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ORIGINAL ARTICLE

Increased expression of Toll-like receptor 3 in intrahepatic biliary epithelial cells at sites of ductular reaction in diseased livers

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Abstract *Background* Toll-like receptors (TLRs) may play active roles in both innate and adaptive immune responses in human intrahepatic biliary epithelial cells (HIBECs). The role of TLR3 expressed by HIBECs, however, remains unclear. *Methods* We determined the in vivo expression of TLRs in biopsy specimens derived from diseased livers immunohistochemically using a panel of monoclonal antibodies against human TLRs. We then examined the response of cultured HIBECs to a TLR3 ligand, polyinosinic–polycytidylic acid (polyI:C). Using siRNAs specific for Toll-IL-1R homology domain-containing adaptor molecule 1 (TICAM-1) and mitochondrial antiviral signaling protein (MAVS), we studied signaling pathways inducing IFN-β expression. *Results* The expression of TLR3 was markedly increased in biliary epithelial cells at sites of ductular reaction in diseased livers, including primary biliary cirrhosis (PBC), autoimmune hepatitis (AIH), and chronic viral hepatitis (CH) as compared to nondiseased livers. Although cultured HIBECs constitutively expressed TLR3 at both the protein and mRNA levels in vitro, the addition of polyI:C to culture media induced only minimal increases in IFN-β mRNA. In contrast, transfection of HIBECs with polyI:C induced a marked increase in mRNAs encoding a variety of chemokines/cytokines, including IFN-β, IL-6, and TNF-α. The induction of IFN-β mRNA was efficiently inhibited by an siRNA against MAVS but not against TICAM-1, indicating that the main signaling pathway for IFN-β induction following polyI:C transfection is via retinoic acid-inducible gene I (RIG-I)/melanoma differentiation-associated gene 5 (MDA5) in HIBECs. *Conclusions* TLR3 expression by biliary epithelial cells increased at sites of ductular reaction in diseased livers; further study will be necessary to characterize it's in vivo physiological role.

Keywords Primary biliary cirrhosis (PBC) · Human intrahepatic biliary epithelial cells (HIBECs) · Interferon beta (IFN-β) · Toll-like receptor 3 (TLR3) Toll-IL-1R homology domain-containing adaptor molecule 1 (TICAM-1) · Mitochondrial antiviral signaling protein (MAVS) · Retinoic acid inducible gene I (RIG-I) · Melanoma differentiation-associated gene 5 (MDA5)

Abbreviations

BEC	Biliary epithelial cell
CK	Cytokeratin
dsRNA	Double stranded RNA
ER	Endoplasmic reticulum
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
HIBEC	Human intrahepatic biliary epithelial cell
HRP	Horseradish peroxidase
IFN	Interferon

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IL	Interleukin
IRF	Interferon regulatory factor
MAVS	Mitochondrial anti-viral signaling protein
MDA5	Melanoma differentiation associated gene-5
MyD88	Myeloid differentiation factor 88
PBC	Primary biliary cirrhosis
PBMC	Peripheral blood mononuclear cells
PolyI:C	Polyinosinic–polycytidylic acid
PRR	Pattern-recognition receptor
RIG-I	Retinoic acid-inducible gene I
RT-PCR	Reverse transcription-polymerase chain reaction
siRNA	Small interfering RNA
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TICAM-1	Toll-IL-1R homology domain containing adaptor molecule 1

Introduction

Epithelial cells are the first barrier against viral infection. Such cells typically express retinoic acid-inducible gene I (RIG-I)/melanoma differentiation-associated gene 5 (MDA5) and Toll-like receptor 3 (TLR3) to sense double-stranded RNAs (dsRNA), hallmarks of viral replication [1–3]. TLR3 is localized to endosomes and/or the cell surface in epithelial cells, while RIG-I/MDA5 resides in the cytoplasm [3–5]. TLR3-expressing epithelial cells are widely distributed throughout the body, with prominent expression in intestinal, cervical, uterine, endometrial, bronchial, and corneal epithelial cells, the central nervous system, and epidermal keratinocytes [6–16]. The function of TLR3 has been intensively studied in some of these epithelial cells; bronchial epithelial cells recognize dsRNA by cell-surface TLR3 and induce cellular responses, including the secretion of type 1 interferon (IFN) via the Toll-IL-1R homology domain-containing adaptor molecule 1 (TICAM-1)-interferon regulatory factor 3 (IRF3) signaling pathway [11, 12]. The intracellular RNA sensors RIG-I/MDA5 also serve as IFN inducers acting via the mitochondrial antiviral signaling protein (MAVS)-IRF3 signaling pathway, thus protecting host cells against the spread of viral invasion [2, 3].

We previously found that the expression of TLR3 and IFN-β mRNAs is significantly increased in both the portal areas and parenchyma of livers diseased with PBC [17]. There was a positive correlation between TLR3 and IFN-β mRNA levels in both areas, indicating that TLR3-type 1 IFN signaling pathway is activated in PBC; the TLR3-expressing and/or IFN-β-producing cells, however, remain unknown [17]. This prompted us to investigate TLR3 expression and IFN-β production in human intrahepatic biliary epithelial cells (HIBECs).

In this study, we used specific monoclonal antibodies against TLRs [4] to determine that intrahepatic bile ducts, but not hepatocytes, in diseased livers strongly express TLR3. TLR3 protein is found in HIBECs at low levels on the cell surface and high levels in endosomes. Our results, however, indicate that the primary signaling pathway for IFN-β induction activated by dsRNA functions via RIG-I/MDA5 in the cytoplasm but not via TLR3 expressed on the cell surface or in endosomes. This is contrary to results obtained for other types of epithelial cells, such as bronchial epithelial cells and endometrial cells, in which surface TLR3 recognizes viral dsRNA to signal the presence of infection via the TLR3-IRF3-type I interferon signaling pathway [9, 11, 12, 15]. Here we discuss dsRNA-sensing system functioning in HIBECs and the role of high expression levels of TLR3 in diseased livers.

Materials and methods

Liver biopsy specimen and immunohistochemical evaluation

Liver needle biopsy specimens, which were derived from seven primary biliary cirrhosis (PBC)-affected, five autoimmune hepatitis (AIH)-affected, and five chronic hepatitis C (CHC)-affected livers, were frozen in OCT compound (Sakura Finetechnical Co, Tokyo, Japan) immediately after the procedure and were stored at −80°C until use. Mouse monoclonal antibodies to human TLR1 (clone TLR1.136, IgG1, *k*), TLR2 (clone TLR2.45, IgG1, *k*), TLR3 (clone TLR3.7, IgG1, *k*), TLR4 (clone TLR4, IgG2a, *k*), and TLR6 (clone TLR6.127, IgG1, *k*) were generated in our laboratory [4]. Among these monoclonal antibodies, the specificity of anti-TLR3 (TLR3.7) was intensively studied. Anti-TLR3 monoclonal antibody specifically binds to the extracellular part of native TLR3 but not to denatured form of TLR3 or other TLRs, including TLR2 and TLR4. Furthermore, TLR3.7 inhibits dsRNA-induced IFN-β production by inhibiting the interassociation between dsRNA and TLR3 [4, 5]. Mouse monoclonal antibodies specific for cytokeratin (CK) 7 and CK 19 were purchased from DAKO (DAKO Japan, Kyoto, Japan). Frozen sections, 4 mm in thickness, were stained with anti-TLR and anti-CK7 or -CK19 antibodies as described elsewhere [17]. Briefly, frozen sections were first fixed in 50 and 100% acetone for 30 s and 3 min, respectively, followed by treatment with Peroxidase Blocking agent (DAKO) for 10 min. Sections were then incubated with anti-TLR monoclonal antibodies (anti-TLR1, 2, 3, 4, and 6) for 60 min at room temperature. A standard 2-step method with ENVISION+ (DAKO) was used to visualize bound antibody using 3,3′-diaminobenzidine as a chromogen

(DAKO); samples were also counterstained with Mayer's hematoxylin (DAKO). Three frozen liver biopsy specimens, which revealed normal histology, were similarly studied as nondiseased livers.

