Final Report

Project Title: Exploiting the Cre/loxP system to determine a requirement of Tgf-β signaling in Hepatic Stellate Cells during the liver fibrogenesis and regeneration

By Dr.Somyoth Sridurongrit

June 2016

Contract No. TRG5780070

Final Report

Project Title: Exploiting the Cre/loxP system to determine a requirement of Tgf-β signaling in Hepatic Stellate Cells during the liver fibrogenesis and regeneration

Dr.Somyoth Sridurongrit
Department of Anatomy, Faculty of Science
Mahidol University

This project granted by the Thailand Research Fund

Abstract

Project Code: TRG5780070

Project Title: Exploiting the Cre/loxP system to determine a requirement of Tgf-β signaling in Hepatic Stellate Cells during the liver fibrogenesis and regeneration

Investigator: Somyoth Sridurongrit, Ph.D.

E-mail Address: somyoth.sri@mahidol.ac.th

Project Period: 2 years

Abstract:

Development of liver fibrosis is orchestrated by highly proliferative, contractile myofibroblasts (MFs). It is generally believed that MFs are mainly derived from Hepatic Stellate Cells (HSCs) thru the process called the HSC activation. This idea has been supported by data obtained in rodent and human studies showing that there is an increase number of cell expressing generic fibroblast/mesenchyme markers (e.g., FSP-1, vimentin, and nestin) in fibrotic livers. However, it has been shown that these markers are not only expressed by HSCs but also by cells of other origin including hematopoietic and epithelial lineage. In addition, since hepatic MFs are currently known to be potentially derived from bone marrow-derived cells and hepatic epithelial cells, it remains to be determined whether activation of HSCs is an essential process in the generation of collagen-producing MFs and, therefore, a major propeller of liver fibrogenesis *in vivo*. To shed more light on this cellular mechanism of liver fibrosis, this study utilized the Cre/loxP system to abrogate Tgf-β signaling, a key inducer of HSC activation *in vitro*, in HSCs. Here, we report that lack of Tgf-β signaling in HSC leaded to reduced collagen deposition and decreased number of myofibroblasts in livers of mice treated with thioacetamide (TAA). There was a significant decrease in the expression of pro-fibrogenic factors (Pdgf-A, Pdgf-B and Tgf-)-β as well as inflammatory cytokines (Tnf-α and IL-6) in *Alk5/GFAP-Cre* mutant livers. CD3 and myeloperoxidase immunostaining showed a reduction in immune cell infiltration in mutant livers, compared to those of control livers. Associated with reduced fibrosis and inflammation in mutant livers, there was marked attenuation of liver injury, as indicated by serum alanine/aspartate aminotransferase. In conclusion, our results indicate that Alk5-mediated Tgf-β signaling is necessary for the HSC-mediated liver fibrosis and accelerates the response to hepatic damage thru the amplification of liver inflammation.

Keywords : fibrogenesis, HSC activation, Tgf-β signaling, hepatic inflammation, liver damage

