When LPS was added, a decreased secretion of pro-inflammatory cytokines was observed. Intriguingly, the effect was abolished if LPS and TLR2 ligands were added at the same time to the cells. Li *et al*[106] showed that the expression of TLR4 in human peripheral blood mononuclear cells in mild AP, raises in the beginning of the disease to return to baseline levels after a week, when the patients recovered. As TNF-α and IL-6 showed the same pattern, the authors concluded that TLR4 might play an important role in the pathogenesis of AP.

In summary, there is strong data indicating that TLR4 plays an important role in experimental AP in rodents. Nevertheless, contrary results have been published. While most studies emphasize the role of TLR4 in the pancreas in AP, its role in AP-associated ALI is controversial. There is some evidence linking TLR4 levels and tissue

damage in the liver in AP. Likewise, the general opinion is that TLR4 is very important in the development of endotoxemia in AP.

In humans, DNA mutations in *TLR4* genes do not seem to be of importance for the development or the clinical course of AP, but TLR4 expression changes in monocytes and macrophages may be of significance. Unfortunately, the latter is only based on a very limited number of studies. There are some promising reports about the use of herbs from traditional Chinese medicine in AP that may act through TLR4 inhibition[107-109]. *Salvia miltiorrhizae* (also known as danshen) appears to inhibit the binding of LPS to TLR4 in the rat liver in SAP, which may reduce bacterial translocation and liver injury. Moreover, treatment with *emodin* and/or *baicalin* reduced serum levels of amylase, IL-6 and TNF-α in AP-induced rats. Pancreatic damage and ALI were also ameliorated. The effect of *emodin* and *baicalin* appears to be mediated by TLR4, since decreased TLR4mRNA expression and protein levels were found in the pancreas and lungs in treated animals. Its implication in experimental AP in rodents makes TLR4 a potentially very promising therapeutic target in AP, its utility though to be demonstrated in humans in future studies.

TLR9-UNEXPLORED IMPLICATIONS

As for TLR2 and TLR4, TLR9 is expressed intracellularly (endosomes) in several immune cells, including B cells and dendritic cells[110]. TLR9 recognises unmethylated *CpG* dinucleotides found in *bacteria* and *virus*[111,112]. *CpG* motifs are rare in vertebrate DNA (less 1% in human genome), but they are common in bacterial DNA. Besides, if present in vertebrate DNA, *CpG* motifs use to be methylated. *CpG* DNA directly stimulates B cells, macrophages and dendritic cells to secrete cytokines[113]. TLR9 can also recognize haemozoin, which is a disposal product formed from the digestion of erythrocytes by some parasites (*e.g.*, *Plasmodium spp.*)[114].

Upon activation, TLR9 signals through a MyD88-dependent pathway, leading to the production of pro-inflammatory cytokines (see previous sections). This pathway is mainly observed in macrophages, B cells, conventional dendritic cells and plasmacytoid dendritic cells. However, in plasmacytoid dendritic cells, TLR9 can induce the production of type I IFN through a different pathway. After the recruitment of Myd88 and IRAK-4, these interact with TRAF6, TRAF3, IRAK-1, IKKα and osteopontin. IRAK-1 and IKKα phosphorylate and activate IRF7, culminating in the production of type I IFN (Figure 2B)[26]. Apart from its role in bacterial, viral or malaria infection; TLR9 has been associated with SLE and cancer[115,116].

TLR9 and AP

To date, there are only two publications in which the role of TLR9 in AP has been investigated. Zeng *et al*[117] reported that TLR9 was expressed in rat pancreas in cerulein-induced AP. TLR9 staining was detected both in the pancreas from AP-induced rats, as in controls. The epithelium of the pancreatic duct and pancreatic microcirculation were the main sites for TLR9 staining in AP-induced animals. Controls showed staining in the vasculature, but not in the pancreatic duct. Moreover, no staining was detected in pancreatic acinar cells in either group. Interestingly, a similar pattern of staining has been reported for TLR4 in AP[64]. TLR9mRNA expression was increased after 30 min, peaked at 1 h, and remained high for the first eight hours after cerulein challenge.