Isolation and culture of human intrahepatic biliary epithelial cells

Human intrahepatic biliary epithelial cells (HIBECs) were isolated from noncancerous liver tissues of three patients who had undergone hepatic resection for intrahepatic cholangiocarcinoma [18]. Briefly, liver specimens were digested with type IV collagenase (100 U/ml) (Sigma–Aldrich, St. Louis, MO). HIBECs were isolated immunomagnetically using Dynabeads conjugated with an epithelium-specific antibody, BerEp4 (Dynal Biotech, Norway). HIBECs were expanded in HIBEC culture medium (DMEM containing 5 μg/ml insulin, 10 ng/ml epidermal growth factor [EGF], 1.0 ng/ml hepatocyte growth factor [HGF], 4×10^{-8} M dexamethasone and 10% fetal bovine serum). All experiments were performed using HIBECs between 5 and 10 passages, which were performed using PBS containing 0.05% trypsin and 0.53 mM EDTA.

We obtained three different HIBECs (BEC3, BEC4, and BEC5) from the three different donors; each cultured HIBEC demonstrated spindle to polygonal epithelial cell morphology, with 100% positivity for CK7 and CK19 as determined by immunostaining with anti-CK7 and -CK19 monoclonal antibodies (DAKO).

Immunostaining and flow-cytometric analysis of HIBECs

HIBECs were cultured to semiconfluence in a tissue culture-treated 8-chamber glass slides (BD Biosciences, Bedford, MA) in HIBEC culture medium. Immunostaining of these cultured cells was then performed in a similar manner as that described for frozen sections of liver biopsies [17]. In brief, HIBECs were fixed with acetone, treated with peroxidase-blocking agent, and incubated with anti-TLR (anti-TLR1, -2, -3, -4, and -6) and anti-CK7 or anti-CK19 monoclonal antibodies followed by visualization of bound antibodies using a standard 2-step method with ENVISION+ (DAKO).

For flow-cytometric analysis, HIBECs were first suspended in PBS containing 0.1% sodium azide and 0.1% bovine serum albumin before incubating with 5 μg mAb (clone TLR3.7, IgG1, *k*) for 30 min at 4°C. Cells were washed and counterstained with FITC-conjugated goat antimouse IgG $F(ab')_2$ for 30 min at 4°C. We then determined fluorescence intensity and mean fluorescence shifts by flow cytometry (FACSCalibur; Becton-Dickinson).

Stimulation of HIBECs with polyI:C

Polyinosinic–polycytidylic acid (PolyI:C) was purchased from Sigma–Aldrich and reconstituted in endotoxin-free PBS. Transfection reagents, Lipofectamine 2000 and DOTAP, were purchased from Invitrogen (Carlsbad, CA) and Roche (Basel, Switzerland), respectively.

Twenty-four hours prior to the start of polyI:C stimulation, we changed the culture medium from HIBEC culture medium to basal medium (1:1 mixture of Ham's F12 and DMEM supplemented with 10% FBS without insulin, EGF, HGF, and dexamethasone). HIBECs were then incubated in the presence of polyI:C (40 μg/ml) or transfected with polyI:C using Lipofectamine 2000 or DOTAP according to the manufacturer's instructions. Optimal conditions for transfection by Lipofectamine 2000 and DOTAP were 0.8 μg/well and 1.0 μg/well polyI:C, respectively, in a 12-well plate (Becton Dickinson, Franklin Lakes, NJ) (data not shown).

RNA extraction and quantitation of mRNA

Total RNA was isolated from HIBECs using an RNeasy MiniKit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Following RNase-free DNase I (QIAGEN) treatment, we synthesized first-strand complementary DNAs (cDNA) from 1.0 μg total RNA using a SuperScript First-Strand Synthesis System (Invitrogen). PCR amplification utilized FAST DNA SYBR Green I (Roche), which allows for automated quantification of amplified products in real-time using a Light-Cycler (Roche). We purchased primer sets specific for IFN-γ, IL-6, TNF-α, IL-8, and TLR3 from Roche. One microliter of each reverse-transcribed cDNA was used for real-time PCR analysis. Initial denaturation was performed at 95°C for 10 min followed by 40 amplification cycles of denaturation at 95°C for 10 s, annealing at 68°C for 10 s, and extension at 72°C for 16 s. We performed a standard melting curve analysis for every quantitation. Results were expressed as the ratio of cytokine/chemokine cDNA to GAPDH cDNA copy numbers in individual samples. Changes in mRNA levels were expressed as fold induction.

Enzyme-linked immunosorbent assay (ELISA)

HIBEC culture supernatants were assessed for cytokine/chemokine secretion using ELISA kits specific for IFN-β (PBL Biomedical Laboratories, Piscataway, NJ) and TNF-α, IL-6, and IL-8 (Beckman Coulter, Fullerton, CA), according to the manufacturers' instructions. Absorbance at either 405 or 450 nm was measured using a microplate reader (Multiskan JX, Thermo electron corporation, Vantaa, Finland).

Effect of siRNA on IFN-β mRNA induction by polyI:C

Oligonucleotides used for siRNA knockdown analysis were purchased from Proligo (St. Louis, MO) (GFP: sense, gcagcacgacuucuucaagtt, and antisense, cuugaagaagucgugc ugctt, MAVS: sense, ccaccuugaugccugugaaca, and antisense, uucacaggcaucaagguggua, TICAM-1: sense, gaccaga cgccacuccaactt, and antisense, guuggaguggcgucugguctt).

BEC3 cells (2.5×10^5 per well) were plated on 12-well plates using basal medium 24 h before siRNA transfection. On day 0, we transfected each siRNA oligonucleotide into BEC3 using Lipofectamine 2000. After 24 h (on day 1), the culture medium was replaced with fresh basal medium. Forty-eight hours after siRNA transfection (on day 2), cells were stimulated with polyI:C (final concentration 40 μg/ml in phosphate-buffered saline), Lipofectamine 2000 (0.8 μg/well in 12-well plates) or DOTAP (1.0 μg/well in 12-well plates). Six hours after polyI:C stimulation, we purified total RNA from BEC3 cells using an RNeasy Mini kit (Qiagen). RT-PCR was performed using M-MLV Reverse transcriptase (Promega, Madison, WA). Quantitative PCR analyses were carried out on an iCycler iQ Real-Time detection system (Bio-Rad, Hercules, CA) using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) using the following primers; β-actin: forward, cctggcacccagcacaat, and reverse, gccgatccacacggagtact; IFN-β: forward, caacttgcttggattcctacaaag, and reverse, tattcaagcctcccattcaattg; MAVS: forward, ggtacccgagtctcgtttcct, and reverse, ttgtcttcagcaaacggcatt; TICAM-1: forward, agcgccttcgacattc taggt, and reverse, aggagaaccatggcatgca [19]. Quantitative PCR data was analyzed by the $2^{-\Delta\Delta CT}$ methods [20].

Ethics board

This study was approved by the Ethics Board of our institute. We obtained written informed consent from each subject for use of their biopsy and operation samples to advance knowledge on the cause of PBC.

Results

HIBECs strongly express TLR3 molecule in vitro

Previous analysis of the expression of TLRs mRNA in HIBECs by RT-PCR revealed that mRNAs encoding TLR1, -2, -3, -4, -5, -6, and -9, but not TLR7 and -8, are constitutively expressed in HIBECs [18]. The expression of TLR proteins, however, has not been examined. In this study, we studied the expression of TLR proteins in HIBECs using mouse monoclonal antibodies against TLR1, -2, -3, -4, and -6 [4]. TLR3 was strongly expressed on HIBECs (Fig. 1c) at an intensity comparable to that of CK19, a marker of biliary epithelial cells in the liver (Fig. 1f). In contrast, TLR1, -2, and -4 were weakly expressed on HIBECs (Fig. 1a, b, d). Flow-cytometric analysis revealed that while TLR3 was strongly expressed in the cytoplasm (Fig. 2a), it was only weakly expressed at the cell surface of HIBECs (Fig. 2b).

