CCL5 is induced by TLR 3 signaling in HuCCT1 human biliary epithelial cells：possible involvement in the pathogenesis of biliary atresia
（HuCCT1 ヒト胆管上皮細胞において CCL5 は TLR 3 シグナルにより誘導される：胆道閉鎖症の発症機序への関与の可能性 ）

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ABSTRACT

Biliary atresia (BA) is a disease of the newborn that is characterized by progressive, inflammatory and sclerosing cholangiopathy. Innate immune responses to viral components are thought to be involved in the pathogenesis of BA. It is also reported that some chemokines, such as CCL5, are possibly involved in the pathogenesis of experimental animal model of BA. We treated human biliary epithelial HuCCT1 cells with polyinosinic-polycytidylic acid (poly IC), an authentic double-stranded RNA (dsRNA) which mimics viral RNA, and analyzed the CCL5 expression by quantitative reverse transcription-PCR (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA). To examine the regulation mechanisms of CCL5, we subjected the cells to RNA interference (siRNA) against Toll-like receptor 3 (TLR3), interferon (IFN)-β, NF-κB p65 and IFN regulatory factor (IRF) 3. Immunohistochemical staining for CCL5 was also performed in tissues from patients with BA. Poly IC induced CCL5 expression in HuCCT1 cells. CCL5 expression induced by poly IC was inhibited by the knockdown of TLR3, p65 or IRF3, but it was not affected by knockdown of IFN-β. Immunohistochemical staining showed that CCL5 was strongly expressed in

biliary epithelial cells of patients with BA. The current study suggests that TLR3 signaling induces CCL5 expression via NF-κB and IRF3 in bile duct cells, and this pathway may be involved in the pathogenesis of BA.

Biliary atresia (BA) is an infantile disease resulting from a severe cholangiopathy, and is characterized by progressive obstruction of bile ducts, devastating liver damage and cirrhosis. The cause of BA may be multifactorial and the exact pathogenesis of BA is not clear yet. There is a theory that viral infection and the following inflammation lead to progressive bile duct damage and obliteration (4, 9).

When the cells are infected with viruses, pathogen-associated molecular patterns of viruses are recognized by pattern recognition receptors, and innate immune reactions against viruses are initiated. Innate immune reactions and subsequent inflammation are important for host defense, but dysregulated inflammation may lead to tissue injury. It has been reported that reovirus, a kind of double-stranded RNA (dsRNA) virus, was detected in hepatobiliary tissues from patients with BA (15). Furthermore, the infection of newborn mice with rhesus rotavirus, a member of reoviruses, leads to sclerosing cholangiopathy resembling human BA (11). These findings suggest that infection with dsRNA viruses and following immune reactions may play an important role in the etiology of BA. However, the mechanisms by which

dsRNA virus infection cause BA are still not known well.

Toll-like receptors (TLRs) are members of pattern recognition receptors, and TLR3 is a receptor for dsRNA. TLR3 is expressed in biliary epithelial cells and is involved in biliary innate immune reactions (4). It is well known that activation of TLR3 signaling induces the expression of various cytokines and chemokines. Interferon (IFN)-β is a key cytokine in innate immune reactions, and chemokines induce inflammation by recruiting leukocytes. Although these reactions are essential for host defense against viruses, excessive expression of cytokines and chemokines may leads to inflammatory diseases.

In the liver or bile ducts from experimental BA model mice, upregulation of multiple genes including chemokines has been reported by three research groups (6, 8, 17). Among various chemokines, CCL5 (also named as RANTES; regulated on activation, normal T cell expressed and secreted) was the only chemokine upregulated in all of above three studies. This suggests that CCL5 may play a pivotal role in the pathogenesis of experimental BA model animals. However, the role of CCL5 in human BA is not fully elucidated.

Polyinosinic-polycytidylic acid (Poly IC) is an authentic dsRNA which mimics viral RNA. In the present study, we treated cultured HuCCT1 cells, a

human biliary duct carcinoma cell line, with poly IC and examined the role of TLR3 signaling in the expression of CCL5.

MATERIALS AND METHODS

Reagents. RPMI-1640 medium, Lipofectamine RNAi MAX reagent and M-MLV reverse transcriptase were purchased from Invitrogen (Frederick, MD, USA). Poly IC was from Sigma (St. Louis, MA, USA). SsoAdvenced SYBR Green DNA polymerase mix was from Bio-rad (Herculus, CA, USA). Oligonucleotide primers for PCR were custom-synthesized by Greiner Japan (Atsugi, Japan). An enzyme-linked immunosorbent assay (ELISA) kit for CCL5 was obtained from R&D systems (Minneapolis, MN, USA). An siRNA against p65 (6534) and an anti-NF-κB p65 antibody (8242S) were from Cell Signaling Technology (Danvers, MA, USA). A negative control non-silencing siRNA (21027281) and siRNAs against IFN regulatory factor (IRF) 3 (03117359) or TLR3 (02655166) were from Qiagen (Hilden, Germany). An siRNA against IFN-β was described previously (5). dNTP mix was from Thermo Fisher Scientific (Asheville, MA, USA). An antibody against IRF3 (18781) was from Immuno-biological Laboratories (Gunma, Japan). Anti-actin rabbit antibody (110564) was from

GeneTex (Irvine, CA, USA). Mouse monoclonal anti-CCL5 antibody (ab9666) was from Abcam (Cambridge, UK).

