Implication of Innate Immunity in the Pathogenesis of Biliary Atresia

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Biliary atresia (BA) is a complex disorder for which the etiology is still far from clear. Newborn infants that develop BA may carry certain genetic defects, resulting in susceptibility to uncertain pathogens with characteristic pathogen-associated molecular patterns (PAMPs). The pathogens with their characteristic PAMPs in turn lead to activation of the innate immune system by triggering pattern recognition receptors on the immune cells. Toll-like receptors (TLRs) are the most recognized pattern recognition receptors and TLR signaling is the telltale sign of activation of innate immunity. The activation of TLR and the innate immune system in BA is demonstrated by the up-regulation of TLR7 and by the association of promoter polymorphism of CD14 with BA. The antimicrobial peptide hepcidin and MxA, a protein downstream of TLR7 signaling, which is also known as a highly specific marker for type I IFN signaling, are also found highly expressed in the early stage of BA. This review examines the known components of innate immunity involved in BA and outlines the potential role of the innate immune system, in cooperation with adaptive immunity, in the pathogenesis of BA. *(Chang Gung Med J 2006;29:240-50)*

Key word: biliary atresia, innate immunity, CD14, toll-like receptor.

Biliary atresia (BA) is a complex disorder characterized clinically by progressive obstructive jaundice and acholic stools, and pathologically by complete obliteration of the extrahepatic bile duct. Despite Kasai's breakthrough correction of the "uncorrectable type" of BA,[1] most cases today still meet with disappointing results. About half of patients with BA require liver transplantation for long-term survival, despite the fact that most of them obtain good bile flow immediately after Kasai's procedure.[2-5] With increasing evidence of the involvement of genetics, epigenetics, and the environment, the pathogenesis of BA has begun to be understood at the dawn of the new century.[6]

It is estimated that around 80% of BA cases are the perinatal or acquired form, while the remaining minority are the embryonic or fetal form.[7] The latter form is believed to be caused by genetically dysregulated ductal plate morphogenesis.[8] Mutations of several laterality genes such as INV, CFCI and ZIC3 have been suggested to be associated with the embryonic form of BA, but more studies are needed to prove this association.[9-12] This article will not encompass any detail of these studies and the readers may refer to a more comprehensive review.[7] We will focus on the implication of innate immunity in the pathogenesis of the perinatal form of BA.

Linking innate immunity to BA via interferon signaling

Innate immunity is based on the genetic constitution of the individual.[13] The innate immune system is composed of macrophages, neutrophils, natural killer cells, mucosal epithelial cells and endothelial

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cells, all of which are able to seek out and destroy invading microorganisms at their first encounter without an antigen-specific adaptation. Innate immunity and adaptive immunity coexist and complement each other to form a complete immune system. Because the function of the innate immune system is immediate and direct, its presence in the living organism is considered to be primitive and ancient.[14,15] However, the importance of innate immunity in the host defense against infection was not recognized until very recently, when the toll-like receptor (TLR) family came to the stage, far behind our understanding of adaptive immunity.

Although no infectious pathogens have been convincingly identified in BA, the development of extra-hepatic biliary atresia in newborn Balb/c mice after intraperitoneal injection of rhesus rotavirus supports an infectious etiology for BA in humans.[16,17] Furthermore, the highest incidence of virally induced extrahepatic BA occurs within the first 12 h postpartum, after which the incidence of the disease decreases.[18] The latter implies a temporal immunological gap in the infectious animal model for BA. Interestingly, genetic knockout of interferon-γ (IFN-γ) in newborn Balb/c mice completely prevents inflammatory and fibrotic obstruction of extrahepatic bile ducts after rotavirus infection.[19] Administration of recombinant IFN-γ to the above IFN-γ-deficient mice leads to recurrent development of bile duct obstruction. Interestingly, in infants with BA, induction of IFN-γ by osteopontin is implicated in the proinflammatory response.[20] On the contrary, single-dose IFN-α therapy protects all rhesus rotavirus-infected pups from cholestatic diseases, implying a probable protective effect against the virus-induced mural BA by maternal antibodies.[21]

IFNs were first recognized for their ability to impede viral replication. IFN signaling is now known as an emerging bridge between TLR-mediated innate immunity and microorganisms.[22,23] A broad range of viruses, bacteria and even parasites express ligands capable of stimulating an array of signaling pathways that all lead to induction of IFN.[24] The signaling pathways are associated with TLRs.