Biliary epithelial cells strongly express TLR3 at sites of ductular reaction in vivo

We examined the expression of TLRs in vivo using frozen sections of liver needle biopsy specimens using the monoclonal antibodies to TLR1, -2, -3, -4, and -6. Again, TLR3 was strongly expressed by intrahepatic biliary epithelial cells, especially at sites of ductular reaction in all diseased livers affected by PBC (Fig. 3b), AIH (Fig. 3c), and CHC (Fig. 3d). TLR3 was only weakly expressed on small bile ducts in normal livers (Fig. 3a). TLR3 was not expressed in hepatocytes of either diseased or normal livers. Minimal expression of TLR1, -2, -4, and -6 was observed in either diseased or normal livers (data not shown).

Induction of IFN-β by polyI:C stimulation in HIBECs

We examined the induction of IFN-β by HIBECs cultured for 24 h in basal medium containing 0–160 μg/ml of polyI:C, a ligand for TLR3. IFN-β secretion was consistently undetectable in culture supernatants, as determined by ELISA (data not shown). IFN-β mRNA copy numbers were calculated as 0.00024 ± 0.00017/GAPDH (mean value $\pm$ SD, $n = 3$) in the basal state, which increased by 2.7- to 5.5-fold (0.0012 ± 0.0010/GAPDH, $n = 3$) after 4–8 h of polyI:C stimulation (Fig. 4a). The increase in IFN-β mRNAs, however, was not statistically significant ($P = 0.195$). In contrast, IFN-β mRNA expression was markedly increased by polyI:C transfection using Lipofectamine 2000 (Fig. 4b). Maximal induction reached approximately 150-fold 4–8 h after transfection. Approximately 250 pg/ml IFN-β was secreted into culture medium over a 24-h period after polyI:C transfection. These results suggest that TLR3, even when expressed on the cell surface of HIBECs, does not recognize extracellular polyI:C; instead, TLR3 localized to endosomes and/or RIG-I/MDA5 in the cytoplasm sense polyI:C in HIBECs.

We also examined the induction of other chemokine/cytokine mRNAs. We observed the potent induction of mRNAs encoding for IL-6 (basal level 0.019–0.052/GAPDH) and TNF-α (basal level 0.00072–0.00081/GAPDH) to levels 50- and 120-fold greater than baseline, respectively (Fig. 5b, c). We detected 4- to 6-fold induction of mRNA for IFN-α (basal level 0.0059–0.0128/

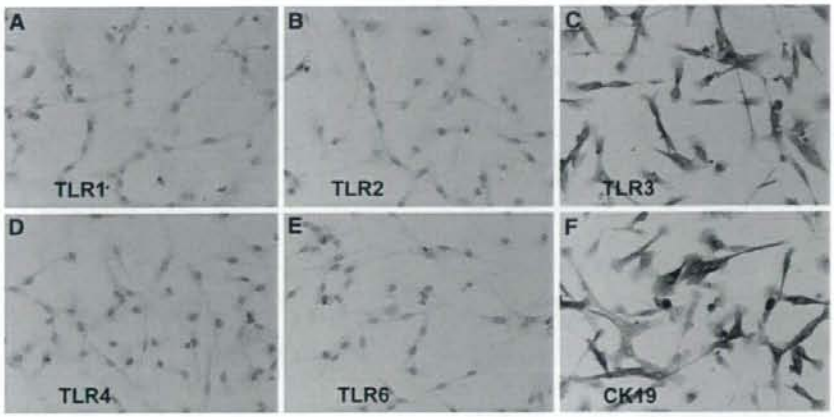

Fig. 1 TLRs immunostaining in cultured human intrahepatic biliary epithelial cells (HIBECs). BEC3 cells were stained with mouse monoclonal antibodies: (**a**) TLR1.136 (diluted 1/80); (**b**) TLR2.45 (diluted 1/100); (**c**) TLR3.7 (diluted 1/100); (**d**) HTA125 (diluted 1/70); (**e**) TLR6.127 (diluted 1/80); (**f**) anti-CK19 (diluted 1/200) for TLR1, TLR2, TLR3, TLR4, TLR6, and CK19, respectively, as described in the text. BEC3 cells stained strongly with TLR3.7 but only weakly with TLR1.136, TLR2.45, and HTA125

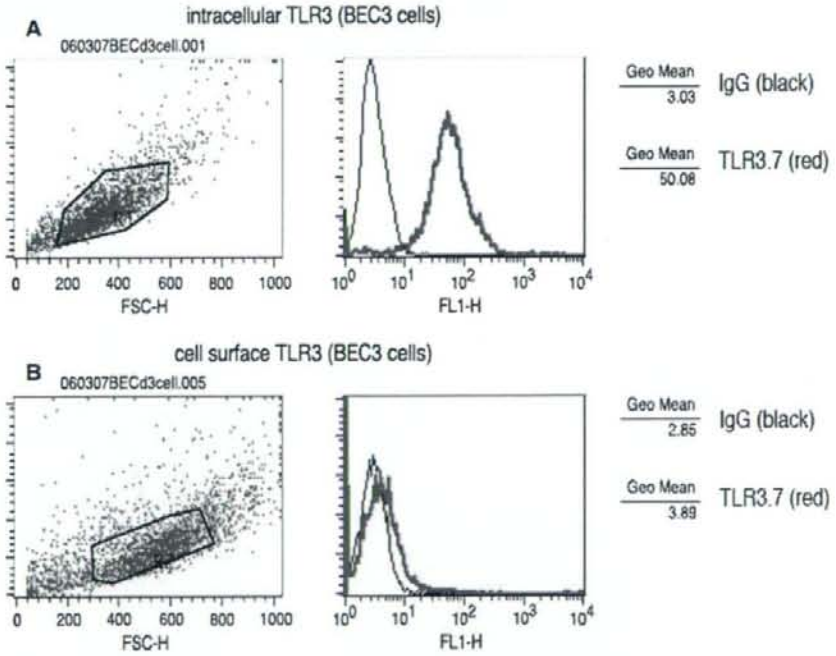

Fig. 2 Flow-cytometeric analysis of TLR3 in cultured human intrahepatic biliary epithelial cells (HIBECs). BEC3 cells were stained with TLR3.7 monoclonal antibody intracellularly (**a**) or extracellularly (**b**). BEC3 cells exhibited strong intracellular staining with TLR3.7 but only weak cell surface staining

GAPDH), IL-8 (basal level 0.241–0.859/GAPDH), and TLR3 (basal level 0.0064–0.0081/GAPDH) (Fig. 5d–f). This upregulation in gene expression is also attributable to intracytoplasmic polyI:C recognition, since addition of polyI:C to culture medium did not induce any increase of mRNA levels for IL-6, TNF-α, IFN-α, IL-8, and TLR3 (data not shown).

Induction of IFN-β mRNA by polyI:C transfection depend on MAVS pathway but not on TICAM-1 pathway in HIBECs

To further confirm the functional role of TLR3 in the induction of IFN-β mRNA in HIBECs, we performed knockdown experiments using siRNA specific for TICAM-

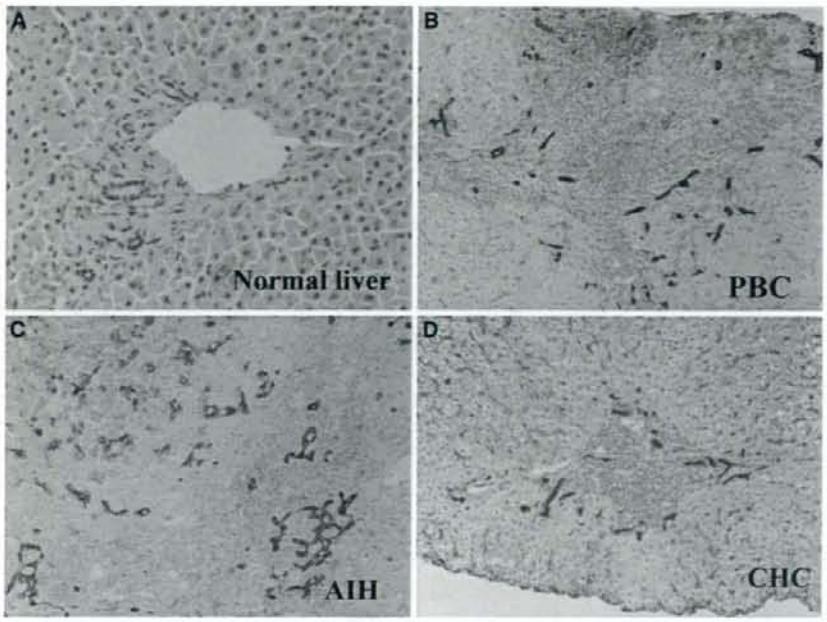

Fig. 3 In vivo expression of TLR3 in intrahepatic biliary epithelial cells. TLR3 was strongly expressed on intrahepatic biliary epithelial cells, especially at sites of ductular reactions, in livers from patients with PBC (**b**), AIH (**c**), and CHC (**d**). In contrast, TLR3 was weakly expressed on small bile ducts in normal liver (**a**)