บทคัดย่อ

กระบวนการเกิดพังพืด (fibrosis) ถูกควบคุมโดยเซลล์ myofibroblasts (MFs) ที่มีความสามารถในการหดสั้นและที่มีอัตราการแบ่งตังสูง ความเข้าใจในปัจจุบันคือว่า MFs เกิดมาจาก Hepatic Stellate cells (HSCs) โดยผ่านกระบวนการ HSC activation ความเข้าใจนี้มีหลักฐานสนับสนุนมาจากการศึกษาตับของคนและสัตว์ทดลองที่แสดงให้เห็นถึงการเพิ่มขึ้นของจำนวน MFs ที่วัดจากการแสดงออกของยีนที่เป็นตัวบ่งชี้ของ fibroblsts เช่น FSP-1 Vimentin และ Nestin ในตับที่เกิดพังพืด อย่างไรก็ตามนักวิจัยในปัจจุบันพบว่าตัวบ่งชี้เหล่านั้นไม่ได้แสดงออกเฉพาะใน fibroblasts เท่านั้นแต่ยังแสดงออกในเซลล์เม็ดเลือด (hematopoietic cells) และเซลล์เยื่อบุผิว (epithelial cells) อีกด้วย นอกจากนี้เนื่องจากเป็นที่ทราบกันแล้วว่า MFs สามารถพัฒนามาจากเซลล์ไขกระดูก (bone marrow cells) และเซลล์เยื่อบุผิว โดยกระบวนการ EMT (Epithelial-to-Mesenchyme Transition) ความเชื่อที่ว่า HSC activation เป็นการกระบวนการหลักที่จำเป็นต่อการสร้าง MFs ระหว่างการเกิดพังพืดจึงควรได้รับการพิสูจน์และยืนยันอีกครั้ง โครงการนี้ศึกษากลไกการเกิดพังพืดโดยใช้ระบบ Cre/loxP ในการยับยั้ง Tgf-β signaling ที่เป็นตัวเหนี่ยวนำที่สำคัญของ HSC activation ผลการทดลองของเราแสดงให้เห็นว่าการยับยั้ง Tgf-β ใน HSCs นำไปสู่การลดลงของคอลลาเจนและจำนวน MFs ที่สะสมอยู่ในตับของหนูที่ได้รับสาร thioacetamide (TAA) เรายังพบอีกว่าปริมาณของสารก่อพังพืด (เช่น Tgf-β Pdgf-A และ Pdgf-B) และสารส่งเสริมการอักเสบ (เช่น TNF-α และ IL-6) ในตับของหนู mutant นั้นต่ำกว่าในหนูกลุ่มควบคุม การย้อมเนื้อเยื่อตับด้วยแอนติบอดี้ที่จำเพาะต่อ CD3 และ myeloperoxidase แสดงให้เห็นว่าเซลล์ T-lymphocytes และ Neutrophils ที่ก่อการอักเสบในตับของหนู mutant มีปริมาณน้อยกว่าปริมาณเซลล์ก่อการอักเสบในตับของกลุ่มควบคุม การลดลงของของการเกิดพังพืดและการอักเสบในตับของหนู mutant นั้นมีความสัมพันธ์กับระดับการบาดเจ็บที่น้อยลงดังที่แสดงให้เห็นโดยการลดลงของระดับ serum alanine/aspartate aminotransferase (ALT และ AST) ในเลือดของหนู mutant โดยสรุปแล้วผลงานของเราแสดงให้เห็นว่า Tgf-β เป็น signaling ที่จำเป็นต่อกระบวนการเกิดพังพืดและสามารถเพิ่มระดับความบาดเจ็บโดยการส่งเสริมการบาดเจ็บของตับ

Final report content:

1.Executive summary

Chronic Liver Disease (CLD) is a major health problem not only in the western world but also in Thailand. CLD is often caused by a damage of liver parenchyma as a result of virus/ liver fluke/ malaria infections, alcohol/drug abuses, food poisons and high-fat diets. While there have been progresses in improving public sanitation and in educating general population about the malediction of consuming uncooked fish, alcoholism and imbalanced diet, too many Thais develop CLD each year resulting in substantial economic costs for health care. The only treatment available for patients with advanced stage of CLD is liver transplantation; however, a shortage of suitable donor organs and complications after the surgery means that alternative therapies for CLD are urgently required. In CLD, persistent fibrosis impairs liver function and causes cirrhosis which predisposes to the development of hepatocellular carcinoma (HCC) and liver failure. An inhibition of chronic fibrogenesis could result in decreased organ injury, improved liver function and enhanced organ regeneration. Thus, this anti-fibrotic approach is currently recognized as a promising therapy for patients with CLD.

To enable the rational development of preventive/therapeutic agents for CLD, it is important to understand how different types of hepatic cells cooperate to trigger the healing response that leads to fibrosis, inflammation and organ repair. In past decades, a significant amount of studies using cell culture-based experiments have proposed several factors as important regulators for cell-to-cell communication during the progression of CLD. However, for most of these factors, their roles cannot be validated in animal model, therefore, impeding the translation of knowledge from basic research to clinical use. This highlights the limitation of popular *in vitro* experiments and the demand of a superior experimental model to study the function of candidate factors that play a key role in the disease progression. Currently, advance in genetic engineering technology using the Cre/loxP system make it possible for scientists to turn on/off a gene in a living organism and in cell-type specific manner. This technique will provide more accurate information about *in vivo* functions of tropic factors that regulate organ injury and repair. Better understanding of this molecular mechanism controlling liver damage will lead to the development of new treatments that can efficiently improve the organ repair and, consequently, reduce the risk of liver-injured patients to develop CLD. In the present study, we applied the Cre/loxP system to address a specific function of Tgf-β signaling in Hepatic stellate cells (HSCs) during liver damage and regeneration. This site-directed mutagenesis technique was used in this study to determine whether Tgf-β type I receptor Alk5 mediates HSCs activation *in vivo* leading to liver fibrosis and influence inflammation and organ damage. The proposed study has given us a more accurate description of how Tgf-β signal via Alk5 regulates HSCs during chronic liver injury; the knowledge that is important for the development of better anti-Tgf-β therapy which could effectively enhance organ regeneration with the less possible side effect.

2. Objective

To determine whether Tgf-β signaling in HSCs is required for liver fibrogenesis and mediates inflammation and organ injury.