Cell culture. HuCCT1 cells were cultured using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) as described (10). The cells were treated with 2 – 50 μg/mL poly IC for up to 48 h. In RNA interference experiments, the cells were transfected with siRNA against TLR3, IFN-β, p65, IRF3 or control siRNA and were incubated for 48 h before treating with poly IC. Then the cells were treated with poly IC and additionally incubated as indicated. The additional incubation time was decided according to pilot studies (data not shown).

Quantitative real-time reverse transcription (RT)-PCR analysis. Total RNA was extracted from the cells after the incubation as indicated. Subsequently, single-strand cDNA was synthesized using M-MLV reverse transcriptase. The expression levels of mRNAs for TLR3, IFN-β, CCL5 and 18S rRNA were examined with a quantitative real-time RT-PCR system. Values were normalized to 18S rRNA and expressed as relative fold increase. The primers

used in this studies were shown in Table 1.

Western blot analysis. In western blot analysis, the cells were lysed with Laemmli's reducing lysis buffer after incubation. The lysates were denatured and subjected to electrophoresis on a 10% polyacrylamide gel. The proteins on the polyacrylamide gel were transferred to a polyvinylidene difluoride membrane. The membrane was incubated with an antibody against IRF3 (1:2500), p65 (1:5000) or β-actin (1:15000), followed by incubating with a secondary antibody labeled with horseradish peroxidase. A chemiluminescence substrate was used for immunodetection.

ELISA. After the incubation indicated above, the cell-conditioned medium was collected and centrifuged. The concentration of CCL5 protein in the supernatant was quantified with an ELISA kit according to the manufacturer's protocol.

Immunohistochemical staining. The surgically resected specimens were obtained from archives of pathology files maintained at the Department of

Pathology and Molecular Medicine, Hirosaki University. Hilar tissues were procured from two patients with BA and non-inflamed hepatic tissues from another patient with hepatoblastoma were used as a control.

All specimens were fixed with 10% formalin and embedded in paraffin. The study conformed to the previsions of the Declaration of Helsinki and the ethical Declaration of the Japanese society of Pathology. Expression of CCL5 in biliary epithelial cells was evaluated by immuhistochemical staining. Briefly, 4 μm-thick paraffin embedded sections of each specimen were deparaffinized, immersed in citrate buffer (pH 6.0) at 123 ºC for 3 min for antigen retrieval, and treated with Background Sniper for 10 min. Then, the specimens were incubated with a primary antibody against CCL5 (1:300) at 4 ºC overnight. Finally, the sections were incubated with biotinylated anti-mouse IgG-IgA-IgM antibody (H1601) and the reaction was visualized using a Metal Enhanced DAB Substrate Kit, followed by light counterstaining with hematoxylin. The specificity of the staining was confirmed by omitting primary anti-CCL5 antibody (data not shown).

Statistics. The results of real-time RT-PCR and ELISA are expressed as mean ± SD (n=3). Data was analyzed by *t*-test and differences were considered

significant at $P < 0.01$.

RESULTS

Poly IC induces the expression of CCL5 in HuCCT1 human bile duct epithelial cells

Poly IC induced the expression of CCL5 mRNA and protein in a concentration-dependent manner in HuCCT1 cells (Fig. 1A and B). CCL5 mRNA in the cells treated with poly IC began to increase 4 h after the treatment, and its expression reached a maximal level 24 h after the treatment (Fig. 2A). Significant amount of CCL5 protein was detected in the medium of poly IC-treated cells, and the increase of CCL5 protein slightly lagged behind the mRNA expression (Fig. 2B).

TLR3 is involved in the expression of CCL5 in HuCCT1 cells treated with poly IC

Transfection of HuCCT1 cells with a specific siRNA against TLR3 inhibited the expression of CCL5 mRNA (Fig. 3A, upper panel) and protein (Fig. 3B) induced by poly IC. Effective knockdown of TLR3 was confirmed by real-time PCR (Fig. 3A, lower panel). On the other hand, the expression of CCL5 mRNA was not

inhibited by a specific siRNA against IFN-β (Fig. 3C, upper panel). Effective knockdown of IFN-β was confirmed by real-time PCR (Fig. 3C, lower panel).