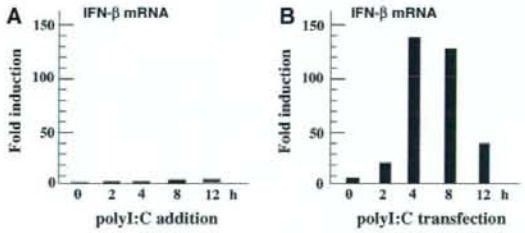

Fig. 4 Induction of IFN-β mRNA by polyI:C in HIBECs. BEC3 cells were either cultured in basal medium containing polyI:C (**a**) or transfected with polyI:C using Lipofectamine 2000 (**b**). mRNA encoding IFN-β was strongly induced by polyI:C-transfection, while IFN-β mRNA induction was minimal following the addition of polyI:C to culture medium

1 or MAVS. We first evaluated the efficiency of knockdown. Knockdown significantly reduced the mRNA levels of MAVS and TICAM-1 in HIBECs to approximately 30% of baseline using the corresponding siRNA (Fig. 6a). We then examined the effect of MAVS or TICAM-1 knockdown on the induction of IFN-β mRNA. As more efficient targeting of nucleotides to the endosomal compartment was reported by using DOTAP in comparison to Lipofectamine 2000 [21], we utilized DOTAP for the induction of IFN-β mRNA in knockdown experiments.

Interferon-β induction following polyI:C stimulation using Lipofectamine 2000 was largely dependent on MAVS/IPS-1, but not on TICAM-1 (Fig. 6b left side). Unexpectedly, similar results were obtained following polyI:C stimulation using DOTAP (Fig. 6b right side). These results suggested that the RIG-I/MDA5 (sensors of dsRNA in the cytosol)-MAVS signaling pathway plays a major role in the induction of IFN-β mRNA in HIBECs. Abundant expression of TLR3 in endosomes does not appear to participate significantly in polyI:C-mediated IFN-β induction in HIBECs.

Discussion

In this study, we provide the first data demonstrating that TLR3 is expressed in vitro in the cultured HIBECs; in these cells, IFN-β mRNA is strongly induced by polyI:C transfection, but only weakly induced by extrinsic polyI:C. Antibody blocking of TLR3 on HIBECs did not result in abrogation of IFN-β promoter activity, suggesting that cell-surface TLR3 participates only minimally in IFN-β promoter activation on dsRNA recognition (data not shown). These results suggested that endosomal, not cell surface, TLR3 is actively involved in type I IFN production by HIBECs. The results obtained by siRNA knockdown of TICAM-1 or MAVS, however, indicated that cytoplasmic RNA sensors like RIG-I/MDA5, not endosomal TLR3, are the major receptors initiating type I IFN induction in HIBECs.

To limit the growth of commensal organisms on their surface and to defend underlying tissues from invading pathogens, epithelial cells have both innate immune

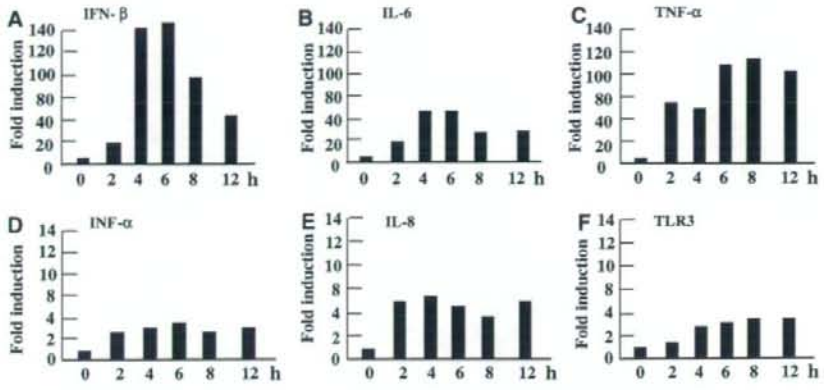

Fig. 5 Induction of chemokine/cytokine mRNAs in HIBECs following polyI:C-transfection. We observed strong induction of mRNAs encoding IFN-β (**a**), IL-6 (**b**), and TNF-α (**c**), but only weak induction of mRNAs for IFN-α (**d**), IL-8 (**e**), and TLR3 (**f**)

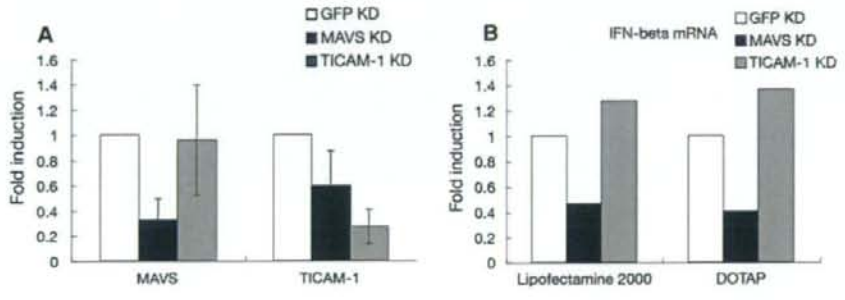

Fig. 6 Effect of MAVS or TICAM-1 knockdown on the induction of IFN-β mRNA following polyI:C transfection. mRNA levels of MAVS and TICAM-1 in HIBECs significantly decreased to 30% of baseline levels by knockdown using an appropriate siRNAs in HIBECs (**a**). The induction of IFN-β mRNA in HIBECs after polyI:C transfection was efficiently inhibited by MAVS but not by TICAM-1 knockdown (**b**)

antimicrobial functions and the ability to modulate the recruitment and activity of innate and adaptive immune system [1, 3]. Human fibroblasts, colon epithelial cells, lung epithelial cells, corneal epithelial cells and keratinocytes, as well as the respective cell lines, express TLR3 on their cell surfaces [4, 5, 7, 12, 14, 16]. Recent analyses of TLR3 subcellular localization, however, have suggested that TLR3 is localized to endoplasmic reticulum (ER) and early endosomes in most human epithelial cell types [5]. A similar localization of TLR3 was observed in HIBECs in the present study; the HIBECs express TLR3 on both the cell surface and within intracellular organelles.

Unexpectedly, surface TLR3 in HIBECs exerted only a weak ability to induce type I IFN in response to polyI:C. As polyI:C must be internalized and delivered to the ER or early endosomes, in which TLR3 is abundant, to activate TLR3, it was speculated that the capacity of HIBECs to internalize polyI:C is weak. Intracellular polyI:C that was internalized into cells by lipofection, however, did not play a major role in activating the type I IFN promoter via TLR3. These results indicate that even if the bile fluid contains dsRNA that may be derived from the gastrointestinal tract via the portal vein, hepatocytes or cholangiocytes infected with virus, or apoptotic cell debris, bile fluid only minimally stimulates TLR3 on the surface or in endosomes to induce type I IFN, although it is also possible that bile fluid may contain as yet unknown TLR3-ligand to induce type I IFN. Further studies of TLR3 in HIBECs will be needed to identify the functional specificities of the surface-expressed and endosome-expressed TLR3.