3. Research methodology

Conditional inactivation of *Alk5* in hepatocytes:

To determine whether Tgf-β signaling is essential for hepatic healing response following live injury, we developed *Alk5/GFAP-Cre* mice mutant mice lacking Tgf-β type I receptor, Alk5 in HSCs. *GFAP-Cre* transgenic mice [1] which is heterozyous for $Alk5^{flox}$ were crossed with $Alk5^{flox/flox}$ mice [2], as shown in Fig. 1, to generate *Alk5/GFAP-Cre* mice which lack endogenous Alk5 in HSCs. One out of four offspring was expected to be the mutant inheriting one *Alk5* floxed allele from the female and one *Alk5* floxed allele along with the *GFAP-Cre* transgene from the male. From this crossing, $GFAP\text{-}Cre^{WT/WT}$ littermates were used as control. Six 3-4 month-old mutant and control littermate were undergone TAA-induced liver injury and their livers were harvested and evaluated for the degree of damage/regeneration using immunohistochemistry.

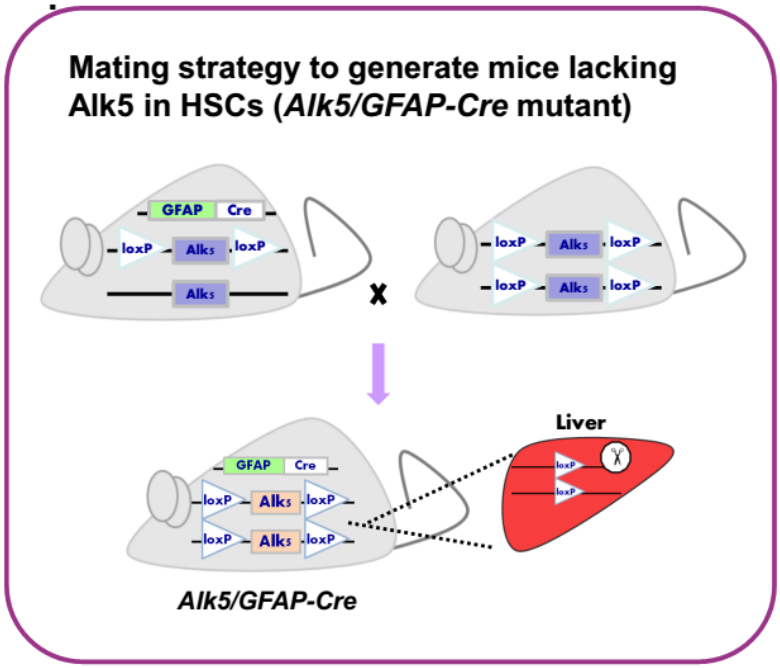


Figure 1. Generation of mutant mice lacking Alk5 in HSCs (Alk5/GFAP-Cre

Induction of liver fibrosis by thioacetamide (TAA):

TAA (sigma) solution (25 mg/ml) was injected intraperitoneally (IP) into control and *Alk5/GFAP-Cre* mutant mice three times a week for 8 weeks. This dosage has been proven to be sufficient to induce centrilobular hepatocyte death which leads to liver fibrosis. Six three-month-old control and mutant mice were subjected to TAA administration. Then, all mice are sacrificed and whole livers are harvested. Each of harvested livers was divided into two parts. One part was fixed in Bouin's fixative for pathological examination and immunohistochemistry. Livers from the second part were frozen in liquid nitrogen for RNA purification and reverse transcription–PCR.

Histological analysis:

To assess histological features of livers architecture and fibrosis progression, mice were sacrificed and liver tissues were collected. The tissues were washed in phosphate-buffer-saline and cut in cross sections of 3 mm. The samples were fixed in Bouin's fixative for 4 days, and embedded in paraffin. Paraffin-embedded tissues were cut into 5-µm thick cross-section, and the sections were stained with hematoxylin and eosin (H&E) histopathological examination. Sections were stained with Sirius red solution kit and Masson's trichrome to visualize collagen deposition.

Immunohistrochemistry:

Paraffin-embedded tissues were cut into 5-µm thick cross-section. The liver sections were deparaffinized, rehydrated by using standard xylene/alcohol protocol and microwaved in Antigen Unmask solutions according to the manufacturer's recommendation. After blocking, the sections were incubated with primary antibody mouse anti-α-smooth muscle actin (Dako), rabbit anti-CD3 (Dako) and rabbit anti-myeloperoxidase (Sigma), at 4 ºC overnight. After incubation, the sections were washed with Tris-buffered saline before incubated with secondary antibody, peroxidase-conjugated goat anti-mouse IgG (Dako) and peroxidase-conjugated goat anti-rabbit IgG (Dako). Visualization was performed using VECTOR NovaRED substrate kit for peroxidase (Vector). For quantification of α-SMA-, Cd3- and myeloperoxidase-positive cells, 5 high-power field images were taken around a periportal area of each animal and counted using Zeiss Axioversion software. All counts were averaged among each group (mutant or control).

