gutjnl-2020-322540-R1
Manuscript entitled "Neutrophils interact with cholangiocytes to cause cholestatic changes in alcoholic hepatitis"

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. Quantification of neutrophils in contact with bile ducts. (A) The number of neutrophils in contact with bile ducts measured by H&E staining or with the neutrophil-specific stain chloroacetate esterase (CAE) is the same. Counting was performed in a blinded fashion by a liver pathologist with liver biopsy specimens from patients with alcoholic hepatitis (n=6, Student's paired *t-test*). The frequency of neutrophil-bile duct contacts was not related to serum levels of **(B)** ALT, **(C)** AST, or **(D)** AST/ALT. **(E)** A weak but significant ($p<0.02$) correlation was observed between the frequency of neutrophil-bile duct contacts and MELD score. No correlation was observed between the frequency of neutrophil-bile duct contacts and serum levels of either **(F)** CRP or **(G)** ferritin. Data were analyzed using the Pearson correlation coefficient.

Supplementary Figure S2. Neutrophils are in contact with bile ducts in primary sclerosing cholangitis. There is a positive correlation between the frequency of neutrophil-bile duct contacts and serum alkaline phosphatase ($R^2=0.647$, $p<0.02$). Data reflect blinded observations from liver biopsies of patients with PSC (n=10). Data were analyzed using the Pearson correlation coefficient.

Supplementary Figure S3. Ethanol plus fructose feeding with LPS injections induce steatohepatitis and cholestatic liver injury. (A) H&E staining of liver sections from control (*left*) and ethanol-fructose-LPS (*right*) mice reveals steatosis and inflammation in the latter. **(B)** Body weight, **(C)** Liver/body weight ratio, **(D)** Serum ALT, **(E)** Serum AST, and **(F)** Serum ALP of mice given a control diet (n=5) all are significantly lower than in mice given ethanol-fructose-

LPS (n=7). Data represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 compared to control diet-fed mice.

Supplementary Figure S4. Accumulation of neutrophils and loss of ITPR3 from bile ducts of ethanol-fructose-LPS treated mice. (A) Representative confocal immunofluorescence images of Ly6G staining of neutrophils (*green*) from livers of mice given the control diet (*top*) or the ethanol diet plus fructose and LPS injection (*bottom*). Nuclei are stained with Hoechst (*blue*). Neutrophils accumulate around the bile ducts in ethanol-fructose-LPS mice but not in control mice (see *insets*). Scale bar, 10 μm. **(B)** Representative confocal immunofluorescence staining of ITPR3 (*red*), CK19 (*green*) and Hoechst (*blue*) from livers of control mice (*top*) and of mice treated with ethanol-fructose-LPS (*bottom*). ITPR3 is expressed in the apical region in bile ducts of control mice but is absent in ethanol-fructose-LPS mice. CK19 is a marker of bile ducts and Hoechst 33342 labels nuclei.

Supplementary Figure S5. NET formation and gap junction communication have minimal effect on the downregulation of ITPR3 in the NHC cell line. (A) Neutrophils form NETs in the presence of NHCs. *Left*: Neutrophils (PMN) were stained with CellTracker (*red*), then co-cultured with NHCs in the presence of the cell-impermeant DNA dye SYTOX Green (*green*). LPS or PMA were added to the co-culture for positive controls of NET formation. Co-cultures were visualized every 5 minutes for 15 hours by time-lapse, swept-field confocal microscopy. *Right*: NET formation was quantified for each condition by calculating the ratio of SYTOX Green intensity (*green*) to CellTracker (*red*) intensity. The number of neutrophils per field was measured in 5 randomly chosen images per experimental group. Data represent mean ± SEM (n=3). *p<0.05, **p<0.01 compared to neutrophils alone (one-way ANOVA). **(B)** Extracellular DNA (ExDNA), a mediator released during NET formation, does not inhibit ITPR3 expression in NHCs. Representative immunoblot (*left panel*) and quantitative blot analysis (*right panel*) of

ITPR3 expression in NHCs after treatment with extracellular DNA for 18 hours. Data represent mean ± SEM (n=3, one-way ANOVA). **(C)** TaqMan real-time PCR analysis of connexin 43 (CX43) shows that CX43 mRNA expression was knocked down by CX43 siRNA in NHCs. mRNA expression levels were normalized to GAPDH. Results are expressed as fold change compared to NHCs transfected with scrambled siRNA. Data represent mean ± SEM (n=3). $^{**}p<0.01$, compared to scrambled siRNA.