Knockdown of p65 and IRF3 reduces the expression of CCL5 in HuCCT1 cells

To examine the signaling molecules involved in poly IC-induced CCL5 expression, HuCCT1 cells were transfected with siRNAs against p65 or IRF3. Knockdown of either p65 or IRF3 inhibited the expression of CCL5 mRNA (Fig. 4A and D) and protein (Fig. 4B and E). Effective knockdown of p65 and IRF3 was confirmed by western blotting (Fig. 4C and F).

CCL5 is expressed in biliary epithelial cells of patients with BA

Immunohistochemical evaluation disclosed that expression of CCL5 was observed in cytoplasm of hilar biliary epithelial cells in both patients with BA. In contrast, almost no CCL5 expression was found in controls (Fig. 5).

DISCUSSION

In the present study, we found that the treatment of HuCCT1 cells with poly IC induced the expression of CCL5. CCL5 is a member of C-C chemokine family,

and induces chemotaxis and activation of various types of leukocytes including monocytes, T lymphocytes, eosinophils and NK cells (2, 12). Therefore, CCL5 may be involved in inflammatory reactions in bile duct triggered by the infection with dsRNA viruses.

We found that knockdown of TLR3 inhibited the induction of CCL5 in HuCCT1 cells treated with poly IC, suggesting that activation of TLR3 and subsequent signaling is involved at least partly in poly IC-induced CCL5 expression. Retinoic acid-inducible gene-I (RIG-I) is another pattern recognition receptor for dsRNA, and RIG-I is involved in CCL5 expression induced by poly IC in human astrocytes (16) and normal human mesangial cells (13). However, knockdown of RIG-I did not affect poly IC-induced CCL5 expression in HuCCT1 cells (data not shown). Signaling pathway in response to dsRNA may be different among cell types.

Activation of TLR3 signaling is known to induce the expression of IFN-β, a key cytokine in innate immune reactions. IFN-β induces the expression of numbers of IFN-stimulated genes (ISGs) and ISGs mediate various antiviral and inflammatory reactions. In the present study, knockdown of IFN-β did not affect CCL5 production in poly IC-treated HuCCT1 cells. This suggests that CCL5 is

induced in this cell type in an IFN-β-independent manner.

In the signaling pathway following TLR3 activation, NF-κB and IRF3 are known to be important transcriptional factors. NF-κB is a key molecule in the expression of various inflammatory genes and IRF3 is an important transcriptional factor in antiviral innate immune reactions. Both of NF-κB and IRF3 are thought to be implicated in the pathogenesis of BA (3, 4). In RNA interference experiments, we found that both of NF-κB p65 and IRF3 are involved in CCL5 expression in HuCCT1 cells treated with poly IC. A TLR3-NF-κB/IRF3-CCL5 pathway may at least partly contribute to the dsRNA virus-induced inflammatory reactions in the bile ducts.

Enhanced expression of inflammation-related factors, such as NF-κB, IRF3 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) etc was reported in human biliary epithelial cells of BA (4). However, there have been no information of the CCL5 expression in human biliary epithelial cells of BA. We first confirmed the expression of CCL5 not only in infiltrated inflammatory cells, but also in biliary epithelial cells of BA. CCL5 was not expressed in biliary epithelium of non-inflamed liver tissue.

The mechanisms by which CCL5 contribute to the pathogenesis of BA was

not clarified yet in the present study. Epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose their polarity and cell-cell adhesion (7). It is known that EMT occurs not only in various biological reactions such as wound healing and tissue development but also in pathological situations including metastasis of cancer (14). EMT phenomenon is also reported to be closely related to the pathogenesis of sclerosing cholangiopathy leading to BA (1). Therefore, we hypothesized that the TLR3-NF-κB/IRF3-CCL5 pathway may be involved in EMT in biliary epithelial cells. In the process of EMT, the expression of E-cadherin, a marker of epithelial cells, decreases. However, the expression of E-cadherin was not affected by treatment with poly IC in HuCCT1 cells (data not shown). This suggests that TLR3-NF-κB/IRF3-CCL5 pathway is not involved in EMT at least in our experimental model. Although the details in the pathological role of CCL5 in BA should be further investigated, there is a possibility that the TLR3-NF-κB/IRF3-CCL5 pathway may be a potential target to develop new therapeutic strategies for BA.

We found that CCL5 is induced by poly IC in HuCCT1 cells. TLR3, NF-κB and IRF3 are involved in this reaction. This pathway in biliary epithelial cells may be involved in the pathogenesis of BA (Fig. 6).

CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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Table 1. Oligonucleotide primers for real-time quantitative RT-PCR

cDNA	primers
CCL5	F: 5'- CTACTCGGGAGGCTAAGGCAGGAA -3'
	R: 5'- GAGGGGTTGAGACGGCGGAAGC -3'
IFN-β	F: 5'- CCTGTGGCAATTGAATGGGAGGC -3'
	R: 5'- CCAGGCACAGTGACTGTACTCCTT -3'
TLR3	F: 5'- TGTCTGGAAGAAAGGGACTTTGA -3'
	R: 5'- GTTGAACTGCATGATGTACCTTGA -3'
18S rRNA	F: 5′-ACTCAACACGGGAAACCTCA -3′
	R: 5′ -AACCAGACAAATCGCTCCAC -3′

FIGURE LEGENDS

Fig. 1 Polyinosinic-polycytidylic acid (poly IC) induces the expression of CCL5 mRNA and protein in HuCCT1 human bile duct carcinoma cells in a concentration-dependent manner. The cells were treated with 2-50 μg/mL poly IC and cultured for 24 h. After the incubation, the cell-conditioned medium was collected and RNA was extracted from the cells. The expression of CCL5

mRNA was examined using quantitative real-time RT-PCR analysis (**A**), and CCL5 protein in the conditioned medium was measured with ELISA (**B**). Values are shown as means+/-SD (n=3).

Fig. 2 Poly IC induces the expression of CCL5 mRNA and protein in HuCCT1 cells in a time-dependent manner. The cells were treated with 10 μg/mL poly IC and cultured for up to 48 h, and were subjected to quantitative real-time RT-PCR (**A**) and ELISA (**B**). Values are means+/-SD (n=3).

Fig. 3 TLR3 is involved in the expression of CCL5 induced by poly IC. The cells were transfected with a specific siRNA against TLR3 or IFN-β, or a non-silencing negative control siRNA, and were incubated for 48 h. Then, the cells were treated with 45 μg/mL poly IC. After additional 24 h incubation, real-time RT-PCR (**A**, **C**) and ELISA (**B**) were performed (n=3, ***** P <0.01).

Fig. 4 Knockdown of p65 and IRF3 inhibited the expression of CCL5 in HuCCT1 cells treated with poly IC. The cells were transfected with a specific siRNA against p65 or IRF3, or a control siRNA. After incubating for 48 h, the

cells were treated with 45 μg/mL poly IC. The cells were further incubated for 24 h, and were subjected to real-time RT-PCR (**A**, **D**), ELISA (**B**, **E**) (n=3, ***P* <0.01) and western blotting (**C**, **F**).

Fig. 5 Expression of CCL5 in biliary epithelial cells of BA.

Immunohistochemical staining for CCL5 was performed using non-inflamed part of hepatic tissue surgically resected from a patient with hepatoblastoma (**A**) and hilar tissues from patients with BA (**B**). Significant CCL5 immunoreactivity was detected in bilialy epithelial cells in tissues from patients with BA, but not in control normal tissue. The expression was localized in cytoplasm of epithelial cells (inset). The images for biliary atresia are representative of 2 cases with similar results.

Fig. 6 Proposed role of CCL5 in the pathogenesis of dsRNA virus-mediated BA.