Serum analysis:

Liver damage and death of hepatocytes either necrosis or apoptosis was determined by measurement level of serum enzymes include alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Blood was collected after sacrifice under anesthesia for the assessment of liver function. Blood was transferred to centrifuge for 10 minutes at 3000 rpm to separate serum. After that blood serum was transferred to a fresh tube and kept in -20ºC until use. Serum biochemical measurements were performed by Bangkok RIA laboratory.

mRNA analysis:

RNA was prepared from frozen liver using RNeasy kit (Qiagen) and cDNA was prepared using QuantiNova reverse transcription kit and omniscript reverse transcription kit (Qiagen). Quantitative real-time-PCR (qRT-PCR) was performed using iTaq Universal SYBR Green supermix (Bio-Rad) and 7500 Fast Real-Time PCR system (Applied Biosystems) with PCR primers in Table 1.

Statistical analysis:

All experiments were performed with three or more animals, and representative data are presented. Quantification of positive cells (α-SMA, CD3 and myeloperoxidase) and serum biochemistry measurements were compared for statistic analysis by student's *t*-test 9Microsoft Excel). Statistic significance was determined by non-parametric Kruskal-Wallis One-way ANOVA on Ranks. A p value <0.05 was considered statistic significant.

Table 1

PCR primer	Sequences	Size of PCR product (bp)
Tnf-α	5'-CCCAGGTATATGGGCTCATACC-3'(F) 5'-GCCGATTTGCTATCTCATACCAGG-3'(R)	121
IL-6	5'-CCGGAGAGGAGACTTCACAG-3'(F) 5'-TCCACGATTTCCCAGAGAAC-3' (R)	102
MCP-1 (CCL2)	5'-ATTGGGATCATCTTGCTGGT-'3 (F) 5'-CCTGCTGTTCACAGTTGCC-'3 (R)	108
MIP-2 (CXCL2)	5'-AGACAGAAGTCATAGCCACTCTCAAG-3' (F) 5'-CCTCCTTTCCAGGTCAGTTAGC-3'(R)	126
Tgf-β	5'-TGCGCTTGCAGAGATTAAAA-'3 (F) 5'-AGCCCTGTATTCCGTCTCCT-'3(R)	186
Ctgf	5'-CAAAGCAGCTGCAAATACCA-'3 (F) 5'-GGCCAAATGTGTCTTCCAGT-'3(R)	220
Pdgf-A	5'-CCTGTGCCCATCCGCAGGAAGAGA-3'(F) 5'-TTGGCCACCTTGACGCTGCGGTG-3 (R)	227
Pdgf-B	5'-ATCGCCGAGTGCAAGACGCG-3'(F) 5'-AAGCACCATTGGCCGTCCGA-3' (R)	582
α-SMA	5'-GTCCCAGACATCAGGGAGTAA-'3(F) 5'-TCGGATACTTCAGCGTCAGGA-'3(R)	102
mCol1a1	5'-GTCCCTGAAGTCAGCTGCATA-3'(F) 5'-TGGGACAGTCCAGTTCTTCAT-3'(R)	145
Bax	5'-GATCAGCTCGGGCACTTTAG-'3(F) 5'-TTGCTGATGGCAACTTCAAC-'3(R)	101
Bcl-xl	5'-GCTGCATTGTTCCCGTAGAG-'3(F) 5'-GTTGGATGGCCACCTATCTG-'3(R)	97
β-actin	5'-GTGGGCCGCTCTAGGCACCAA-3'(F) 5'-CGGTTGGCCTTAGGGTTCAGGG-3'(R)	245
Gapdh	5'-TGTTGAAGTCACAGGAGACAACCT-3'(F) 5' -AACCTGCCAAGTATGATGACATCA-3'(R)	111

Results

4.1) Thioacetamide (TAA) administration leaded to hepatocyte degeneration, liver fibrosis and inflammation.

The mechanism of liver damage involves hepatic cell injury, immune cell infiltration and liver fibrosis. As expected, TAA caused hepatic injury after 8-week treatment (Fig.2a) with corresponding increase in serum ALT and AST (Fig.2b). There is significant immune infiltration in periportal area in mice treated with TAA (Fig.2c-e).The accumulated leukocytes include infiltrating monocytes (Fig.2c), neutrophils (Fig.2d) and T-lymphocytes (Fig.2e). This increased immune infiltration is accompanied by higher expression of proinflammatory mediators in liver (Fig.2f). In addition, collagen staining shows that The TAA treatment is sufficient to induce fibrogenesis in mice (Fig.2g).