In a more extensive study, Hoque *et al*[118] found that TLR9 is involved in pancreatic acinar cell death in AP-induced mice. After cerulein-administration, TLR9−/− mice had less oedema, leukocyte infiltration and IL-1βmRNA expression in the pancreas. Even if several cell types express TLR9 in the pancreas, bone marrow-derived CD45+ cells (mainly macrophages) had the highest expression level. Furthermore, the action of a TLR9 antagonist (IRS954) was evaluated. TLR9+/+ animals had IRS954 administered 1 h before inducing AP by cerulein. Pancreatic oedema, leukocyte infiltration and pancreatic cell apoptosis were ameliorated. Moreover, pancreatic pro-IL-1β elevation was also reduced, as serum amylase levels. These results were observed at 1 h after AP-induction.

The authors speculated if IRS954 might have a therapeutic value. Interestingly, the administration of IRS954 after cerulein challenge reduced pancreatic oedema, leukocyte infiltration and pancreatic cell apoptosis. Similar results were observed when another model for AP was used. Pre-treatment with IRS954 at 1 h after AP induction through taurolithocholic acid 3-sulphate challenge in TLR9+/+, reduced serum amylase elevation, pancreatic necrosis and inflammatory cell infiltration in mice lungs. Additionally, *in vitro* experiments were performed. When isolated peritoneal macrophages were exposed to pancreatic homogenate and DNA, significant NFκB activation was observed. Interestingly, pre-treatment with IRS954 reduced NFκB activation to baseline levels.

IRS954 appears to be a good candidate for further investigation. Like TLR2 and TLR4, TLR9 may be important when AP is aggravated by sepsis. Reports indicate that during sepsis, TLR9 is expressed both in murine and human adrenal glands and its stimulation leads to corticosterone release and inflammatory response[119]. Macrophages and NK-cells in the liver mediates liver toxicity and express high levels of TLR9 in murine peritonitis[120]. Chloroquine inhibits TLR9 and may prevent sepsis-induced acute kidney injury in mice[121]. Additionally, TLR9−/− mice have reduced mortality in polymicrobial sepsis[122].

Still, the number of studies is very limited. Hence, it is impossible to conclude if TLR9 plays an important role in AP with a clinical course complicated with our without sepsis.

Closing remarks

An increasing number of publications suggest that TLRs

Table 5 Toll-like receptors and acute pancreatitis

	Observed change	Tissue/cell	Ref.
TLR2	Increased mRNA expression	Pancreas	[35]
		Lungs	[40]
		Liver	[48,49]
	Increased protein levels	Pancreas	[35]
		Liver	[48]
	Decreased mRNA expression	Pulmonary macrophages	[43]
TLR4	Increased mRNA expression	Pancreas	[35,64,73,92]
		Lungs	[40,88]
		Liver	[48,94,96]
		Kidney	[96]
	Increased protein levels	Small intestine	[96]
		Pancreas	[35,92]
		Lungs	[88]
		Liver	[48,49]
		Intestine	[65]
		Blood monocytes	[106]
	Decreased mRNA expression	Pulmonary macrophages	[43]
TLR9	Increased mRNA expression	Pancreas	[117,118]
	Increased protein levels	Pancreas	[118]

TLR: Toll-like receptor.

Table 6 Toll-like receptor intervention in acute pancreatitis

	Substance	Ref.
TLR2	Chloroquine	[40,49]
	L-Arg	[40,49]
	WY14643	[35]
TLR4	Anti-MIF antibody	[88]
	Baicalin	[107]
	Chloroquine	[40,49]
	Emodin	[107]
	Eritoran	[83]
	L-Arg	[40,49]
	Salvia miltiorrhizae	[108,109]
TLR9	IRS954	[118]

TLR: Toll-like receptor; MIF: Migration inhibitory protein.

are involved in the pathophysiology of several important medical conditions. In acute pancreatitis, TLRs appear to play a role, but a general consensus has not been achieved, since contradictory results have been presented.

The main candidate for targeting seems to be TLR4, which recognizes numerous DAMPs associated to AP[123,124]. TLR2 has also been linked to AP, but there are few studies that exclusively has studied its role in AP. There is data suggesting that TLR9 also can play a role in AP. The associations found between TLRs and AP, as possible novel types of therapy are presented (Tables 5 and 6). There is also some evidence indicating that TLR4 and TLR3 may be involved in chronic pancreatitis[125,126]. This is of particular importance since chronic pancreatitis may develop following repeated AP episodes, as chronic pancreatitis is to some extent a risk factor for pancreatic cancer[127].

CONCLUSION

To our knowledge, this is the first time the current understanding of the role of TLRs in acute pancreatitis is summarized. Of course, further research and elucidation of involved mechanisms is warranted, hopefully stimulated by the present review giving an update and state-of-the-art concerning the role of TLRs in acute pancreatitis and its potential future clinical implications.

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