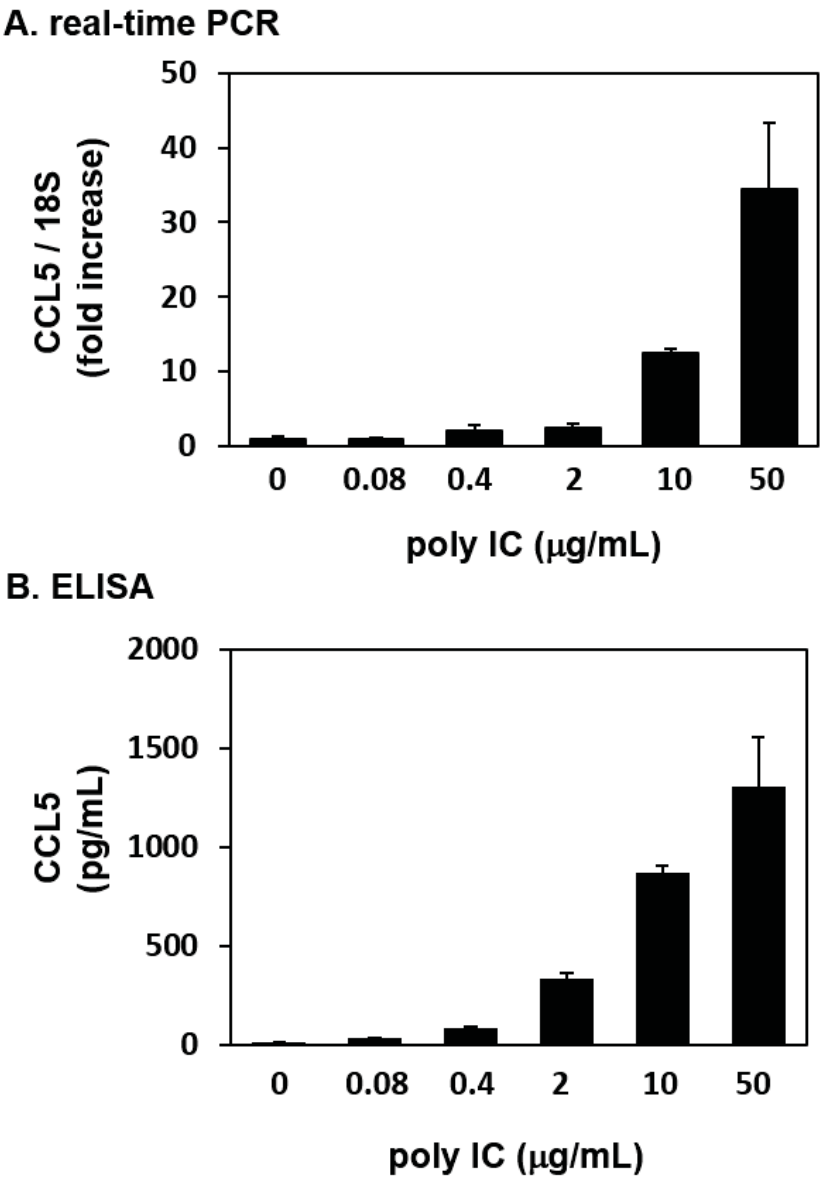


Fig.1 Shimada, et al

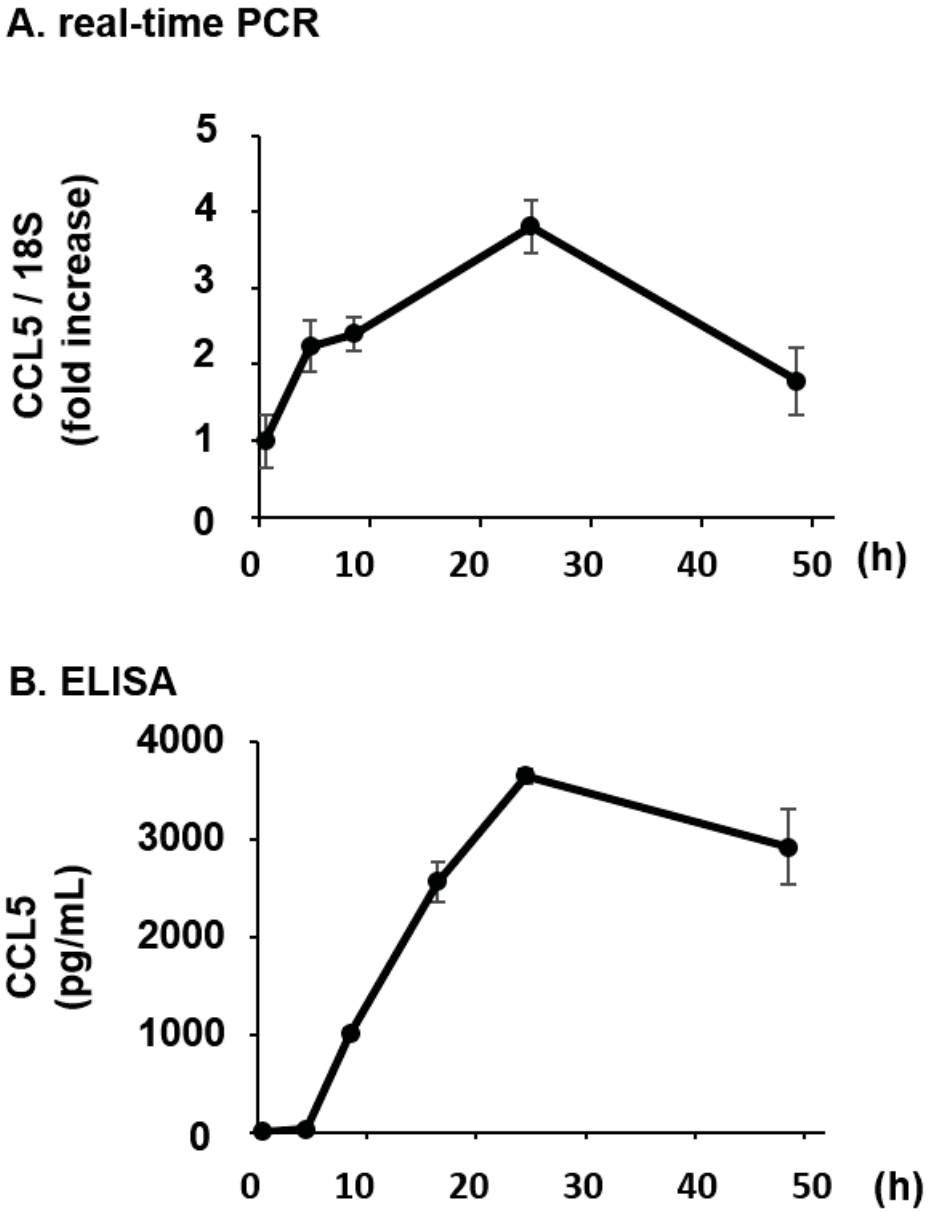