In this study, we also provide the first evidence that the expression of TLR3 by intrahepatic biliary epithelial cells is markedly increased at sites of ductular reaction in diseased livers, including those affected by PBC, AIH, and CHC. TLR3 protein expression increased in synovial tissues from patients with RA. In addition, cultured RA synovial fibroblasts were activated by the TLR3 ligand polyI:C and by RNA released from necrotic synovial fluid cells, suggesting that necrotic cells may act as an endogenous TLR3 ligand leading to the stimulation of proinflammatory gene expression and autoimmunity [22–24]. The overexpression of TLR3 in thyrocytes is associated with the development of Hashimoto's autoimmune thyroiditis [25]. TLR3 activation can drastically

enhance susceptibility to immune destruction of solid organs, as seen in autoimmune hepatitis [26]. Exposure of pancreatic β cells to the combination of dsRNA and IFN-α, -β, or -γ significantly increases apoptosis [27, 28]. TLR3 can directly trigger apoptosis in human umbilical vein endothelial cells and cancer cells [29, 30]. TLR3 plays a role in the development of hepatitis C-associated glomerulonephritis through the induction of mesangial cell apoptosis [31]. Thus, enhanced TLR3 expression in intrahepatic biliary epithelial cells may play a critical role in the induction and maintenance of inflammation, immune destruction, and/or biliary epithelial cell apoptosis in vivo in diseased liver such as PBC, whereas enhanced TLR3 expression in biliary epithelial cells in CHC may play a critical role for protecting them from hepatitis virus infection.

TLR3 in the nervous system induces the expression of a range of neuroprotective mediators and angiogenic factors, chemokines, and anti-inflammatory cytokines that regulate astrocyte cellular growth, differentiation, and migration [32]. Activation of TLR3 protects against DSS-induced acute colitis [33]. Thus, it is possible that high TLR3 expression in HIBECs at sites of ductular reaction may protect against cell death or stimulate tissue repair and regeneration by inducing the production of as yet unknown protective and/or growth factors. The strong expression of TLR3 at ductal plate in human fetal liver indicates the importance of TLR3 in the regeneration and/or development of biliary epithelial cells (data not shown). Therefore, it is also considered possible that as yet unknown TLR3-ligand is involved in the development of ductular reaction in diseased livers including PBC, AIH, and CHC.

Enhanced expression of various molecules, including MHC-class I and class II antigen, adhesion molecules (ICAM-1, VCAM-1, LFA-3, etc.), chemokines (MCP-1, SDF-1, Fractalkine, etc.), cytokines (IL-6, IL-8, TNF-α, etc.), costimulatory molecules (B7, PD-L1, PD-L2, etc.), and TLR4, have also been reported in biliary epithelial cells in livers affected by PBC [34–38]. In addition to these molecules involved in innate and acquired immune response, we here demonstrated for the first time that RIG-I/MDA5–MAVS signaling pathway is operative in the strong induction of IFN-β by dsRNA stimulation in HIBECs. TLR3 and RIG-I/MDA5 expression increase in the presence of IFN-α, IFN-β, IFN-γ, and TNF-α in vitro [39–43]. These results may indicate that intrahepatic biliary epithelial cells are involved as an immunoregulatory organ in various liver diseases, including PBC, AIH, and CHC. In addition, the portal inflammation is closely associated with ductular reaction in periportal areas. As hepatic stem cells are speculated to reside alongside biliary epithelial cells in canal of Hering [44, 45], the existence of multiple IFN-inducing pathways, including TLR3 and RIG-I/MDA5, may suggest the importance of this innate immune effector pathway in the protection of putative hepatic stem cells from viral infection.

In conclusion, we demonstrated for the first time the increased expression of TLR3 at sites of ductular reaction in diseased livers including PBC, AIH, and CHC. Since cytoplasmic RNA sensors like RIG-I/MDA5, not TLR3, seem to be the major receptors initiating strong type I IFN induction in biliary epithelial cells, we speculate that there is another important role in TLR3 that is highly expressed in biliary epithelial cells. Further study will be necessary to characterize its in vivo physiological role.

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TLR3: Interferon induction by double-stranded RNA including poly(I:C) ☆

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Abstract

Toll-like receptor 3 (TLR3) recognizes viral double-stranded RNA and its synthetic analog polyriboinosinic:polyribocytidylic acid (poly(I:C)) and induces type I interferon (IFN), inflammatory cytokine/chemokine production and dendritic cell (DC) maturation via the adaptor protein TICAM-1 (also called TRIF). TLR3 is expressed both intracellularly and on the cell surface of fibroblasts and epithelial cells, but is localized to the endosomal compartment of myeloid DCs. Several studies in TLR3-deficient mice demonstrate that TLR3 participates in the generation of protective immunity against some viral infections. Involvement of TLR3-TICAM-1 in activation of NK cells and CTLs by myeloid DCs suggests that TLR3 serves as an inducer of cellular immunity sensing viral infection rather than a simple IFN inducer. In this review, we summarize the current knowledge on TLR3 and discuss its possible role in innate and adaptive immunity.

Keywords: Adjuvant; Cross-priming; Double-stranded RNA; Innate immunity; NK cell activation; Toll-like receptor; Type I interferon; Viral infection

Contents

1. Introduction

Type I interferon (IFN) was first discovered in the 1950s from the independent studies by Nagano and Isaacs, in which it was demonstrated that animal cells infected with viruses

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released anti-viral factors [1,2]. Both natural and synthetic double-stranded (ds) RNAs elicit IFN production [3,4]. Several studies have suggested that human cells recognize particular spatial and steric organizations of dsRNA via putative cell membrane receptors and produce type I IFN [5–7]. Among the synthetic dsRNAs, polyriboinosinic:polyribocytidylic acid (poly(I:C)) was found to be the most potent IFN inducer [5]. In in vivo studies in mice, intraperitoneal injection of poly(I:C) elicited IFN-α/β production and NK cell activation [8,9]. However, the mechanisms by which cells recognize dsRNA and produce IFN-α/β remain largely unknown.

At the beginning of this century, Toll-like receptor 3 (TLR3), a member of the TLR family proteins, was identified as a receptor for dsRNA [10,11]. Upon recognition of dsRNA, TLR3 transmits signals via the adaptor protein Toll-IL-1 receptor (TIR) domain-containing adaptor molecule-1 (TICAM-1) (also called TIR-domain-containing adapter inducing IFN-β [TRIF]). This activates the transcription factors interferon regulatory factor 3 (IRF-3), NF-κB, and AP-1 (a complex of activating transcription factor 2 (ATF2) and JUN), leading to the induction of type I IFN (especially IFN-β), cytokine/chemokine production and dendritic cell (DC) maturation [12,13]. Although TLR3 participates in the dsRNA-induced production of type I IFN, TLR3-null cells still produce IFN-α/β in response to viral infection [14]. Additional cytosolic dsRNA receptors, retinoic-acid inducible gene-I (RIG-I) and melanoma differentiation associated antigen 5 (MDA5), have been recently identified [14,15]. RIG-I and MDA5 recognize 5′-triphosphate-containing ssRNA/dsRNA and poly(I:C), respectively and induce IFN-α/β production [16,17]. Studies using knock-out mice clearly showed that RIG-I is essential for the production of IFN-α/β in response to RNA viruses whose genomes contain 5′-triphosphate, whereas MDA5 is involved in the detection of picornaviruses [18,19]. Based on the different subcellular localization of cytosolic RNA receptors and TLR3, these receptors seem to play distinct roles in anti-viral immune responses. In this review, we summarize the current knowledge on TLR3 and discuss its possible role in innate and adaptive immunity.

2. Properties of TLR3

2.1. Structure of TLR3

Human TLR3 consists of an extracellular domain containing 23 leucine rich repeats (LRRs) and N- and C-terminal flanking regions, the transmembrane domain, and the intracellular TIR domain [20]. It possesses 15 putative carbohydrate-binding motifs in the extracellular domain. Recent structural analyses of the human TLR3 ectodomain revealed that the LRRs form a large horseshoe-shaped solenoid of which one face is largely masked by carbohydrate, while the other face is glycosylation-free [21,22]. TLR3 molecules are arranged as dimers in the crystals, and the C-terminal highly-conserved surface residues and a TLR3-specific LRR insertion at LRR20 forms a homodimer interface [21]. In addition, there are two patches of positively charged residues on the glycosylation-free surface [21]. Bell et al. demonstrated that H^{539} and N^{541} in TLR3-LRR20, located on the glycan-free lateral face, are critical amino acids for ligand binding and signaling [23]. The positively charged residues in the two patches and the carbohydrate moiety are not involved in TLR3 function [23]. Based on these results together with the structure of dsRNA, a dsRNA-induced symmetrical receptor crosslinking model has been proposed for TLR3 [23,24]. An LRR-deletion study on TLR3 suggests that the C-terminal LRRs (LRR20-LRR22) control the receptor dimerization and signaling [25]. However, it is not yet understood how extracellular receptor-receptor interactions induce structural reorganization of the cytoplasmic TIR domain, necessary for TICAM-1 binding and activation.

