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Toll-like receptor 5 tunes hepatic and pancreatic stellate cells activation

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ABSTRACT

Objective Stellate cells are responsible for liver and pancreas fibrosis and strictly correlate with tumourigenesis. Although their activation is reversible, an exacerbated signalling triggers chronic fibrosis. Toll-like receptors (TLRs) modulate stellate cells transition. TLR5 transduces the signal deriving by the binding to bacterial flagellin from invading mobile bacteria.

Design Human hepatic and pancreatic stellate cells were activated by the administration of transforming growth factor-beta (TGF-β). TLR5 was transiently knocked down by short-interference RNA transfection. Reverse Transcription-quantitativePCR and western blot were performed to analyse the transcript and protein level of TLR5 and the transition players. Fluorescence microscopy was performed to identify these targets in spheroids and in the sections of murine fibrotic liver.

Results TGF-β-activated human hepatic and pancreatic stellate cells showed an increase of *TLR5* expression. *TLR5* knockdown blocked the activation of those stellate cells. Furthermore, TLR5 busted during murine liver fibrosis and co-localised with the inducible Collagen I. Flagellin suppressed *TLR5*, *COL1A1* and *ACTA2* expression after the administration of TGF-β. Instead, the antagonist of TLR5 did not block the effect of TGF-β. Wortmannin, a specific AKT inhibitor, induced *TLR5* but not *COL1A1* and *ACTA2* transcript and protein level.

Conclusion TGF-β-mediated activation of hepatic and pancreatic stellate cells requires the over-expression of TLR5. Instead, its autonomous signalling inhibits the activation of the stellate cells, thus prompting a signalling through different regulatory pathways.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Toll-like receptors (TLRs) are involved in hepatic and pancreatic fibrogenesis.

WHAT THIS STUDY ADDS

⇒ TLR5 exerts a key role in the transactivation of the hepatic and pancreatic stellate cells.
⇒ TLR5 contributes to hepatic and pancreatic fibrosis.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The involvement of TLR5 highlights a possible contribution of the middle gut microbiota to the fibrogenesis of liver and pancreas.
⇒ TLR5 represents a valid target for the therapy of liver and pancreas fibrosis.

INTRODUCTION

Liver cirrhosis and chronic pancreatitis are major causes of death in western countries. Chronic inflammation leads to progressive organ fibrosis as the main reason for malignant transformation and chronic organ failure. Stellate cells are the key players as a source of extracellular matrix proteins after being activated by the pro-inflammatory environment.[1–3]

Stellate cells are a resident population of cells enriching the liver and pancreas[4] parenchyma. The liver ones, also called *ito* cells, have been identified for their involvement in the fibrosis process. Nonetheless, the pancreatic stellate cells are responsible for the pancreas fibrosis. Both liver and pancreatic cells are able to transit from an epithelial quiescent status into a mesenchymal active status characterised by a morphological change of their form, which achieves a so-called stellate form. Once active, the stellate cells do stop accumulating vitamin A and fat. Instead, they proliferate and fulfil the liver[5] and pancreas parenchyma.[6] This process has been extensively investigated in the last years; it has been found that liver injury and hepatic stellate cell activation could be reverted before becoming chronic.[7] Pancreatic stellate cells have been characterised for exerting a key role during the development of pancreatic cancer by sustaining the survival and the proliferation of tumour cells.[8 9]

Toll-like receptors (TLRs) are a type of pattern-recognition receptors and a crucial part of the innate immune system, which participates in endogenous danger signal detection.[10] TLR-mediated inflammatory-signalling pathways are shown to be associated with entire spectrum of liver diseases, from hepatitis, liver fibrosis and cirrhosis to alcoholic and non-alcoholic liver disease, ischaemia/reperfusion injury, liver regeneration and hepatocellular carcinoma.[11–14]

Prolonged or repeated liver injury leads to a maladaptive interplay of hepatocytes, HSCs (Hepatic Stellate Cells) and KCs (Kuppfer Cells) in association with TLR expression, eventually resulting in abnormal extracellular matrix protein deposition in the liver.[5 15 16] Recently, TLR4, which is considered as one of the major responsible for liver fibrosis,[17] could be downregulated by short hairpin RNA thus attenuating the activation of stellate cells and fibrosis of liver and pancreas.[18 19] TLR5, activated by bacterial flagellin, is responsible for the induction of interleukin 1 receptor antagonist, thus ameliorating liver fibrosis.[20] Nonetheless, TLR5 promotes liver regeneration in mice after partial hepatectomy.[21] Gut microbiota dependent activation of TLR5 induces the synthesis of apolipoprotein A1 and high-density lipoprotein-cholesterol in the murine liver.[22] Furthermore, TLR5 expression has been identified in pancreatic stellate cells, thus leading to the activation of pro-inflammatory response.[23]

Up to now, the role exerted by TLR5 in the activation of the hepatic and pancreatic stellate cells has not been fully elucidated yet. Here, we propose to clarify the influence of TLR5 in the activation of hepatic and pancreatic stellate cells and its contribution after the TGF-β (transforming growth factor-beta)-mediated cellular transition.

MATERIALS AND METHODS

Cells

Human hepatic stellate cells LX-2 and pancreatic stellate cells HPSC2.2 were kindly provided by Scott Friedman and Malte Buchholz. The cells were grown in RPMI 1640 (Gibco, Paisley, UK) supplemented with 2% fetal bovine serum (Gibco), penicillin (100 units/mL) and streptomycin (100 µg/mL) (Gibco) at 37°C in a humidified atmosphere containing 5% CO_2. The activation of both stellate cells was performed by 48 hours of incubation with 2.5 ng/mL of recombinant human (rh) TGF-β.

Substances

The rhTGF-β HEK293-derived (7754-BH) was purchased from R&D Systems (Minneapolis, Minnesota, USA). Wortmannin (tlrl-wtm, InvivoGen, Toulouse, France) and Flagellin (tlrl-bsfla, InvivoGen) were purchased from InvivoGen (San Diego, California, USA), dissolved in dimethyl sulfoxide (DMSO) and dissolved in growth culture medium for the final working concentration. TLR5 neutralising monoclonal human antibody (maba2-htlr5) was purchased by InvivoGen.