Fig. 2 Shimada, et al

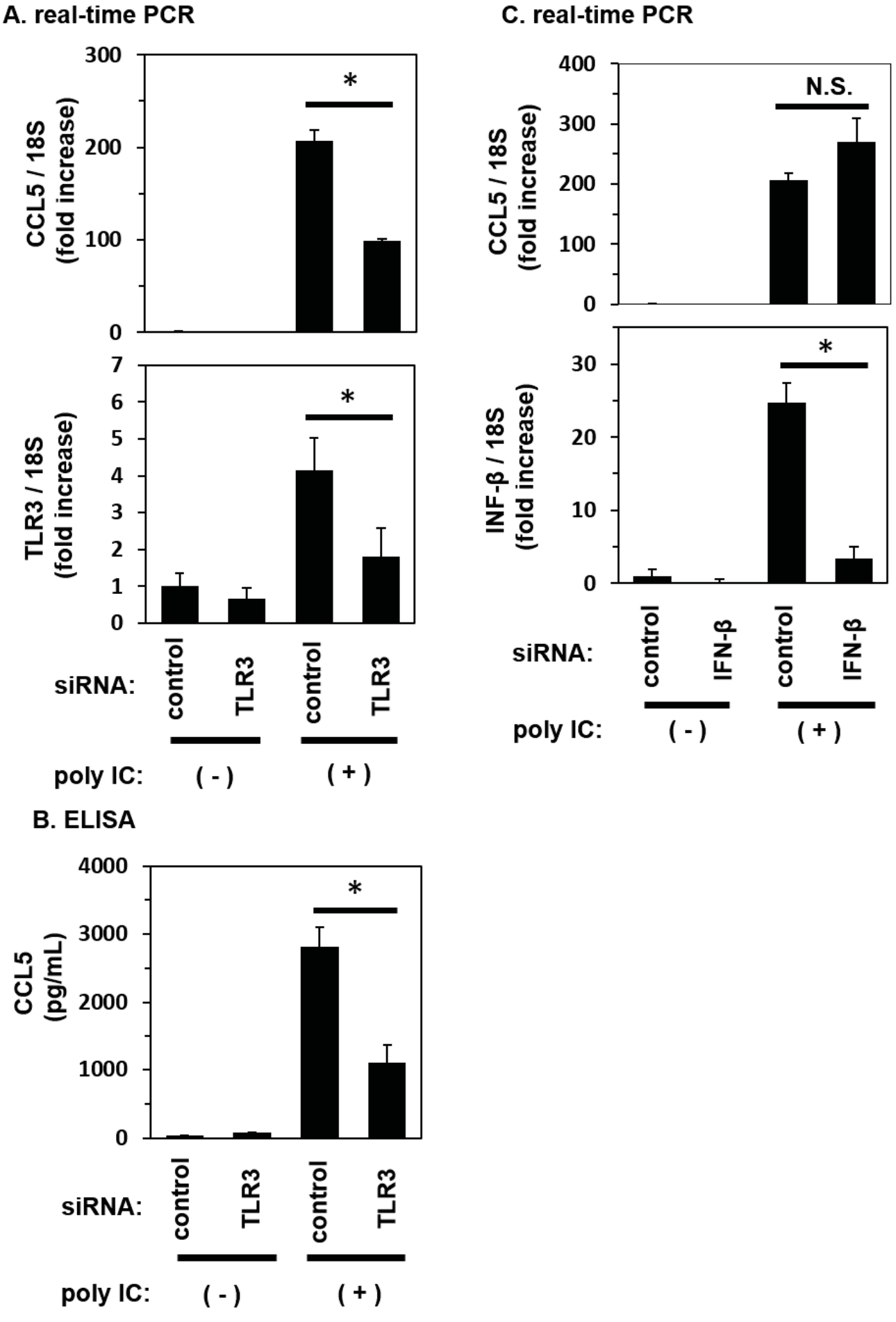


Fig. 3 ABC Shimada, et al (Revised)