2.2. Expression and subcellular localization of TLR3

Human *TLR3* mRNA has been detected in the placenta, pancreas, lung, liver, heart and brain [26]. It is also expressed in myeloid DCs and intestinal epithelial cells but not in monocytes, polymorphonuclear leukocytes, T, B and NK cells, suggesting a physiological role in innate immunity [27–29]. In addition, the second type of DC precursor cell, pre-DC2 (previously known as plasmacytoid DC precursor), which expresses TLR7 and TLR9 and secretes large amounts of IFN-α in response to ssRNA and imidazoquinoline compounds (TLR7 ligands) or CpG DNA (TLR9 ligand), does not express TLR3 [30,31]. These observations suggest that these DC subsets have different roles in anti-viral immune responses.

It has been shown using flow cytometry with anti-human TLR3 mAb (TLR3.7) that human fibroblasts and epithelial cells express TLR3 both intracellularly and on the cell surface while monocyte-derived immature DCs and $CD11c^+$ blood DCs only express TLR3 intracellularly [11,31]. TLR3.7 mAb inhibits poly (I:C)-induced IFN-β production by fibroblasts, indicating that the cell-surface TLR3 participates in the poly(I:C) recognition [31]. However, in both fibroblasts and myeloid DCs, TLR3 signaling arises in the intracellular compartment, requiring endosomal maturation [31]. Immunofluorescent staining and confocal microscopic analysis of myeloid DCs and human embryonic kidney (HEK) 293 cells stably expressing human TLR3 revealed that TLR3 localizes to specific unidentified intracellular vesicles [32]. More recently, it has been shown that some TLR3 molecules localize to the early endosome in epithelial cell lines [33,34]. The structural motifs determining the intracellular localization of TLR3 are located in the 'linker' region between the transmembrane domain and the TIR domain of TLR3 [32,35]. This differs from the regulatory mechanism of intracellular TLR7 and TLR9 [35,36]. Interestingly, murine embryonic fibroblasts (MEFs) do not respond to exogenous addition of poly(I:C), suggesting that cell-surface expression of TLR3 is species-specific in fibroblasts.

In macrophages, DCs and epithelial cells, TLR3 expression is up-regulated by viral infection and exogenous addition of poly (I:C) or type I IFN [37–39]. In addition, TLR3 expression in human astrocytes is increased by poly(I:C) stimulation [40,41]. The IFN-responsive element (ISRE) located at approximately −30 bp on the human *TLR3* promoter region is responsible for viral- and poly(I:C)-induced *TLR3* gene expression [38,39]. Released IFN-α/β acts on DCs in an autocrine manner through

IFN-α/β receptor to induce TLR3 expression. Even in this case, TLR3 expression is up-regulated intracellularly but not on the cell surface in DCs. Importantly, the regulation of murine TLR3 expression is somewhat different from that of human TLR3 [38]. Although lipopolysaccharide strongly induces TLR3 expression in mouse macrophages and DCs [10,38], this effect is not observed in human cells. The sequences of the proximal promoter regions as well as the non-coding 5′-exons are different in these two species [42]. Despite the overall characteristic difference in *TLR3* promoter sequences, mRNA expression of *TLR3* is induced by type I IFN in both mice and humans.

2.3. TLR3 ligands

TLR3 recognizes both in vitro-transcribed dsRNA and poly (I:C), suggesting that the RNA duplex and not 5′-triphosphate is critical for TLR3 activation [10,11,43]. Since TLR3 is predominantly expressed intracellularly, ligands are often transfected into the cells with cationic liposomes such as lipofectin or DOTAP. dsRNA–liposome complexes are thought to be delivered to the endosome where they activate TLR3. Under such experimental conditions, bacterial total RNA and in vitro-transcribed ssRNA but not mammalian total RNA, which is abundant in modified nucleosides, activate HEK293 cells expressing TLR3 [44]. By using modified RNAs, it has been shown that the recognition of in vitro-transcribed ssRNA by TLR3 is suppressed by modification of nucleotides such as methylation probably due to destabilization of RNA duplexes [44]. Although TLR3 appears to recognize the dsRNA structure formed in unmodified RNA, the possibility that the RIG-I/MDA5 pathway participates in the recognition of these RNA cannot be excluded. It would be interesting to determine whether viral RNA derived from negative-stranded or positive-stranded ssRNA viruses are recognized by TLR3. Precise studies using null cells are necessary for identification of natural ligands for TLR3.

2.4. Delivery of dsRNA

TLR3 is activated by extracellular dsRNA. The mechanisms by which extracellular dsRNA is delivered to the TLR3-containing organelle remain unknown. A recent study demonstrated that CD14 enhances dsRNA-mediated TLR3 activation by directly binding to poly(I:C) and mediating cellular uptake of poly (I:C) [45]. The internalized poly(I:C) colocalizes with CD14 and TLR3. Since the extracellular domain of CD14 consists of LRRs [46], poly(I:C) might be transferred from CD14 to TLR3. In human fibroblasts, cell-surface TLR3 is involved in the recognition of dsRNA [11]. Since CD14 is expressed on the fibroblast cell surface, it may cooperate with TLR3 to internalize dsRNA. On the other hand, in the case of CD14-negative myeloid DCs, extracellular dsRNA must be internalized with the putative uptake receptor. Remarkably, uptake of dsRNA largely depends on the dsRNA structure. Among various synthetic dsRNAs, poly(I:C) is preferentially internalized and activates TLR3 in myeloid DCs [43]. High dose of poly(I:C) reportedly activates MDA5 and induces type I IFN production [18,19]. However, it is not known how poly(I:C) reaches the cytosol. Physiologically, TLR3 may encounter viral RNAs in the endosome where viruses enter through the endocytic pathway or by uptake of the apoptotic bodies derived from virally infected cells. Cells infected by positive-stranded RNA viruses and DNA viruses contain virus-derived dsRNA [47]. Indeed, Ebihara et al., recently reported that myeloid DCs phagocytosed the apoptotic bodies derived from Hepatitis C virus-infected cells containing HCV-derived dsRNA and induced innate immune responses including IFN-β production [48].

3. The TLR3-mediated type I IFN signaling pathway

Among the TLR family members, only TLR3 does not use myeloid differentiation factor 88 (MyD88) as a signaling adaptor [49]. TLR3 mediates signals via the adaptor protein TICAM-1/TRIF [12,13] (Fig. 1). TICAM-1 activates the transcription factors IRF-3, NF-κB and AP-1, leading to the induction of type I IFN, cytokine/chemokine production and DC maturation. The TIR domain of TLR3 is responsible for signaling and recruiting the adaptor protein. A substituted mutant A795H, in which an alanine residue in the B–B loop of the TIR domain of TLR3 is replaced with a histidine residue [50], does not activate NF-κB and the IFN-β promoter in HEK293 cells upon poly(I:C) stimulation because of its failure to bind TICAM-1 [12]. In addition, phosphorylation of two specific tyrosine residues (Tyr^{759} and Tyr^{858}) in the TIR domain of TLR3 is essential for

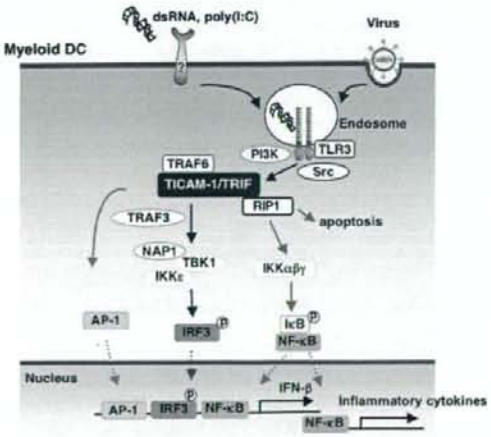

Fig. 1. TLR3-TICAM-1-mediated type I IFN signaling pathway. In myeloid DCs, TLR3 is expressed in the endosomal compartments and recognizes extracellular viral dsRNA and its synthetic analog poly(I:C). Once TLR3 is dimerized by internalized dsRNA, it recruits the adaptor protein TICAM-1/TRIF. After the transient association of TLR3 with TICAM-1 through the TIR domains, TICAM-1 dissociates from TLR3 to form a speckle-like structure containing RIP1, TRAF3 and NAP1 where TICAM-1-mediated signaling is initiated. RIP1 associates with TICAM-1 via the PHIM domain in the C-terminal region and acts as an NF-κB activator and apoptosis mediator in TICAM-1-mediated signaling. TRAF3 and NAP1 participate in the recruitment and activation of the IRF-3 kinases TBK1 and IKKε. Phosphorylated IRF-3 translocates into the nucleus and together with NF-κB and AP-1 induces IFN-β gene transcription. The TICAM-1-mediated AP-1 activation pathway is unclear.