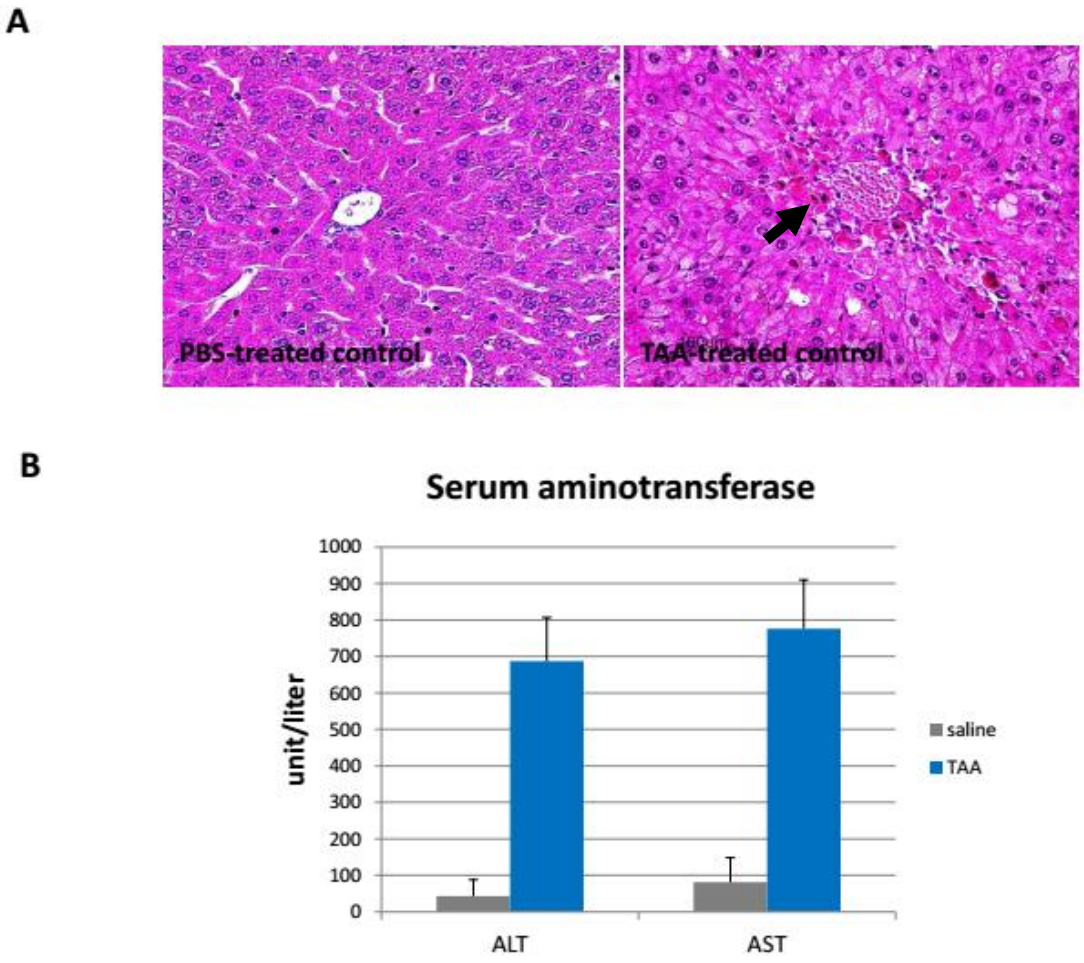


Figure 2. A liver fibrosis, inflammation and injury were observed in mouse livers after TAA exposure. A. Representative Photomicrographs for H&E staining of livers from mice treated with TAA. An arrow indicates centrilobular necrosis in livers of TAA-treated mice. B. Quantification of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) showed an increased SLT and ALT in mice treated with TAA.