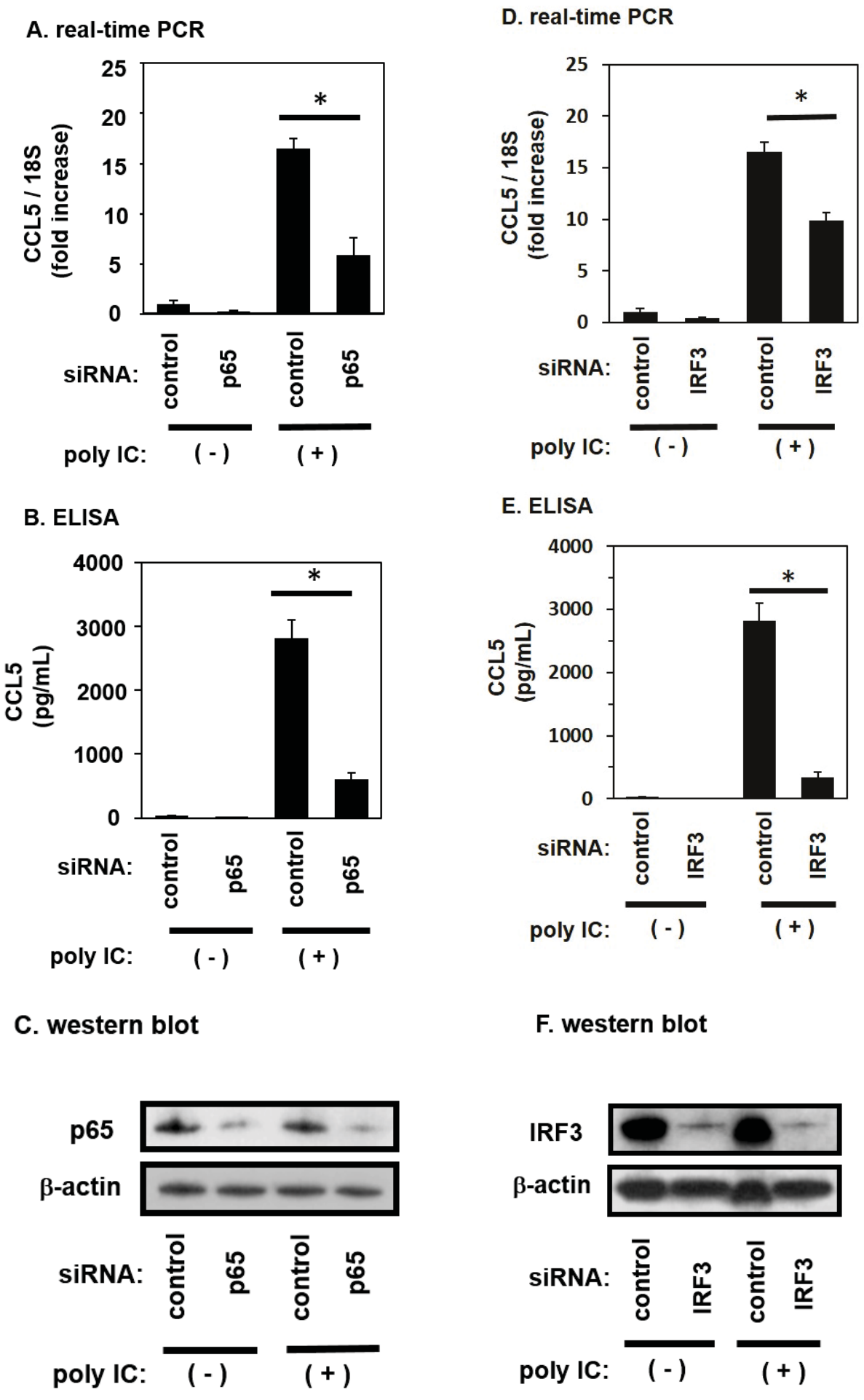
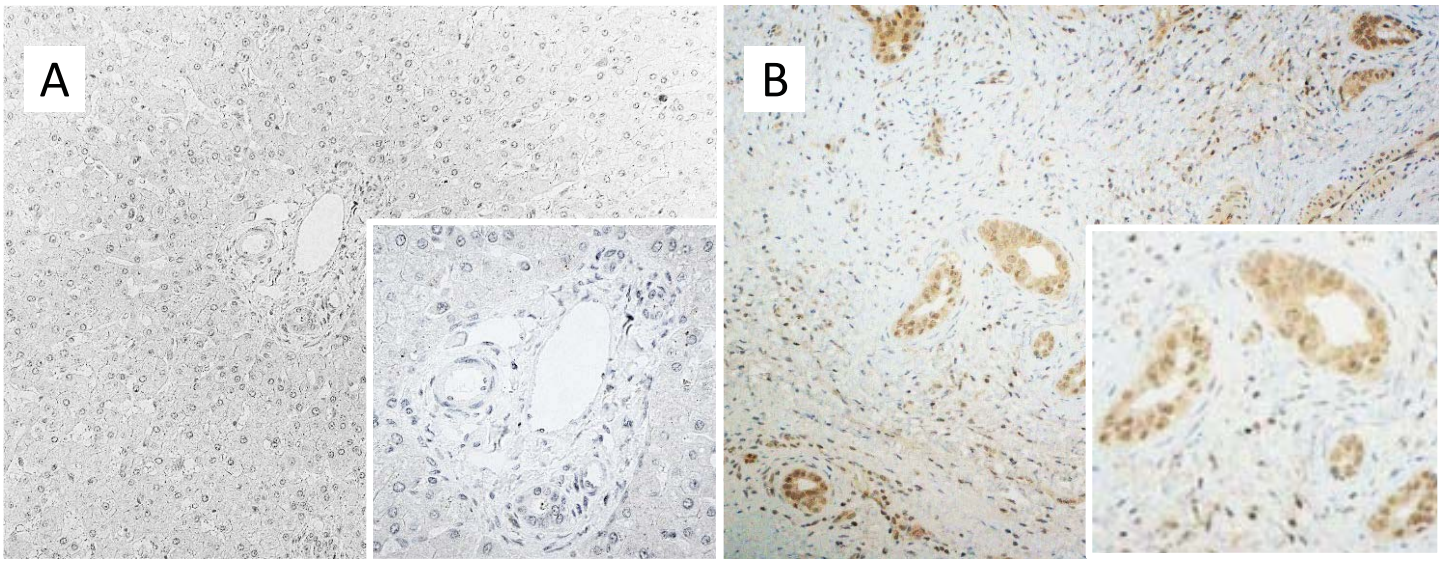