TLR5 knockdown

TLR5 expression was knocked down by the use of specific short-interference RNA (siRNA) purchased from QIAGEN (Hilden, Germany). Four different siRNAs (1027416) targeting *TLR5* messenger RNA (mRNA) were tested and the one showing the best efficiency, based on Reverse Transcription-quantitativePCR analysis, was used for all further experiments.

Spheroids cultivation

LX-2 and HPSC2.2 spheroids were formed on 50 µl 1,5% peqGOLD Universal Agarose (PEQLAB Biotechnologie GmbH, Erlangen, Germany) in a flat-bottom 96-well plate (SARSTEDT AG and Co. KG, Nümbrecht, Germany) for 6 days as previously described.[24] 5000 LX-2 and 2000 HPSC2.2 cells were plated in 200 µl medium in a humidified atmosphere containing 5% CO_2 at 37°C and placed on an orbital shaker with a shaking speed of 40 rpm overnight.

Western blot analysis

Whole cell lysates were prepared with Jie's Buffer (10 mM NaCl, 0.5% NonidetP40, 20 mM Tris-HCl pH7.4, 5 mM $MgCl_2$, 1 mM PMSF, cOmplete Protease Inhibitor and Phosphatase Inhibitor (Roche, Basel, Switzerland)). The proteins were separated through SDS-PAGE (NP0342, Life Technologies, Carlsbad, California, USA) and transferred to nitrocellulose membranes (10600009, GE Healthcare Life Sciences, Chicago, USA) by semi-dry-blotting with Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). The membranes were further sliced according to the required molecular weight of the proteins of interest, blocked in 4% bovine serum albumine (BSA) (23208, Thermo Fisher Scientific, Waltham, Massachusetts, USA) in TBS-Tween20 (0.5%) and incubated with primary antibodies against Collagen I (ab34710, Abcam; Boston, Maine, USA), α-Smooth Muscle Actin (IC1420G, R&D Systems; Minneapolis, Minnesota, USA), Toll-like receptor 5 (ab13876, Abcam), AKT1 (ab54752, Abcam), Phospho-AKT1 (Ser473) (9271S, Cell Signaling Technology). β-actin (A5441 Sigma-Aldrich, St. Louis, Missouri, USA) was detected as equal loading control.

RNA isolation and Reverse Transcription-qPCR

Total RNA was isolated with the RNeasy Mini Kit (74106, QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Reverse Transcription of mRNA was performed with iScript cDNA Synthesis Kit (170–8891, Bio-Rad, Hercules, California, USA) on FlexCycler (Analytik Jena AG, Jena, Germany). QIAGEN primers for human *GAPDH* (QT01192646), *ACTA2* (QT00088102), *COL1A1* (QT00037793), *SNAI1* (xxxxxxxxxx), *TLR5* (QT01009596), *VIM* (QT00095795) and primers for mouse *Gapdh* (QT01658692), *Tlr5* (QT02328221), *Acta2* (QT00140119), *Col1a1* (QT00162204) were used with GoTaq qPCR Master Mix (Promega, Madison, USA) on RT-qPCR thermocycler CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, California, USA). Results were analysed with the Bio-Rad CFX-Manager (Bio-Rad Laboratories) and normalised with *GAPDH* mRNA content for each sample. Raw data were further analysed with REST 2009 (Relative Expression Software Tool V.2.0.13., QIAGEN).

Fixation and paraffin embedding of cells and spheroids

Spheroids were collected, washed twice with phosphate-buffered saline (PBS) and immobilised in plasma-Thromborel S (Siemens Healthineers, Erlangen, Germany) 1:1 mixture clot and fixed in 4% formaldehyde before paraffin embedding.

Immunofluorescence on paraffin embedded cells/spheroids/tissue

5 µm thin sections of 4% formaldehyde fixed paraffin embedded spheroids and C3H liver were cut, rehydrated and deparaffinised. Antigen retrieval was performed in citrate buffer (pH=6) in a microwave with 480 Watt for 10 min. The endogenous peroxidase was blocked with 3% H2O2 for 10 min. The sections were permeabilised by 0,5% Triton X-100 (Carl Roth Gmbh & Co. KG) in PBS Buffer (Life Technologies) for 10 min. Unspecific bindings were blocked through 30 min incubation in 10% immunised serum. The slides were then incubated with the 1 µg/mL primary antibodies to α-Smooth Muscle Actin (IC1420G, R&D Systems; Minneapolis, Minnesota, USA), Collagen I (ab34710, Abcam; Boston, Maine, USA), SNAIL (C15D3, Cell Signaling; Frankfurt am Main, Germany), Toll-like receptor 5 (ab13876, Abcam), Vimentin (D21H3, Cell Signaling). The bound primary antibodies were labelled with 2 µg/mL Alexa Fluor 568 F(ab')2 fragment of goat anti-rabbit IgG (H+L) (A21069, Invitrogen)/Alexa Fluor 488 goat anti-mouse IgG (H+L) secondary antibodies. Nuclei were stained with 1 µg/mL Hoechst 33342 (Sigma-Aldrich) in 1% BSA-PBST. After 90 min incubation with secondary antibodies and Hoechst, the spheroids slides were washed and mounted with Fluoromount (Sigma) and the tissue slides of Rip1Tag2 were processed with Vector TrueVIEW Auto fluorescence Quenching Kit (Vector Laboratories, Burlingame, California, USA) and mounted with VECTASHIELD Vibrance Antifade Mounting Medium (Vector Laboratories). LAS AF and LAS X software (Leica Microsystems, Wetzlar, Germany) were used for the analysis of fluorescence images acquired with the wide field fluorescence microscope Leica DM5500.