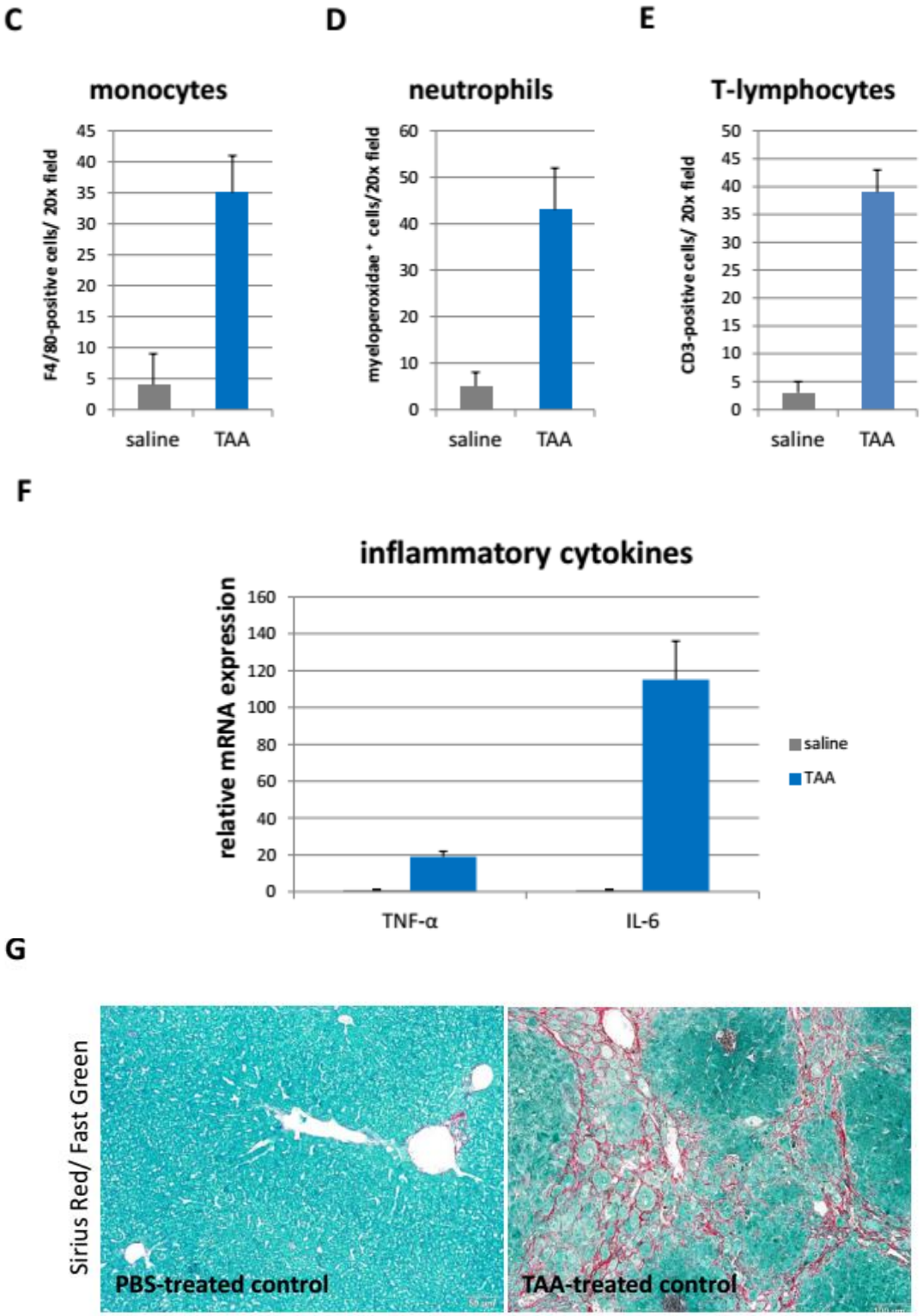


Figure 2 (continued). **C-E**. Quantification of F4/80-, CD3- and myeloperoxidase-positive cells shows a significant increase of monocytes (**C**), neutrophils (**D**) T lymphocytes (**E**) in livers of mice treated with TAA. **F**.Relative mRNA expression analysis of Tnf-α and IL-6 (by qRT-PCR) demonstrates an increased expression of inflammatory cytokines in mice treated with TAA. **G**. Increased collagen deposition in livers of TAA-treated mice, as assessed by Sirius Red/Fast green staining.

4.2) Attenuation of TAA-induced liver fibrosis in Hepatic Stellate Cell (HSC)-Alk5-deficeint mice (*Alk5/GFAP-Cre* mice).

Since Tgf-β signaling is known to promote HSC differentiation into collagen-producing myofibroblast, we assessed fibrosis by collagen morphometry in liver of mutant mice Alk5 in HSCs. After 8 week treatment of TAA, collagen deposition is significantly decreased in *Alk5/GFAP-Cre* mice, compared to control littermates, as indicated by Sirius red/fast green staining and morphometry (Fig.3a-b). To determine whether the amount of myofibroblasts coincide with a decreased fibrosis in mutant livers, we immunostain liver sections using α-smooth muscle actin (α-SMA) antibodies. Our results show a fewer number of α-SMA-positive cells in periportal and centrilobular areas of mutant, compared to those of controls (Fig.3c-d). Quantitative RT-PCR (qRT-PCR) is performed to confirm a reduction in α-SMA and collagen expression in mutant liver (Fig.3e).