Fig. 4 ABCDEF Shimada, et al (Revised)



Control **BA**

Fig. 5 Shimada, et al

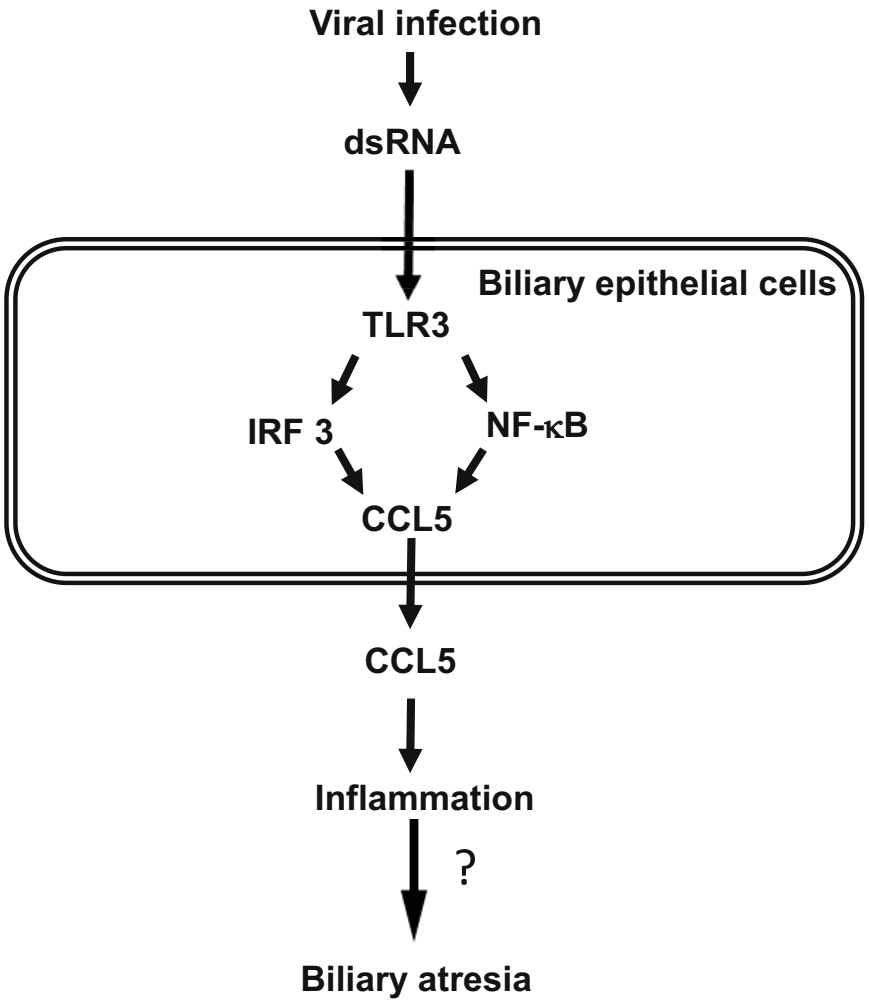


Fig. 6 Shimada, et al