TLR and proinflammatory cytokine induction

Recognizing and defeating microorganisms is essential for the survival of an organism. Conserved molecular patterns that are invariant among an entire class of pathogens that are detected by the host are called pathogen-associated molecular patterns (PAMPs). The PAMP receptors are called pattern recognition receptors (PRRs). The TLR family is the best characterized class of PRRs in mammalian species.[25] TLRs are the human counterparts of Toll receptors in the fruit fly, Drosophila. To date, 11 TLRs have been identified and demonstrated to recognize a variety of PAMPs, including lipopolysaccharide (LPS) (detected by TLR4), bacterial lipoproteins and lipoteichoic acids (detected by TLR2), flagellin (detected by TLR5), the unmethylated CpG dinucleotides in the DNA of bacteria and viruses (detected by TLR9), double-stranded RNA (detected by TLR3) and single-stranded viral RNA (detected by TLR7).[25] With growing numbers of known TLRs, understanding the engagement of these receptors by pathogenic components and the subsequent induction of specific genes has become a rapidly expanding field in the biomedical sciences.

Many crucial factors in the induction of TLR-responsive genes belong to the IFN-regulatory factor (IRF) family. For induction of inflammatory cytokines through TLR signaling, either a myeloid differentiation factor88 (Myd88)-dependent pathway to activate NF-κB or a Myd88- and IL-1R-associated kinase-1 (IRAK-1)-dependent pathway to activate IRF5 is essential. Activation of IRF3/7 through TLR3 or TLR4 signaling or IRF7 through TLR7/9 signaling induces type I interferon genes or co-stimulatory molecules.[23] These findings suggest that the inductions of type I interferon and proinflammatory cytokines occur via different IRF-signaling pathways. The signaling cascade that eventually leads to the production of proinflammatory cytokines, such as TNF, IL-1, and IL-6, is very complex, involving more than a dozen molecules.[23]

TLR signaling in human inflammatory diseases

Cardiovascular diseases

The clinical significance of TLR signaling is demonstrated by a recent report which shows that TLR9-induced type I IFN protects mice from experimental colitis.[26] *In vitro* and *in vivo* evidence also supports the possible contribution of TLR signaling and innate immunity to vascular pathologies and atherogenesis.[27] For example, recognition of minimally modified LDL, a proinflammtory and proatherogenic lipoprotein, by TLR4 on endothelial

cells leads to the secretion of the chemokine IL-8.[28] Several TLR gene polymorphisms have been described, including $Asp^{299}Gly$ and Thr^{399} Ile, that map to the extracellular domain of TLR4. Individuals with the $Asp^{299}Gly$ or Thr^{399} Ile polymorphism are hyporesponsive to challenge with LPS,[29] indicating a protective effect against chronic inflammatory disorders such as atherosclerosis. Subjects that carry the $Asp^{299}Gly$ polymorphism are found to be protected from atherosclerosis and cardiovascular diseases.[30] But one report with contradictory findings showed that men but not women with both $Asp^{299}Gly$ and Thr^{399} Ile polymorphisms have increased susceptibility to myocardial infarction.[31] A large clinical study enrolling a sufficient number of subjects or haplotyping the TLR family is required to clarify these discordant findings.

Liver diseases

The irregular and focal distribution of TLRs in liver biopsy specimens from children infected with hepatitis C virus but not from controls suggests a role for TLR in the pathogenesis of chronic viral hepatitis, at least in children.[32] TLR-3 and type I IFN signaling pathways are active in both the portal tract and the liver parenchyma of early-stage primary biliary cirrhosis (PBC), indicating a role for TLR signaling in the pathophysiology of PBC.[33] Peripheral blood monocytes taken from patients with PBC are more sensitive to selective TLR signaling, resulting in the secretion of pro-inflammatory cytokines integral to the breakdown of self-tolerance.[34] In a murine model of hepatic ischemia/reperfusion injury (IRI), disruption/absence of the TLR4 pathway increased anti-oxidant heme oxygenase-1 mRNA and protein expression and reduced IRI, compared to that in wild type or TLR2-deficient mice. This study implies the importance of cross talk between heme oxygenase-1 and the TLR system in the mechanism of hepatic IRI.[35] Recent studies have identified some members in the TLR-4 dependent signaling pathways, including CD14 and the mitogen-activated protein kinase family members, ERK1/2 and p38, in the development of alcoholic liver disease.[36,37]

One important and intriguing finding is the ability of TLR ligands to induce antiviral cytokines and to inhibit hepatitis B virus (HBV) replication, suggesting that TLR activation could become a powerful tool in the treatment of chronic HBV infection.[38] Whether this type of antiviral strategy is applicable to hepatitis C viral infection or to other types of viral hepatitis is currently unknown.