Murine liver fibrosis model

C57/BL6 mice were housed under standard conditions. Approval for animal experimentation was granted before by the Regional Government of Lower Franconia, Würzburg, Germany (No. 54–2531.31-18/06). Fibrosis was induced by a modified protocol of 3×/week intraperitoneal application of 0.15 mg/kg thioacetamide (TAA) and continuous feeding of 10% (v/v) ethanol in sweetened drinking water for 18 weeks.[25] Untreated control groups received regular food and water ad libitum. Each of the four experimental groups consisted of eight animals. Animals were killed by cervical dislocation and liver samples were snap frozen in liquid nitrogen for isolation of mRNA and protein, or fixed in 4% phosphate-buffered formalin for paraffin embedding. Sirius red staining was used to visualise fibrosis under normal and polarised light microscopy. Collagen fibres I-III were

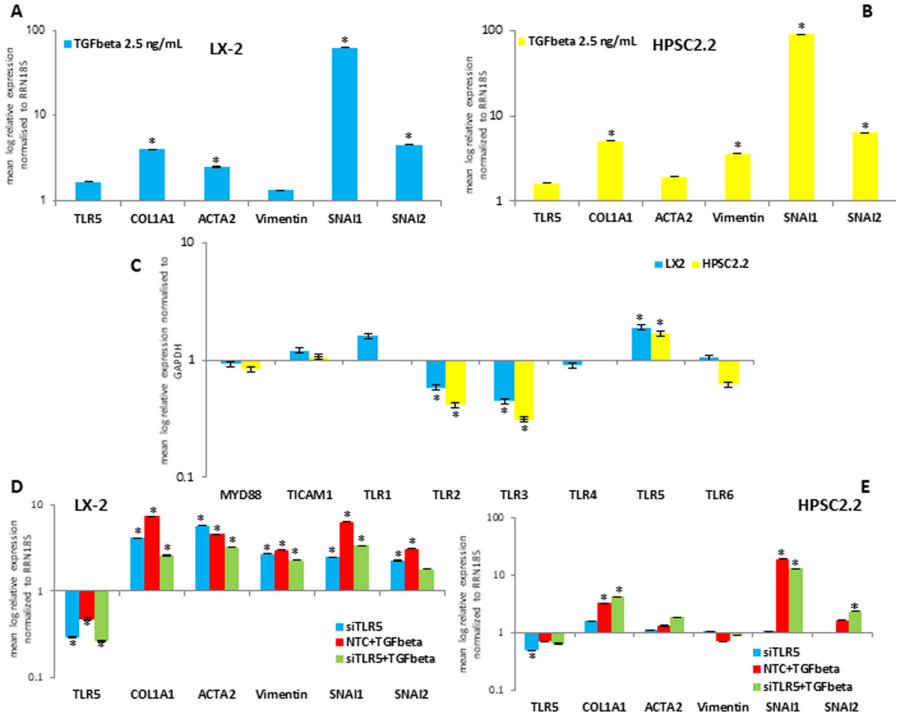

Figure 1 Expression of trans-activation markers after TGF-β and TLR5 knockdown. Transcript level of the transactivation markers *COL1A1, ACTA2, VIM, SNAI1, SNAI2* and *TLR5* in LX-2 (A) and HPSC2.2 (B) cells after 48 hours of treatment with 2.5 ng/mL of rhTGF-β. (C) Transcript level of *MYD88, TICAM TLR(1-6)* in LX-2 and HPSC2.2 cells treated for 48 hours with 2.5 ng/mL of rhTGF-β. Transcript level of the transactivation markers *COL1A1, ACTA2, VIM, SNAI1, SNAI2* and *TLR5* in LX-2 (D) and HPSC2.2 (E) cells after 48 hours of treatment with 2.5 ng/mL of rhTGF-β and TLR5 knockdown with specific siRNA. The expression was normalised to untreated cells. *GAPDH* was detected as a housekeeping gene. Shown are mean triplicate values±SEM. *p<0.05 regarded as significant for untreated/NTC versus rhTGF-β/TLR5 kd cells. NTC, negative transfection control, kd, knockdown; rh, recombinant human; siRNA, short-interference RNA; TGF-β, transforming growth factor-beta; TLR, Toll-like receptor.

visualised by red (light microscopy) and yellow–orange (polarised light) colours. The muscle, vascular fibres were evidenced by yellow (light microscopy) and green (polarised light microscopy).

Statistical analysis

Statistical analysis was performed using Excel 2017 for Windows (Microsoft, Redmond, Washington, USA). Whisker and box plots were assessed by Excel 2017. Significance was calculated using the t-test for paired samples, *p<0.05 was regarded as significant.

RESULTS

Expression of TLRs and activation factors in hepatic and pancreatic stellate cells

Human hepatic (LX-2) and pancreatic (HPSC2.2) stellate cells were treated for 48 hours with 2.5 ng/mL of rhTGF-β. The activation of both cells was confirmed by the over-expression of the inducible factors *COL1A1* and *ACTA2*.[26] Additionally, the expression of *VIM*, *SNAI1* and *SNAI2* was busted by the treatment with rhTGF-β.The expression of *TLR5* was also monitored, thus resulting in slight upregulation (figure 1A, B). Activated LX-2 and HPSC2.2 cells were further processed by microarray that detected a significant suppression of *TLR2*, *TLR3* and a significant over-expression of *TLR5*. *MYD88*, *TICAM* and *TLR6* were stably expressed in both stellate cells, whereas *TLR1* and *TLR4* were detectable only in LX-2 cells (figure 1C). Surprisingly, *TLR5* knockdown (kd) caused in the LX-2 hepatic stellate cells an over-expression of *COL1A1*, *ACTA2*, *VIM*, *SNAI1* and *SNAI2* transcripts. Instead, the HPSC2.2 pancreatic stellate cells showed a stable expression of these transcripts. However, the kd of *TLR5* in LX-2 cells was able to reduce the rhTGF-β–mediated over-expression of the activation factors (figure 1D). Instead, no inhibitory effect was observed in rhTGF-β-treated HPSC2.2 cells after the kd of *TLR5* (figure 1E). Immunofluorescence micrographs evidenced that *TLR5*-kd was able to suppress Collagen I, TLR5, SNAIL and Vimentin in LX-2 and HPSC2.2 cells, even after treatment with rhTGF-β (figure 2A, B). The protein bands of the inducible Smooth Muscle Actin, Collagen I, TLR5 and SNAIL were even not detectable after *TLR5*-kd (LX-2) and the administration of rhTGF-β (LX-2 and HPSC2.2) (figure 2C, D).