dsRNA-induced signaling [51]. Phosphatidylinositol 3-kinase (PI3-K) is recruited to these residues, and is required for full phosphorylation and activation of IRF-3. TLR3 also associates with c-Src tyrosine kinase on the endosome in response to dsRNA [52]. dsRNA-induced IRF-3 activation is abolished in Src kinase deficient cells, suggesting a critical role for Src kinase in dsRNA-TLR3-mediated signaling [52]. The Src kinase inhibitor markedly inhibits dsRNA-elicited phosphorylation of Akt, a downstream target of PI3-K. Although these data suggest that IRF-3 activation via the PI3-K-Akt-pathway is dependent on c-Src, the precise role of c-Src requires further elucidation.

TICAM-1 consists of an N-terminal proline-rich domain, a TIR domain and a C-terminal proline-rich domain. The TIR domain of TICAM-1 is essential for binding to the TIR domain of TLR3 and also to the TLR4 adaptor TICAM-2 (also called TRIF-related adaptor molecule [TRAM]) [53,54]. Once TICAM-1 is oligomerized, the serine-threonine kinases, TANK-binding kinase 1 (TBK-1; also called NAK or T2K) and IκB kinase-related kinase ε (IKK-ε; also called IKK-i), are activated and phosphorylate IRF-3 [55,56]. Phosphoprylated IRF-3 translocates into the nucleus and induces *IFN-β* gene transcription [57]. The N-terminal deletion mutant of TICAM-1 abolishes the *IFN-β* promoter activation while sustaining the NF-κB activating ability, suggesting an important role for the N-terminal region in TICAM-1-mediated IRF-3 activation [12]. It is reported that NF-κB activating kinase (NAK)-associated protein 1 (NAP1) participates in the recruitment of IRF-3 kinases to the N-terminal region of TICAM-1 [58]. In addition, TRAF3 is involved in the TLR3-TICAM-1-mediated IRF-3 activation [59,60]. Cells lacking TRAF3 are defective in IFN-β production but not NF-κB activation in response to poly(I:C). Although both NAP1 and TRAF3 associate with TICAM-1 and serve as a critical link between TICAM-1 and downstream IRF-3 kinases, there is no evidence that they bind directly to TICAM-1.

Whereas the N-terminal region is crucial for TICAM-1-mediated IRF-3 activation, the C-terminal region of TICAM-1 is involved in NF-κB activation and apoptosis. Receptor-interacting protein 1 (RIP1), a kinase containing a death domain, associates with TICAM-1 via the RIP homotypic interaction motif (RHIM) domain in the C-terminal region and acts as an NF-κB inducer and apoptosis mediator in TICAM-1-mediated signaling [61–63]. TRAF6 has also been implicated in NF-κB activation by TICAM-1 [64]. TRAF6 directly binds to the N-terminal region of TICAM-1 through the TRAF domain. Although TRAF6 is required for NF-κB activation in MEFs [65], poly(I:C)-induced NF-κB activation is not impaired in TRAF6-deficient macrophages [66]. The participation of TRAF6 in TICAM-1-mediated signaling may depend on the cell types.

Recently, Funami et al. reported subcellular localization of TICAM-1 and its dynamics in response to dsRNA [33]. TICAM-1 alters its distribution profile from diffuse to a speckle-like structure in response to poly(I:C) stimulation. TICAM-1 is transiently recruited to the endosomal TLR3 in response to poly(I:C), and thereafter moves away from TLR3 to form speckle-like structures. The downstream signaling molecules RIP1 and NAP1 are also recruited to the TICAM-1-positive speckles. Hence, TICAM-1-mediated signaling events are closely associated with the spatiotemporal mobilization and speckle formation of TICAM-1.

TLR3-TICAM-1-mediated signaling is negatively regulated by a fifth TIR adaptor protein SARM [67]. SARM and TICAM-1 have been shown to interact and SARM strongly suppresses NF-κB activation as well as IRF-3 activation by TICAM-1.

4. Anti-viral function

Viral infections result in the stimulation of IFN-α/β and IFN-inducible genes, which play a critical role in anti-viral host defense [68]. Since TLR3 responds to a synthetic dsRNA, poly (I:C), and viral dsRNA and induces IFN-β gene transcription, it is thought that TLR3 plays a key role in anti-viral immune responses. However, a study in TLR3-deficient ($TLR3^{-/-}$) mice showed that the immune response to different viruses including lymphocytic choriomeningitis virus (LCMV, a positive sense single-stranded (ss) RNA virus), vesicular stomatitis virus (VSV, a negative sense ssRNA virus), murine cytomegarovirus (MCMV, a dsDNA virus) and reovirus (a dsRNA virus), was unaffected compared to wild-type mice [69] (Table 1). In contrast, Wang et al. demonstrated the important role of TLR3 in infection by West Nile virus (WNV, a positive sense ssRNA virus) [70]. $TLR3^{-/-}$ mice had impaired cytokine production and enhanced viral load in the periphery, whereas in the brain, viral load, inflammatory responses and neuropathology were reduced compared to wild-type mice [70] (Table 1). TLR3-mediated peripheral inflammatory cytokine production is critical for disruption of the blood–brain barrier, which facilitates viral entry into the brain causing lethal encephalitis.

Table 1
Representative results from TLR3-deficient mice studies on viral infection

Virus	Genome structure	Phenotype in $TLR3^{-/-}$ mice	Reference
MCMV	dsDNA	Survival ↘ Vial load in spleens ↑ IFN-α/β, IL-12 and IFN-γ in serum ↓ NK and NKT cell activation ↓	[76]
LCMV	ssRNA (−)	Normal $CD4^+$ or $CD8^+$ T cell responses (IFN-γ production) Normal T cell memory function	[69]
VSV	ssRNA (−)	Normal $CD4^+$, $CD8^+$ T cell responses (IFN-γ production)	
MCMV	dsDNA	Normal $CD4^+$, $CD8^+$ T cell responses (IFN-γ production)	
Reovirus	dsRNA	CNS injury, mortality (no difference)	
WNV	ssRNA (+)	Survival ↑ Viral load in blood ↑ IL-6, TNF-α, IFN-β ↓ Viral load in brain ↓ Neuropathology ↓	[70]
RSV	ssRNA (−)	Pulmonary mucus production ↑ Pulmonary IL-13 expression ↑	[73]
IAV	ssRNA (−)	Survival ↑ Viral load in lungs ↑ IL-6, IL-12 and RANTES ↓ $CD8^+$ T cells in lung ↓	[74]

Therefore, TLR3$^{-/-}$ mice are more resistant to lethal WNV infection. In this case, TLR3 responds to viral infection but does not display a protective role.

In other RNA viral infections such as respiratory syncytial virus (RSV), influenza A virus (IAV), and phlebovirus, TLR3-dependent inflammatory cytokine and chemokine production also appears to affect the virally induced pathology and host survival [71–75]. TLR3$^{-/-}$ mice infected by IAV had reduced inflammatory mediators leading to increased survival [74]. It is notable that these experimental conditions that use higher viral doses may lead to over-production of inflammatory cytokines and chemokines.

Interestingly, a TLR3-mediated anti-viral response has been demonstrated in MCMV infection [76]. TLR3$^{-/-}$ mice are hypersusceptible to MCMV infection. Cytokine (type I IFN, IL-12p40, and IFN-γ) production, and NK cell and NKT cell activation are impaired in TLR3-deficient mice. Thus, the role of TLR3 in the anti-viral response appears to be dependent on the viral genome structure, entry route into the cells, viral affecting sites, and property of the host anti-viral effector functions.