As most cases of BA are the acquired form, it is reasonable to assume that the development of BA follows a pattern characteristic of an innate immune reaction. This assumption is corroborated in the following section.

Constitution of innate immunity in BA (Table)

The mRNA expression levels of 10 toll-like receptors (TLRs) and 21 related genes are detectable in all human tissues including the liver.[39] In a study of ontogeny of TLR2 and TLR4 in mice, the levels of TLR2 and TLR4 were high in the liver regardless of age, but were barely detectable in immature fetal lung (d 14-15). Levels of TLR2 and TLR4 in the lung were increased several-fold during prenatal and perinatal development, indicating an organ-specific expression of TLRs that depends on the stage of differentiation.[40] Like other inflammatory disorders described above, TLR signaling is expected to play a role in the pathophysiology of BA. In our unpublished observation, mRNAs of TLR-2, -3, -4, -7, and -9 are present in the liver tissue of patients with BA. However, only liver TLR-7mRNA in BA increased significantly over that in the controls and in patients with choledochal cyst. Other known components of innate immunity in BA include CD14, macrophages and other cells of the innate immune system and their intracellular and extracellular protein products, which coordinate to provoke an inflammatory reaction of the hepatobiliary system.

CD14

The innate immune system can be divided into the afferent (sensing) and the efferent (effector) arm.[41] CD14 was one of the earliest-known sensing molecules and was first recognized as a monocyte differentiation marker expressed on the surface of macrophages, neutrophils and other myeloid lineage cells.[42] CD14 plays an integral role in the activation of cells by lipopolysaccharide.[43] Strong sinusoidal expression of immunoreactive CD14 was shown in all patients of BA with clinically evident cholestasis, but not at an earlier stage when cholestasis had not developed.[44] The dynamic change of CD14 expression in BA was also demonstrated in a study that

Table. Constitution of Innate Immune System in Biliary Atresia

Components	Presentation	References
TLR signaling		
TLRs	TLRs in BA	N/A
Type I IFN	IFN-α protects murine model of BA	21
Type II IFN	IFN-γ mRNA induced by osteopontin in BA	20
	Loss of IFN-γ by knockout protects, while administration of IFN-γ resumes murine BA	18,19
CD14	Reactive CD14 immunostaining	44,45
	CD14 in murine model of BA or BDL	46,47
	Promoter polymorphism of CD14	48
Cells of the innate immune system		
Macrophages	Proliferation in BA with severe cholestasis	44
	Release proinflammatory cytokine IL-18	49
	Express HLA-DR and ICAM-1	50
	Infiltration affects outcome	50,51
Neutrophils	Inconsistent findings for neutrophils in BA	52-54
Natural killer cells	Mentioned, but not firmly established	50,58
Bile duct epithelium	Expresses HLA-DR	59-61
	Expresses ICAM-1 and TGF-β2	50,63,64
Sinusoidal endothelium	Expresses CD14	44,45
Effector molecules/cytokines		
Reactive oxygen/nitrogen species	Superoxide dismutase and NO production	65,66
TNF-α	In cell culture and murine model of BA/BDL	67,68
IL-6	Serum IL-6 and IL-1ra levels in BA	69
IL-8	Increased serum IL-8 in late stage of BA	56,57
IL-18	High blood level after Kasai procedure	49
IP-10	High serum IP-10 predicts hepatocyte death	72

Abbreviations: BA: biliary atresia; BDL: bile duct ligation; IFN: interferon; ICAM-1: intercellular adhesion molecule-1; IL-1ra: interleukin-1 receptor antagonist; IP-10: interferon-inducible protein-10; N/A: not available; NO: nitric oxide; TGF-β2: transforming growth factor-β2; TLRs: toll-like receptors; TNF-α: tumor necrosis factor-α.