Expression of TLR5 and activation factors in three-dimensional model of stellate cells

The expression of *TLR5* and the above-mentioned activation factors was further analysed in LX-2 and HPSC2.2 spheroids (figure 3). LX-2 spheroids were characterised by a stable level of *TLR5* protein and transcript even after treatment with rhTGF-β (figure 3A, B). *TLR5*-kd was able to suppress, significantly (p value<0.05), *TLR5* transcript level. The protein level of TLR5 was suppressed after TLR5-kd and the administration of the transactivation inducer rhTGF-β. Additionally, the transcripts of the activation factors *COL1A1*, *VIM*, *SNAI1* and *SNAI2* were downregulated by *TLR5*-kd, whereas *ACTA2* was slightly upregulated (figure 3B). Focusing on the protein abundance detected by immunofluorescence, it was observed that LX-2 spheroids showed a basal low expression of the inducible Collagen I, which was altered neither by the addition of rhTGF-β nor after *TLR5*-kd. Instead, the inducible alpha smooth muscle actin (αSMA), Vimentin and SNAIL were induced by the treatment with rhTGF-β. *TLR5*-kd hampered the efficacy of rhTGF-β by causing a reduction of the expression of αSMA and Vimentin, whereas SNAIL protein level was not altered (figure 3A). *TLR5*-kd was not efficient in HPSC2.2 spheroids (data not shown), which were employed to show the efficacy of the treatment with rhTGF-β. As shown in figure 3D, rhTGF-β caused a significant over-expression of *COL1A1* and *SNAI1* transcripts. The protein level of TLR5, αSMA and Vimentin was strongly over-expressed. Collagen I and SNAIL proteins were stably low even after the addition of rhTGF-β (figure 3C).

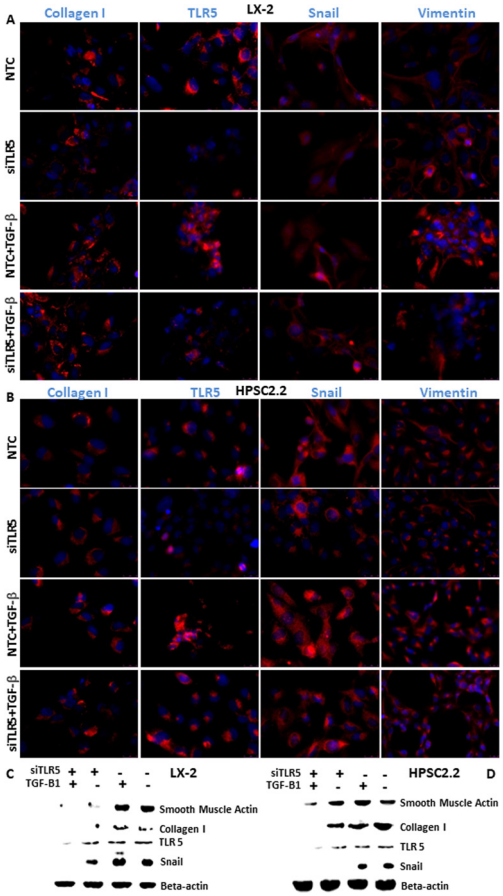

Figure 2 Protein level of the transactivation markers in monolayer cells. Immunofluorescence detection of Collagen I, TLR5, Vimentin and SNAIL in LX-2 (A) and HPSC2.2 (B) cells treated for 48 hours with 2.5 ng/mL of rhTGF-β and siRNA for TLR5. Western blot detection of the protein level of the transactivation markers smooth muscle actin, Collagen I, TLR5 and SNAIL in LC-2 (C) and HPSC2.2 (D) cells treated for 48 hours with 2.5 ng/mL of rhTGF-βand siRNA for TLR5. B-actin was detected as equal loading control. rh, recombinant human; siRNA, short-interference RNA; TGF-β, transforming growth factor-beta; TLR, Toll-like receptor.