5. Induction of adaptive immunity

5.1. Induction of cross-priming by TLR3

Selective TLR3 expression in myeloid DCs but not in plasmacytoid DCs raises the possibility that TLR3 may play a key role in the anti-viral response by induction of the adaptive immune responses rather than primary IFN-α/β production. Myeloid DCs are the best professional antigen presenting cells having several antigen processing and transporting pathways [77]. One of the most notable features of myeloid DCs is a cross-presentation of exogenous antigens to CD8$^+$ T cells (Fig. 2). This pathway is important for effective host CTL induction against viruses that do not directly infect DCs. DCs take up cell-associated antigens and after processing, present peptides bound to MHC class I molecules to CD8$^+$ T cells. Several mechanisms have been proposed to explain cross-presentation [78]. Virus-induced type I IFN also promotes cross-priming of CD8$^+$ T cells through direct stimulation of DCs, although the downstream signal involved in this pathway is unknown [79]. Datta et al. demonstrated that TLR3 and TLR9 ligands induced cross-presentation by bone marrow-derived DCs in a transporter associated with antigen processing (TAP)-dependent manner [80]. Using TLR3-deficient mice, Schiltz et al. clearly showed that TLR3 has an important role in cross-priming [81]. Murine CD8α$^+$ DCs, which express TLR3 and have a central role in cross-presentation, are activated by phagocytosis of apoptotic bodies from virally infected cells or cells containing poly(I:C) in a TLR3-dependent manner. Furthermore, immunization with virally infected cells or cells containing poly(I:C), both carrying ovalbumin (OVA) antigen, induces OVA-specific CD8$^+$ T cell responses, which are largely dependent on TLR3-expressing DCs [81]. In this study virus that could not infect APCs and release vial progeny from infected cells was used to avoid direct CTL priming and the effects of virus-induced IFN-α/β in vivo. In many cases, virally infected cells produce IFN-α/β which activate DCs to promote CD8$^+$ T cell cross-priming [79]. Thus, both TLR3- and IFN-α/β-mediated signaling are likely implicated in licencing DCs for cross-priming of CD8$^+$ T cells.

5.2. TLR3-TICAM-1-mediated NK activation

CTL and NK cells are principal effector cells in anti-viral and anti-tumor immunity. Contribution of TLR3 and TLR9 in anti-viral responses has been shown in MCMV infection [76,82], which partly relies on NK cell activation for virus clearance. Production of type I and type II IFNs as well as NK cell activation after MCMV infection is impaired in TLR3$^{-/-}$ mice and TLR9 mutant mice, suggesting that TLR3- and TLR9-mediated type I IFNs activate NK cells. Recently, a critical role of CD11c^{high} DCs in NK cell activation has been demonstrated in vivo [83]. Recruitment of NK cells to local lymph node after TLR3 stimulation and IL-15 released by DCs in a IFN-α/β-dependent manner are necessary and sufficient for the priming of NK cells, resulting in IFN-γ production and cytotoxicity against target cells [83]. Akazawa et al. subsequently showed that the TLR3 adaptor TICAM-1 is essential for poly(I:C)-induced NK cell-mediated tumor regression in a syngeneic mouse tumor implant model [84]. Remarkably, production of

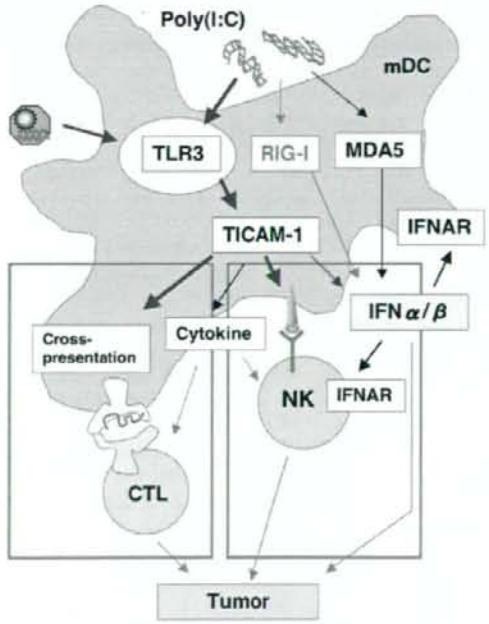

Fig. 2. dsRNA-induced TLR3-TICAM-1-mediated cellular responses in myeloid DCs. Myeloid DCs take up extracellular poly(I:C) or apoptotic bodies from virally infected cells and induce type I IFN and cytokine production, NK cell activation and CTL induction via the TLR3-TICAM-1 pathway. Extracellular poly(I:C) also activates the cytosolic RNA helicase MDA5 and induces robust type I IFN production. Type I IFN participates in NK cell activation and promotion of cross-priming resulting in the CTL induction.

IFN-α is not impaired in TICAM-1$^{-/-}$ mice compared to wild-type mice, after in vivo poly(I:C) injection or in vitro mDC stimulation, whereas IL-12 production is completely dependent on TICAM-1, consistent with previous reports [18]. Furthermore, NK cell activation requires cell–cell contact with mDCs preactivated by poly(I:C) but not IFN-α or IL-12. Thus, the TLR3-TICAM-1 pathway in mDCs facilitates the mDC–NK cell interaction following NK cell activation (Fig. 2). Poly(I:C)-induced TICAM-1-independent IFN-α production, which does not contribute to NK cell activation in this case, probably relies on the cytosolic dsRNA receptor MDA5. It is not presently known whether the RIG-I/MDA5-signaling is implicated in mDC-mediated NK cell priming. Further, identification of putative NK cell activating molecules inducible on mDCs by TICAM-1-signaling remains undetermined.

5.3. Application of the TLR3 ligand to adjuvant vaccine therapy

Until now, application of the TLR3 ligand to adjuvant vaccine therapy has been tried in a syngeneic mouse tumor implant model or in a viral infection mouse model [84–87]. Selective expression of TLR3 in mDCs and TLR3-TICAM-1-induced immune responses (type I IFN, cytokine/chemokine production, DC maturation, CTL and NK cell activation) are advantages associated with using TLR3 ligands as an adjuvant. However, several issues remain unresolved including a suitable transport system for TLR3 ligands. Poly(I:C) intraperitoneally injected in mice activates both TLR3 and MDA5 [18,84], indicating that extracellular poly(I:C) is delivered to endosomal TLR3 and further to cytosolic MDA5 in murine cells. The mechanism of intracellular transport of poly(I:C) has not been analyzed. In human monocyte-derived immature DCs, IFN-β production in response to extracellular dsRNA largely depends on the dsRNA structure [43]. Extracellular addition of DCs fails to produce IFN-β in response to in vitro-transcribed dsRNA, suggesting that the internalization of dsRNA in mDCs depends on the dsRNA structure. Thus, the dsRNA structure and targeting approach of dsRNA to the endosomal TLR3 in mDCs are important matters for generating the innate and adaptive immune responses by TLR3 ligands. Goen et al. showed that poly(I:C_{12}U) induces IFN-β in a TLR3-dependent and MDA5-independent manner, and exhibits protective anti-viral effects in mice [87]. Identification of the putative dsRNA uptake receptor is crucial for analyzing the intracellular transport of dsRNA. Furthermore, clarification of the difference between the RIG-I/MDA5-mediated and TLR3-TICAM-1-mediated signaling pathways is important for assessment of the dsRNA-induced immune responses.

6. Conclusion

Numerous studies on TLR3 have been performed in the past 10 years. Although it is clear that TLR3 recognizes extracellular dsRNA and induces TICAM-1-mediated innate and adaptive immunity, the in vivo role in anti-viral responses is still controversial. In addition, the mechanisms by which endosomal TLR3 and intracellular MDA5 recognize extracellular dsRNA are not fully understood. Identification of the TLR3-TICAM-1-mediated signaling cascades different from those of RIG-I/MDA5, ligand repertoire of TLR3, and dsRNA transport system provides new insights for understanding the TLR3 function in vivo, as well as for development of the TLR3-related vaccine adjuvant for tumor and/or infectious diseases.

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