revealed overexpression of CD14 in the early stage and reduced expression in the late stage of BA.[45] In two studies by Bezerra's group, CD14 was overexpressed throughout the experimental period (3-14 days in experimental biliary atresia induced by rotavirus infection and 1-21 days after bile duct ligation in adult mice). The results are interpreted as a general response of CD14 to impaired bile flow, rather than a specific response to experimental biliary atresia.[46,47]

Nevertheless, in a study by our group, promoter polymorphism of the CD14 endotoxin receptor gene was associated with BA and idiopathic neonatal cholestasis. Furthermore, decreased plasma soluble CD14 from the early stage of BA to the late stage was observed in carriers of the T/T and T/C genotypes but not in carriers of the C/C genotype.[48] These findings suggest that CD14 may not only be implicated in the pathogenesis but also in the progression of BA. However, more studies are required to support this assumption.

Macrophages and other cells of the innate immune system

Immune responses are mediated by leukocytes, which include polymorphonuclear leukocytes and macrophages of the innate immune system and lymphocytes of the adaptive immune system. The innate immune system has been expanded to include natural killer cells, mucosal epithelial cells and endothelial cells.[14] In a study of BA, marked proliferation of CD68-positive cells (resident macrophages or Kupffer cells) was found in five of six patients with BA and in one patient with another liver disease who had severe cholestasis, but not in those without cholestasis.[44] Release of IL-18, a novel proinflam-

matory cytokine that can induce interferon gamma from CD68-positive Kupffer cells, is thought to play a role in progressive inflammation and fibrosis in BA.[49] Interestingly, HLA-DR is strongly expressed on Kupffer cells and to a lesser extent on proliferating bile ducts and sinusoidal endothelium. Intercellular adhesion molecule-1 (ICAM-1) is expressed strongly in remnant bile duct tissue. Reduced expression of CD68 within the liver and biliary remnants and reduced ICAM-1 expression on the infiltrating cells in the biliary remnants is associated with a better prognosis.[50] However, contradictory results from another study revealed that infants with BA with favorable outcomes have more macrophage infiltration in the portoenterostomy core than those with unfavorable outcomes.[51] The contradiction in these two studies implies that the role of macrophages in the pathogenesis of BA has not yet been established.

A 1977 study disclosed that inflammation of the liver with mononuclear cells and neutrophils occurs in BA.[52] In a mathematical model using 49 histologic parameters including neutrophils in the inflammatory infiltrate, obstructive cholestasis (mainly BA) could be differentiated from nonobstructive cholestasis with high accuracy and sensitivity.[53] However, a recent study showed that none of four liver specimens with BA had myeloperoxidase (MPO)-positive cells in the bile ducts or portal area.[54] As MPO is a heme protein that is abundantly expressed in neutrophils and monocytes,[55] the absence of MPO-positive cells in the liver specimens almost denies the role of neutrophils in the pathogenesis of BA. Our unpublished observation shows that mRNA of the C-X-C chemokine interleukin-8 (IL-8), but not the C-C chemokine monocyte chemoattractant protein-1 (MCP-1), is upregulated in the liver of BA compared to the controls. Liver IL-8mRNA increases further in the late stage of BA, which is consistent with two recent reports of increased serum IL-8 in BA particularly in the late stage in patients with persistent jaundice, portal hypertension or an increased histological activity index in the liver.[56,57] Since IL-8 is the chemoattractant for neutrophils and MCP-1 is the chemoattractant for monocytes/macrophages, further studies to explore the interaction of chemokines and inflammatory cell infiltrates in BA are required.

In a study of natural killer (NK) cell expression in BA, more immunoreactive CD56 (+) NK cells were found in the liver of patients with BA than in controls.[50] However, strong immunoreactive CD56 expression was also present in the bile duct epithelium in another study, which questions the specificity of CD56+ cells representing NK cells in BA.[58]

In addition to CD56 expression, the bile duct epithelium, as the prime focus in the pathogenesis of BA, expresses an array of major histocompatibility complex (MHC) Class II antigens and immune-associated proteins. Among the MHC Class II antigens, HLA-DR aberrant expression is most frequently found in the bile duct epithelium of patients with BA and is associated with macrophage infiltration and inversely related to the short-term postoperative outcome.[59-61] Interestingly, in a retrospective analysis of human leukocyte antigens in Japanese BA patients who underwent living donor liver transplantation, the frequency of HLA-DR alone or the haplotype HLA-A24-B52-DR2 was significantly higher in patients with BA than in healthy Japanese volunteers or in the general Japanese populations described in the literature.[62] Our group has identified strong TGF-beta2 immunostaining in the bile duct epithelium, which was remarkably higher in liver transplantation.[63] Although TGF-beta is implicated in fibrogenesis in BA and other liver disorders, it also modulates homing, adhesion, chemotaxis and activation of T cells. Increased expression of intercellular adhesion molecule-1 (ICAM-1) in the bile ducts is associated with the postoperative prognosis.[50,64]