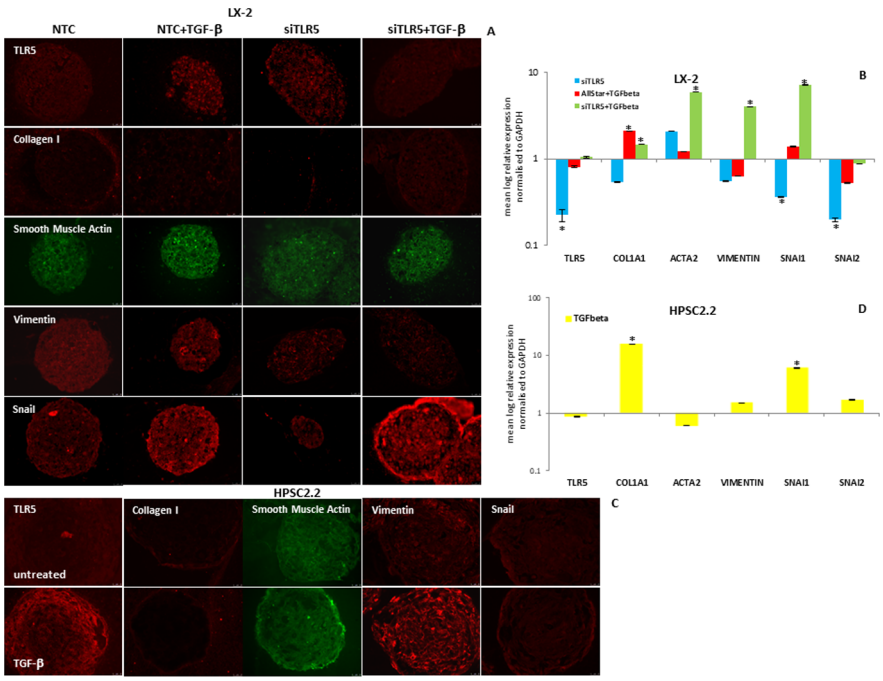

Figure 3 Detection of the transactivation markers in LX-2 and HPSC2.2 spheroids. Immunofluorescence signal (A) and transcript level (B) of the transactivation markers in LX-2 cells treated for 48 hours with 2.5 ng/mL of rhTGF-β and siRNA for TLR5. Immunofluorescence signal (C) and transcript level (D) of the transactivation markers in HPSC2.2 cells treated for 48 hours with 2.5 ng/mL of rhTGF-β. The expression of the transcripts was normalised to untreated cells. *GAPDH* transcript was detected as a housekeeping gene. Shown are mean triplicate values (B, D) ± SEM. *p<0.05 regarded as significant for untreated/NTC versus rhTGF-β/TLR5 kd cells. AllStar/NTC, negative transfection control, kd, knockdown; rh, recombinant human; siRNA, short-interference RNA; TGF-β, transforming growth factor-beta; TLR, Toll-like receptor

Direct modulation of TLR5 does not trigger stellate cells activation

To clarify further the implication of *TLR5* in the activation of hepatic and pancreatic stellate cells, both LX-2 and HPSC2.2 cells were treated for 48 hours with 100 ng/mL of flagellin. LX-2 cells, grown as monolayer, showed a stable expression of *TLR5* transcript and a significant downregulation of the inducible factors *COL1A1* and *ACTA2* (figure 4A). Furthermore, LX-2 spheroids treated with flagellin showed a stable level of *TLR5*, *COL1A1* and *ACTA2* (figure 4B). In HPSC2.2 monolayer and spheroids, flagellin was responsible for pulling down as well *TLR5*, *COL1A1* and *ACTA2* (figure 4C, D). The inhibitory effect of flagellin was neutralised by the addition of

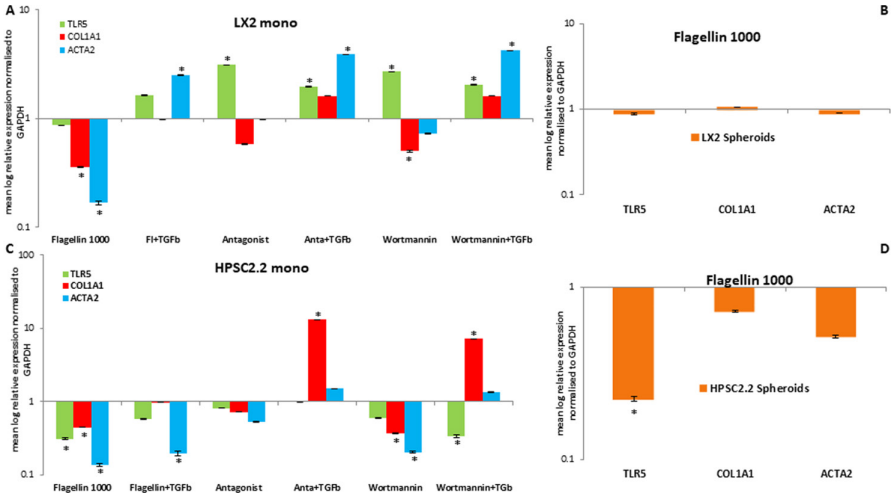

Figure 4 Implication of flagellin in the modulation of *TLR5*, *COL1A1* and *ACTA2* in monolayer and spheroids. Detection of the transcript level of *TLR5*, *COL1A1* and *ACTA2* in LX-2 and HPSC2.2 monolayer (A, C) and spheroids (B, D) treated for 48 hours with solo and combined administration of 1000 ng/mL of flagellin, 2.5 ng/mL of rhTGF-β, 1 ng/ml of TLR5 antagonist and 1 µmol/L of wortmannin. The expression of the transcripts was normalised to untreated cells. *GAPDH* transcript was detected as a housekeeping gene. Shown are mean triplicate values±SEM. *p<0.05 regarded as significant for untreated versus treated LX-2/HPSC2.2 cells. TGF-β, transforming growth factor-beta; TLR, Toll-like receptor

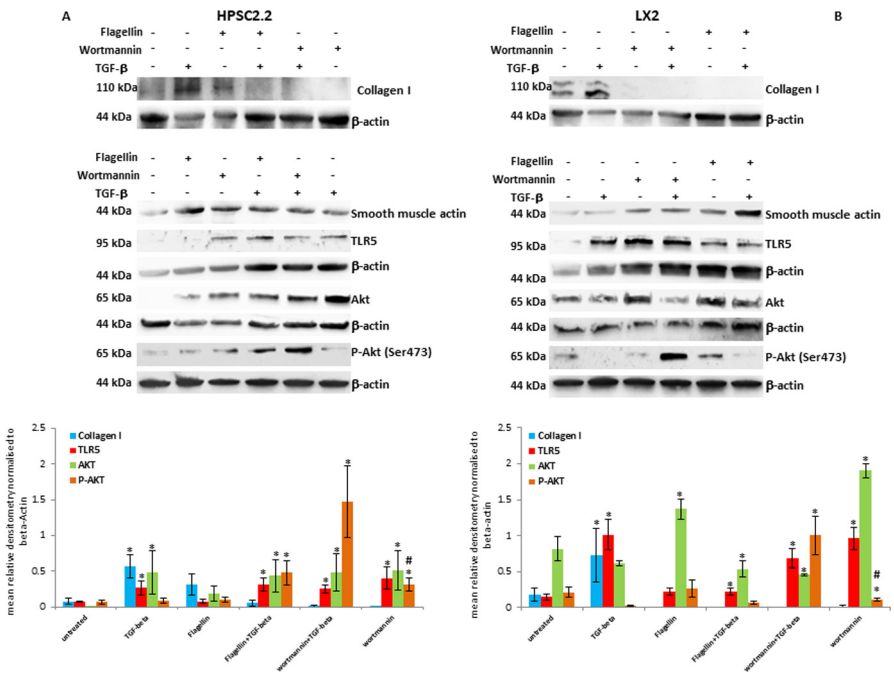

Figure 5 Implication of Akt in the transactivation of the hepatic and pancreatic stellate cells. Detection of the protein level of Collagen I, smooth muscle actin, TLR5, Akt and P-Akt in LX-2 and HPSC2.2 cells treated for 48 hours with single and combined 1000 ng/mL of flagellin, 2.5 ng/mL of rhTGF-β and 1 µmol/L of wortmannin. B-actin was detected as equal loading control. Graphs represent the densitometry analysis of the protein bands. Shown are mean triplicate values±SEM. *p<0.05 regarded as significant for untreated versus treated LX-2/HPSC2.2 cells. rh, recombinant human; TGF-β, transforming growth factor-beta; TLR, Toll-like receptor