Supplementary Figure S6. Decreased expression of ITPR3 induced by neutrophils is not mediated by the LPS/TLR4/NFκB pathway. (A) Treatment of the NHC cell line with the TLR4 inhibitor TAK-242 does not inhibit the decreased expression of ITPR3 induced by neutrophils. Representative immunoblot (*left panel*) and quantitative blot analysis (*right panel*) of ITPR3 in cells treated either with vehicle or TAK-242 for 18 hours in co-culture conditions. Data represent mean ± SEM (n=3); $^{***}p<0.0001$ compared to NHC-vehicle control (one-way ANOVA). **(B)** Real-time PCR demonstrates the efficacy of NFκB-p65 siRNA in reducing the expression of NFκB-p65 mRNA in NHCs. mRNA expression levels were normalized to GAPDH. Results are expressed as fold change relative to NHCs transfected with scrambled siRNA. Data represent mean ± SEM (n=3); $^{***}p<0.0001$ compared to scrambled siRNA. **(C)** Knockdown of NFκB-p65 in NHCs does not reduce the inhibitory effect of neutrophils on ITPR3 expression. Representative immunoblot (*left panel*) and quantitative blot analysis of NFκB-p65 expression (*middle panel*) and of ITPR3 expression (*right panel*) in NHCs transfected with NFκB-p65 siRNA (50 nM) and co-cultured with neutrophils. GAPDH is used as an internal loading control. Data represent mean ± SEM (n=3); $^{*}p<0.05$ relative to NHCs treated with scrambled siRNA (one-way ANOVA).

Supplementary Figure S7. Neutrophil-induced loss of ITPR3 from the NHC cell line is not associated with apoptosis or necrosis. Representative confocal microscopic images are shown of NHCs co-cultured with and without neutrophils for 18 hours. Apoptosis and necrosis

were detected using GFP-CERTIFIED Apoptosis/Necrosis detection kit. Staurosporine was used to induce apoptosis. Co-culture with neutrophils did not elicit either apoptosis or necrosis in the NHC cell line. Scale bars: 10 μm.

Supplementary Figure S8. DNA sequence of the proximal (2.1 kb) region of the human ITPR3 promoter. Potential transcriptional binding sites for c-Jun/AP-1 are in *bold*, and underlined nucleotides are the core sequence of AP-1. Numbered positions refer to the transcription starting sites (nucleotide, +1, arrow).

Supplementary Figure S9. c-Jun mediates the neutrophil-induced-downregulation of ITPR3 expression in cholangiocytes. (A) Representative immunoblot (*left panel*) and quantitative blot analysis (*right panel*) of c-Jun expression in the NHC cell line in the absence or presence of neutrophils. GAPDH is used as an internal loading control. Data represent mean ± SEM (n=6); ***$p<0.0001$, relative to NHCs alone. **(B)** Immunoblot analysis shows that c-Jun expression is knocked down by c-Jun siRNA in NHCs. Data represent mean ± SEM (n=9). ***$p<0.0001$, compared to cells transfected with scrambled siRNA. **(C)** c-Jun is increased and **(D)** ITPR3 expression is decreased in livers of ethanol-fructose-LPS mice. Relative protein expression of c-Jun and ITPR3 in the livers extracted from mice fed control diet (CD) or ethanol-fructose-LPS. *$p<0.05$, compared with CD (n=5-7).

Supplementary Figure S10. Bile acids are not implicated in recruiting neutrophils to bile ducts under cholestatic conditions. CDCA- or GCDCA-activated neutrophils decrease the expression of ITPR3 to a similar extent as non-activated neutrophils. Representative immunoblot (*top panel*) and quantitative blot analysis (*bottom panel*) of ITPR3 expression in the NHC cell line co-cultured either with non-activated neutrophils or CDCA (100 μM)- or GCDCA

(100 μM)-activated neutrophils. GAPDH is used as an internal loading control. Data represent mean ± SEM (n=3); ** $p<0.001$, relative to NHCs alone.