As mentioned previously, strong sinusoidal expression of immunoreactive CD14 in all BA patients with clinically evident cholestasis[44] indicates that sinusoidal endothelial cells are not exempt from immune modulation in BA. Taking together, the review shows that all cells of the innate immune system in the liver might be involved in the pathogenesis of BA.

Cytokines and other effector molecules

Innate immunity is executed through cytokines, antimicrobial peptides, proteases, cell adhesion molecules, reactive oxygen and nitrogen species, acute phase reactants.[41] Superoxide dismutase, a key enzyme in free radical protection, is increased significantly in the liver tissue of patients with BA, suggesting that products of free radical reactions are involved in the pathogenesis of BA.[65] Likewise, nitric oxide (NO) production is elevated in postoper-

ative BA patients and serum NO levels are correlated with serum alanine aminotransferase levels, suggesting a role for NO in liver injury associated with postoperative BA.[66]

The acquired or perinatal form of BA is a Th1 inflammatory disease in which liver osteopontin (OPN) mRNA and protein expressions are significantly increased in BA compared with normal and other cholestatic diseases. Moreover, osteopontin expression by human bile duct epithelial cells in culture is responsive to IL-2 and TNF-alpha.[67] Although TNF-alpha is increased in mice after bile duct ligation and after rotavirus infection,[68] the role of TNF-alpha expression in BA is still unknown. In one study, serum levels of TNF-alpha were not detectable in any postoperative patients with BA regardless of their status. On the contrary, serum levels of IL-6 and antiinflammatory cytokine interleukin-1 receptor antagonist were generally higher in postoperative BA patients with liver dysfunction than in controls.[69] Interestingly, circulating levels of another proinflammatory cytokine IL-18 were higher in BA patients before and after the Kasai procedure than in healthy controls. The high IL-18 level lasted for a long time after the procedure, even in patients without jaundice, but decreased immediately after liver transplantation.[49] Since in situ expression of these pro- and anti-inflammatory cytokines in the liver tissue in BA is generally unknown, the significance of the above findings requires further study.

In a study of graft acceptance after liver transplantation in children, the authors found that a balance toward a Th2 cytokine profile, including IL-4 and IL-10, in infants in the first months of life predisposes to improved graft acceptance.[70] In addition to IL-8, another C-X-C chemokine interferon-inducible protein-10 (IP-10) that targets both T cells and NK cells is up-regulated in multiple murine models of hepatic and bile duct injury, including bile duct ligation and CCl(4), D-galactosamine, and methylene dianiline toxic liver injuries.[71] The findings suggest a role for IP-10 in tissue regeneration after liver injury. Elevation of serum IP-10 levels is found in postoperative BA patients, which predicts hepatocyte death and correlates with progressive liver dysfunction and fibrosis in BA.[72] Thus IP-10, like other cytokines in BA, may actively participate in progressive liver injury, although the exact role is still uncertain.

Integration of innate immunity in the pathogenesis of BA

A recent microarray study to differentiate the embryonic and perinatal forms of BA revealed a unique pattern of expression of genes involved in chromatin integrity/function and overexpression of five imprinted genes in the embryonic form. However, there was no evidence of functional dominance of a specific immunity/inflammatory network between the embryonic and perinatal form.[73] The latter implies a common pathway underlying the pathogenesis of BA.

BA is not a simple familial or hereditary disorder. Genetic variation plays a determinant role in individuals susceptible to the development of BA. Besides the association of promoter polymorphism of CD14 with BA and idiopathic neonatal cholestasis,[48] mutations of human jagged 1 gene are found in severely ill BA patients subjected to liver transplantation at less than 5 years of age. Since the coexistence of human jagged 1 protein in Huh 7 cell cultures may suppress IL-8 production after TNF-alpha induction, the mutation of human jagged 1 gene has functional implications in BA.[74] Likewise, a decrease of plasma soluble CD14 from the early stage to the late stage of BA observed in carriers of the T/T and T/C genotypes but not in carriers of the C/C genotype[48] also indicates that variation in the CD14 gene has functional consequences in BA.