rhTGF-β leading to an over-expression of *TLR5*, *COL1A1* and *ACTA2* in LX-2 cells (figure 4C);whereas the addition of rhTGF-β,to HPSC2.2 cells, was only able to recover the transcript level of *TLR5* and *COL1A1* without perturbing the downregulation of *ACTA2* caused by the administration of flagellin (figure 4D). The administration of TLR5 antagonist antibody (1 ng/mL) to LX-2 and HPSC2.2 cells caused an over-expression of *TLR5* transcript and a stable expression of *COL1A1* and *ACTA2*. Furthermore, TLR5 blocking antibody showed an additive effect to the administration of rhTGF-β inducing a further over-expression of *TLR5*, *COL1A1* and *ACTA2* in both hepatic and pancreatic stellate cells (figure 4C, D). Nonetheless, the administration of wortmannin, a well-known inhibitor of the PI3K/AKT pathway, caused the downregulation of *COL1A1* and *ACTA2* but not of *TLR5* in both cells. Its effect was neutralised by the administration of rhTGF-β, which restored the over-expression of *TLR5*, *COL1A1* and *ACTA2*. *TLR5* transcript was downregulated only in HPSC2.2 cells (figure 4C, D).

Collagen I, expressed at basal level in LX-2 cells, increased significantly after treatment with rhTGF-β. Instead, its protein level was detectable neither after the addition of wortmannin nor after flagellin (figure 5B). In HPSC2.2 cells, Collagen I increased after treatment with rhTGF-β and flagellin. Its level was not detectable after treatment with wortmannin and its combination with rhTGF-β (figure 5A).The αSMA was detected in both LX-2 and HPSC2.2 cells at basal level and after treatment with rhTGF-β, flagellin and wortmannin. TLR5 protein level increased in both cells after administration of rhTGF-β.Interestingly, the addition of wortmannin to the cells caused an upregulation of TLR5 protein level, whereas the treatment with flagellin did not cause any change of TLR5 protein level. The protein level of AKT was stable or upregulated in both cells treated with rhTGF-β, flagellin and wortmannin. Interestingly, the active phosphorylated form of AKT (Ser473) was upregulated in LX-2 cells by the administration of both rhTGF-β and wortmannin. In HPSC2.2, it could be observed an increase of P-AKT even after the combination of rhTGF-β and flagellin (figure 5A, B).The solo administration of wortmannin caused a reduction of the active P-AKT in both cells in comparison with the total AKT protein level (figure 5A, B).

Expression of TLR5 and activating factors in liver fibrosis mouse model

Liver sections of mice treated for up to 18 weeks with TAA and ethanol were stained for the inducible collagen type I and collagen type III (muscle fibres). As shown in figure 6A, inducible collagen was detectable already after 6 weeks of treatment. The prolongation of the treatment up to 18 weeks enhanced the expression of collagen. As shown under polarised microscopy, the collagen fibres were characterised by an accumulation of collagen type I (yellow-orange) and type III (green). Thus confirming a fibrotic process induced by TAA and alcohol in the hepatic tissue of the mice. Liver specimens of mice treated with TAA and ethanol were stained for collagen I and

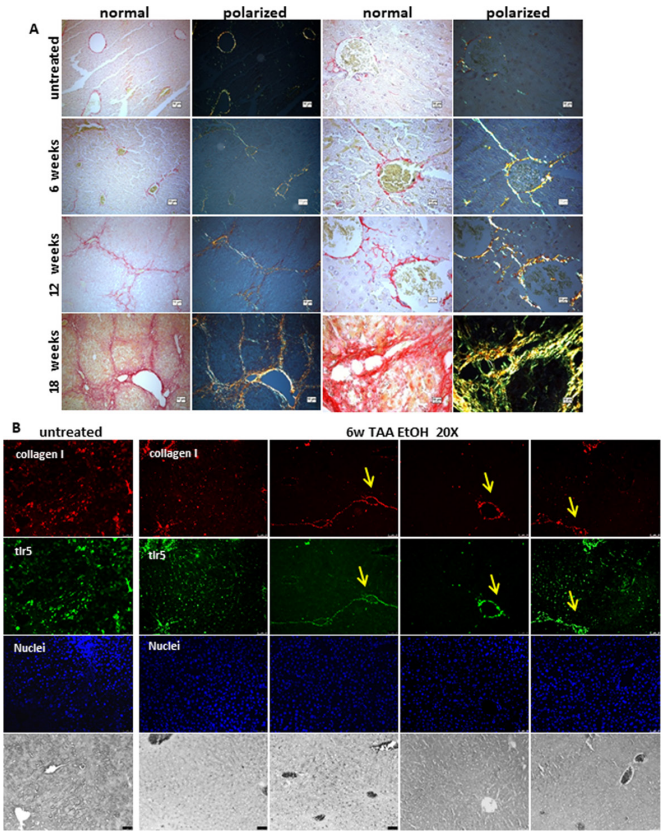

Figure 6 Detection of fibrosis and transactivation markers in murine fibrotic liver tissue. Detection of the three different collagen fibres in thioacetamide/ethanol induced liver fibrosis (A). Picrosirius stained collagen fibres type I are evidenced by the red colour (normal light) and yellow-orange (polarised light). The yellow (normal light) green colour (polarised light) evidence the deposit of muscle/vascular fibers of collagen type III. (B) Immunofluorescence detection of the transactivation marker collagen I (red) and tlr5 (green) in mice treated for 6 weeks with thioacetamide/ethanol. Nuclei were stained with Hoechst 33342 (blue).