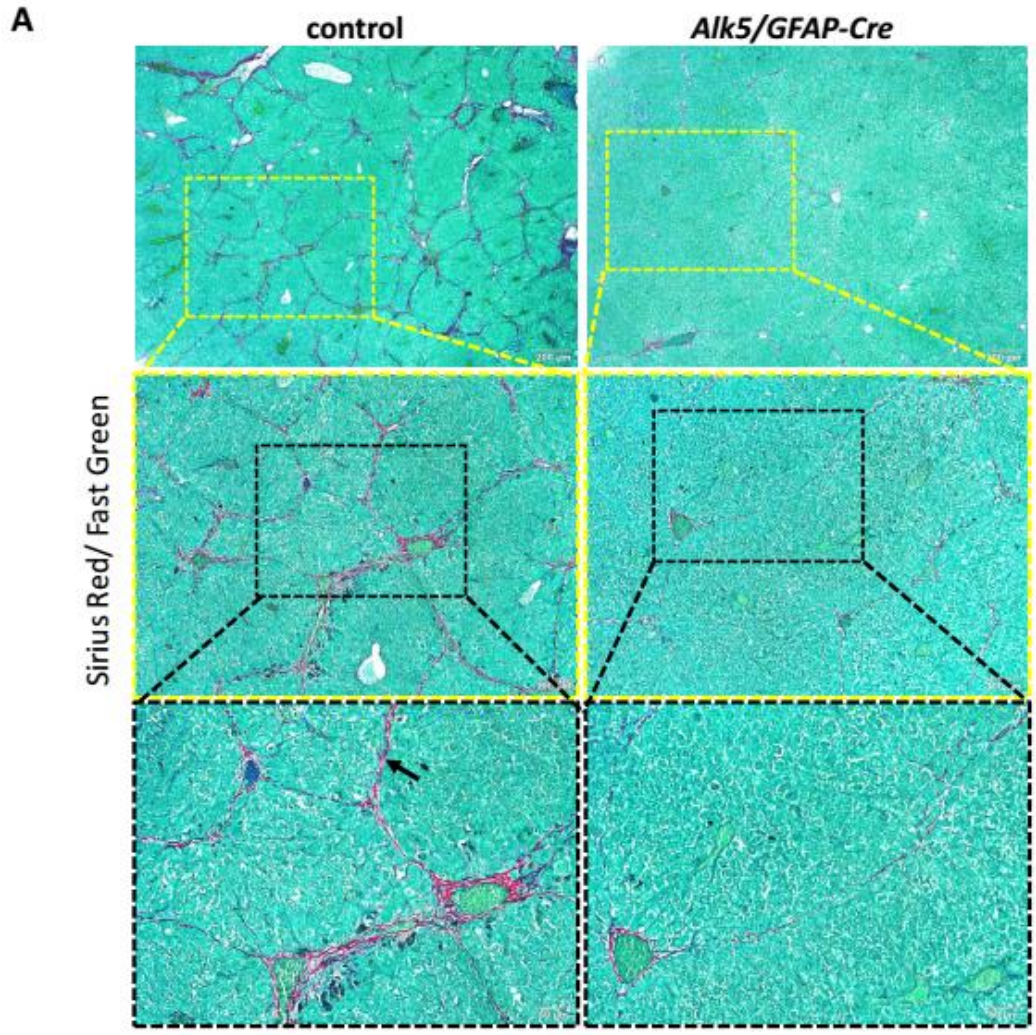


Figure 3. Reduced collagen accumulation in TAA-treated Alk5/GFAP-Cre mutant liver

A . Sirius Red staining was used to analyze an amount of collagen deposit in mutant liver compared to those of control. Control livers display a number of fibrous septa (black arrow) connecting periportal areas whereas mutant livers have fewer number of fibrotic bridging that is stained positive for Sirius Red.