BA may start somewhere in the prenatal and in the early postnatal period. PAMPs from not yet identified pathogens may trigger the TLR signaling pathway, leading to activation of interferons (IFNs) and members of the IFN-regulatory factor (IRF) family in the innate immune system. Inhibition of virus replication by IFNs and TLR signaling may result in failure to detect virus when a full-blown picture of BA is shown. At the same time, the activation of proinflammatory cytokines leads to obliterative inflammation of the biliary system and, consequently, BA (Figure). Interestingly, one of the latest findings from our laboratory shows that MxA, a protein downstream of TLR7 signaling and also known as a highly specific marker for type I IFN signaling,[75] is found highly expressed in the early stage but not in the late stage of BA. The antimicrobial peptide hepcidin is found highly expressed in the early stage of BA and the expression correlates well with iron deposition in the liver. Down-regulation of hepcidin

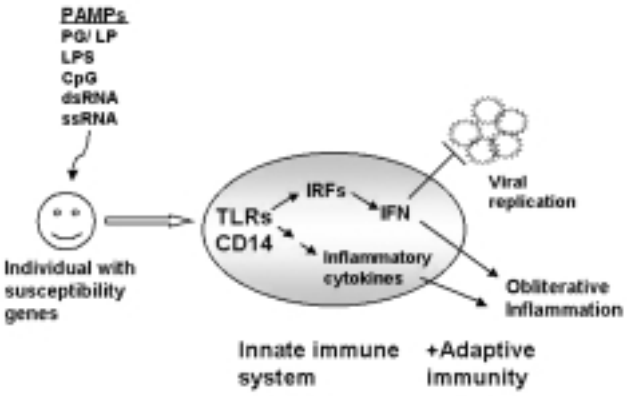

Figure Individuals with susceptibility genes (e.g. CD14) may be infected by pathogens with their particular pathogen-associated molecular patterns (PAMPs), which activate toll-like receptors (TLRs) and possibly CD14. TLR signaling then induces expression of specific members of the interferon (IFN)-regulatory factor (IRF) family and consequently activates IFN and proinflammatory cytokines. In conjunction with adaptive immunity, the process leads to obliterative inflammation of the biliary system characteristic of biliary atresia. PG, peptidoglycan; LP, lipopeptide; LPS, lipopolysaccharide; CpG, DNA with unmethylated CpG motifs; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA.

in the late stage of BA is disease-specific and is not found in HBV-associated liver cirrhosis.[76] Interestingly, increased expression of hepcidin after bacterial infection is TLR4 dependent in myeloid cells but not in hepatocytes.[77]

In summary, the significance of TLR signaling in BA is eminent and the interplay of the innate immune system with adaptive immunity is expected to be the key to uncovering a puzzling disorder like BA. The latter mechanism adds to the current view of BA as a consequence of genetic induction of proinflammatory immunity or an immune response to a perinatal insult (e.g. cholangiotropic viral infection).[7,20]

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天賦免疫參與膽道閉鎖的發病原理

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膽道閉鎖是迄今病因仍不明的一種複雜疾病。罹患此病之新生兒本身即帶有易感基因，致使其容易遭受某種仍不明的病原侵犯。這些病原都帶有可被宿主辨識，但仍屬於它們特有的病原相關分子型，後者透過宿主細胞上的特定受體，啓動天賦免疫系統。托樣受體（toll-like receptor，簡稱 TLR）是其中最關鍵性的受體。在膽道閉鎖，我們可以發現 TLR7 的表現顯著較高，而 CD14 啓動子的多形性和膽道閉鎖相關。抗菌蛋白質 hepcidin 和第一型干擾素調控特定蛋白質 MxA，在膽道閉鎖早期也顯著較高。根據這些資料，吾人推測天賦免疫系統中的 TLR 反應基因，與繼承性免疫攜手合作，啓動第一型干擾素及趨發炎細胞素的產生，進一步導致肝膽系統的發炎反應。本篇綜說揭露至今已知存於膽道閉鎖的天賦免疫的成員，並描述天賦免疫系統可能參與膽道閉鎖的發病原理。(長庚醫誌 2006;29:240-50)

關鍵字：膽道閉鎖，天賦免疫，CD14，托樣受體 (TLR)。

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