tlr5. As shown by immunofluorescence (figure 6B), the protein level of collagen I and tlr5 was unchanged in the 6weeks treated mice. Interestingly, both collagen I and tlr5 distributed differently in treated mice in comparison to untreated mice. It could also observe an accumulation of both proteins in the fibrotic areas (yellow arrows). Prolonged treatment (12 weeks) with TAA and ethanol caused a pronounced fibrosis in murine livers (figure 7). The detection of collagen I and tlr5 evidenced that both proteins localised in the stiffness of fibrotic areas and their expression increased in comparison with untreated and 6 weeks treated mice. Further analysis of mice treated for 18 weeks with TAA and ethanol evidenced a strong fibrosis (figure 8). The expression of collagen I and tlr5 in these liver specimens is extremely high and distributed not only in the fibrotic areas but also in the rest of the tissue (figure 8). Interestingly, liver tissue is characterised for the simultaneous expression of both collagen I and tlr5 in the same areas. Additionally, the transcripts of *col1a1* and *acta2*, detected in 18 weeks treated mice, were significantly overexpressed. The *tlr5* transcript was stable (box and whisker plots, figure 8).

DISCUSSION

There have been many evidences, within the last decades, that bacterial components such as microbiome or bacteria itself are key drivers in liver fibrosis. Flagellin—a part of most entero-bacteria and other intestinal prokaryotes—is the natural ligand to TLR-5. Meanwhile portal-hypertension, flagellin is able to transit from the intraluminal compartment into the portosystemic circulation.[27–30]

This study was set up to analyse the expression of *TLR5* in hepatic and pancreatic stellate cells and its involvement in the TGF-β-mediated transition. Stellate cells express at basal level TLRs, especially TLR3, TLR4 and TLR5 in our proposed model. Active cells evidenced an over-expression of *TLR5*, which exert a key role for the cellular transition as well. This study highlighted a double-edged sword mechanism of *TLR5* relying on the signalling pathways activated in the presence of TGF-β.

Stellate cells of liver and pancreas are responsible for the storage of vitamin A and fatty acids. They are able to transactivate during tissue damage and colonise the

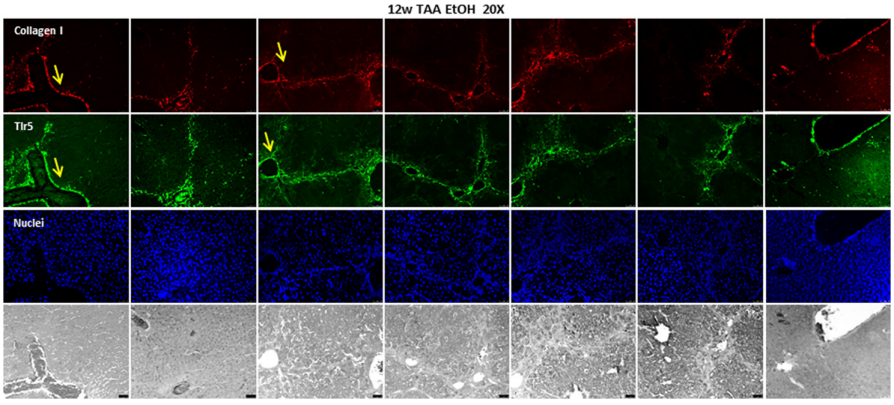

Figure 7 Detection of collagen I and tlr5 in murine fibrotic liver tissue after 12 weeks of treatment. Immunofluorescence detection of the transactivation marker collagen I (red) and tlr5 (green) in mice treated for 12 weeks with thioacetamide/ethanol. Nuclei were stained with Hoechst 33342 (blue).

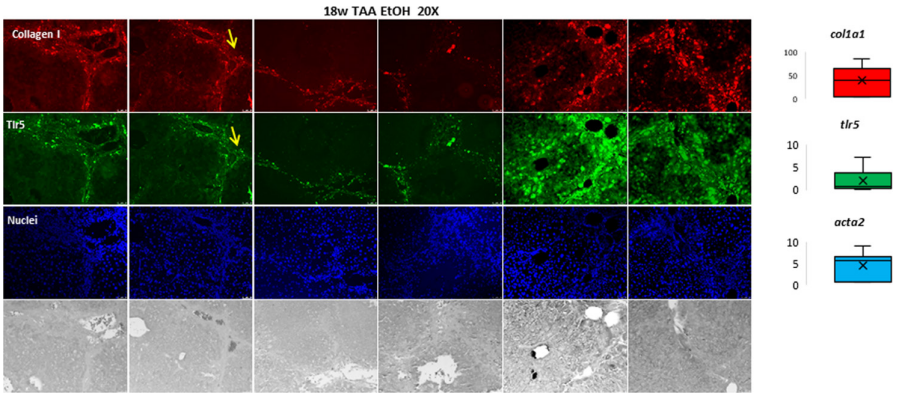

Figure 8 Detection of collagen I and tlr5 in murine fibrotic liver tissue after 18 weeks of treatment. Immunofluorescence detection (left panels) of the transactivation marker collagen I (red) and tlr5 (green) in mice treated for 12 weeks with thioacetamide/ethanol. Nuclei were stained with Hoechst 33342 (blue). Box and whiskers plots of the transcript expression of *col1a1*, *tlr5* and *acta2*. Shown are means of six mice values±SEM.

surrounding tissue exerting a repair process of fibrosis. An exacerbation of such a process has been correlated with pathological alteration of liver and pancreas, thus leading to cirrhosis in the liver and, furthermore, tumourigenesis in both organs.[8 9 11–14]

Both hepatic and pancreatic stellate cells, when incubated with TGF-β[31] showed an over-expression of the transactivation responsible genes *COL1A1* and *ACTA2*[32] and, additionally, an upregulation of *TLR5, VIM and SNAI1*. The protein level of these genes was found upregulated also in monolayer and LX2-derived and HPSC2.2-derived spheroids. Although only a weak suppressing effect of these genes could be observed after knockdown of *TLR5*, its silencing caused a significant downregulation of the protein level of Collagen I, αSMA, Vimentin and *TLR5* protein. Interestingly, it could be evidenced that *TLR5*-kd was able to neutralise the transactivation mediated by the administration of TGF-β thus leading to a suppression of the proteins αSMA, Collagen I, TLR5, SNAIL and Vimentin in both hepatic and pancreatic stellate cells monolayer and spheroids.

The administration of flagellin, the ligand of TLR5,[33] was not able to induce itself the transactivation of the hepatic LX-2 and pancreatic HPSC2.2 stellate cells. Surprisingly, both cells showed a downregulation of *TLR5*, *COL1A1* and *ACTA2*, thus excluding *TLR5* as unique responsible for the transactivation and highlighting an inhibitory effect of TLR5, even after the administration of TGF-β, in the hepatic and pancreatic stellate cells. Instead, antagonising TLR5 did not inhibit the expression of *TLR5*, *COL1A1* and *ACTA2* and prompted the efficacy of TGF-β to further induce the over-expression of such genes. The solo administration of wortmannin, a well-known inhibitor of the PI3K/AKT pathway and able to modulate flagellin-induced gene expression,[34] was able to downregulate *COL1A1* and *ACTA2* but not *TLR5* transcripts. Its inhibitory effect was hampered by TGF-β,which caused the over-expression of *COL1A1* and *ACTA2* transcripts. Analysis of the protein level highlighted the ability of wortmannin to suppress P-AKT and, furthermore, Collagen I. TGF-β was able to upregulate P-AKT and neutralise the inhibitory effect of wortmannin on the expression of Collagen I. Thus, highlighting, for the first time, an involvement of the PI3K/AKT pathway in the mechanisms mediated by TGF-β and TLR5. The development of liver fibrosis, observed in mice treated with TAA,[35] timely correlated with an increase of *col1a1* and *acta2* and the protein level of the inducible collagen I and the αSMA. Interestingly, it was found a significant upregulation of *tlr5* and its encoded protein, especially in the high fibrotic areas of the murine liver tissue after long time exposure to TAA. Thus, highlighting a direct contribution of *tlr5* to the development of liver fibrosis and an active role in the transactivating process of the stellate cells. Such conclusions have been previously observed in carbon tetrachloride treated mice knocked out for *tlr5*[36] and in a bile duct ligation liver fibrosis model.[20]

Based on the current findings, the study highlights that *TLR5* correlates strictly with the transactivation of hepatic and pancreatic stellate cells in vitro and in vivo. The transient inactivation of *TLR5* was able to suppress the expression of the activating genes *COL1A1* and *ACTA2* and their encoded proteins, even after the administration of the most potent activator TGF-β. Even though in contrast with the previously observed effects of TLR5 in a mouse model of partial hepatectomy[21] and hepatotoxin, cholestasis,[20] the current study pointed to find the possible implication of *TLR5* in TGF-β-mediated transactivation of stellate cells. Interestingly, a direct activation of TLR5 by the administration of flagellin[20 21] inhibited the transactivation, which was prompted, instead, by the combination of TGF-β and TLR5 antagonist. The administration of wortmannin inhibited the expression of the transactivation proteins similarly to flagellin but did not modulate the expression of TLR5. To summarise the current findings, it could be assumed that the treatment with TGF-β promoted the transcriptional activity of SMAD (Mothers Against Decapenthaplegic) in the stellate cells.[37–39] Thus prompting the transcription of *COL1A1*, *ACTA2* and *TLR5* genes. Additionally, TGF-β

promotes the activity of PI3K/AKT,[37] which inhibits NF-kB (Nuclear Factor-kappaB). The inhibition of NF-kB impedes the suppression of *TLR5*, *COL1A1* and *ACTA2*. The activation of TLR5 by the administration of flagellin or the inhibition of PI3K/AKT mediated by the application of wortmannin unleash NF-kB thus leading to the suppression of the transcription of such genes and the block of transactivation of the stellate cells.

In conclusion, *TLR5* gene is transcribed after administration of TGF-β and its expression is bound to the stellate cell transactivation process. Instead, a direct activation of TLR5, mediated by its ligand flagellin, would preferably suppress the transactivation of stellate cells, even in cells treated with TGF-β.

Our data are underlining the role of TLR-5 as a druggable target, since new compounds, for example, siRNA/compound loaded nanoparticles, have been found and are under investigation in ongoing clinical trials.[40 41] These in vitro findings conduct a step forward closing the gap between the descriptive observations of *bacteria* and enhanced organ fibrosis, for example, *Helicobacter hepaticus (H. hepaticus)* in patients suffering from inflammatory hepatitis.[42] Furthermore, it has been shown that *H. hepaticus* is responsible for the induction of liver fibrosis by triggering the expression of the interleukin-33 receptor ST2 in BALB/c mice, thus not excluding the involvement of other inflammatory pathways.[43] It is reasonable that *bacteria* are able to orchestrate a pro-inflammatory environment through the flagellin receptor TLR-5, which is cross activated by TGF-β.

Targeting TLR-5 would be the new target to silence organ fibrosis conducting to resolve extracellular matrix and inflammation. Nonetheless, TLR5 represents a druggable target for other human diseases. It has been shown that TLR5 and other TLRs are over-expressed in patients affected by lupus[44] and are diagnostic markers for oesophageal adenocarcinoma.[45] Interestingly, the expression of TLR5 has been correlated with a better prognosis of patients affected by colorectal adenocarcinoma[46] and its expression has been found in nasopharyngeal carcinoma.[47] However, the molecular mechanisms causing its over-expression still need to be clarified in such human diseases. The conclusions raised by our study highlighted not only new aspects regarding the molecular mechanisms underlying the role exerted by TLR5 but also the implication of an interaction within undesirable guests (bacterial pathogens) and human specialised cells of different tissues and organs.

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