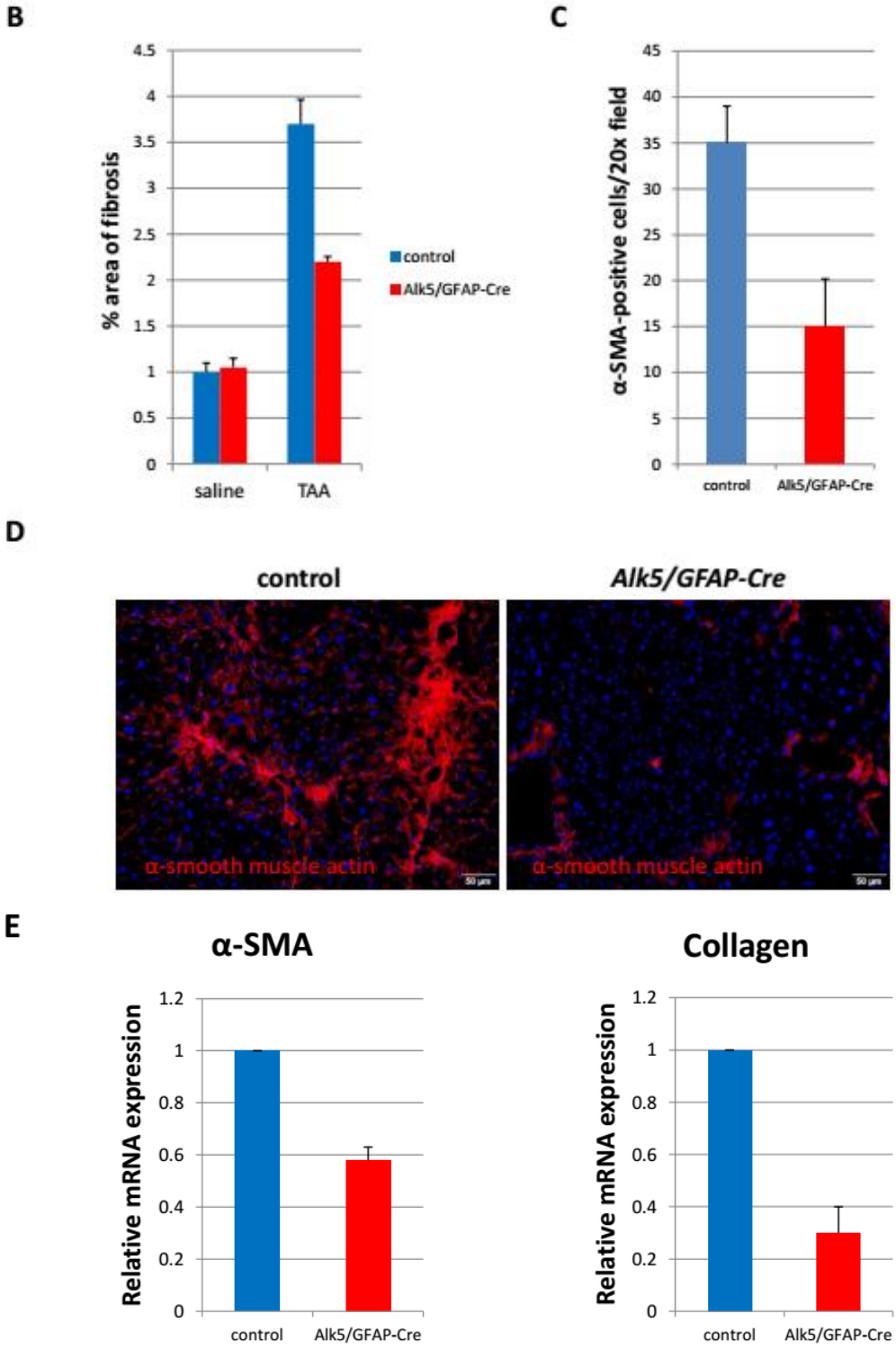


Figure 3 (continued). **B**. Fibrosis content was quantified by Sirius Red/ Fast green staining and computerized morphometry TAA-treated controls and mutants. Less fibrosis was observed in TAA-treated mutant than in control mice. **C-D**. α-SMA-immunostaining in liver showing less intense signals from periportal regions of mutant mice, compared to control (D).Quantification of α-SMA-positive cells shows a significant decrease in mutant, compared with control livers (C). **E**. Relative mRNA expression of α-SMA and collagen 1a1 were reduced in TAA-treated mutants

4.3) Expression of pro-fibrotic factors was decreased in liver of *Alk5/GFAP-Cre* mice.

Because (1) activated HSCs/myofibroblasts secrete various mediators of hepatic healing response and (2) Tgf-β signaling has shown to play a regulatory role in the production of many factors that are important for organ damage and regeneration, we next analyze gene expression of several pro-fibrotic factors as well as inflammatory cytokines in mutant livers. Connective tissue growth factor (CTGF), Platelet-derived growth factor-A and –B (PDGF-A and -B) is recognized as key mediator of liver fibrogenesis. qRT-PCR analysis shows that expression of these several pro-fibrotic factors are reduced significantly in mutant livers (Fig.3a) . We also find that expression of Tgf-β in mutant livers is lower than that of control livers (Fig.3a). In addition, hepatic expression of pro-inflammatory cytokines, TNF- , IL-6, but not MIP and MCP, is decreased in mutant livers, compared to control livers (Fig.3b).

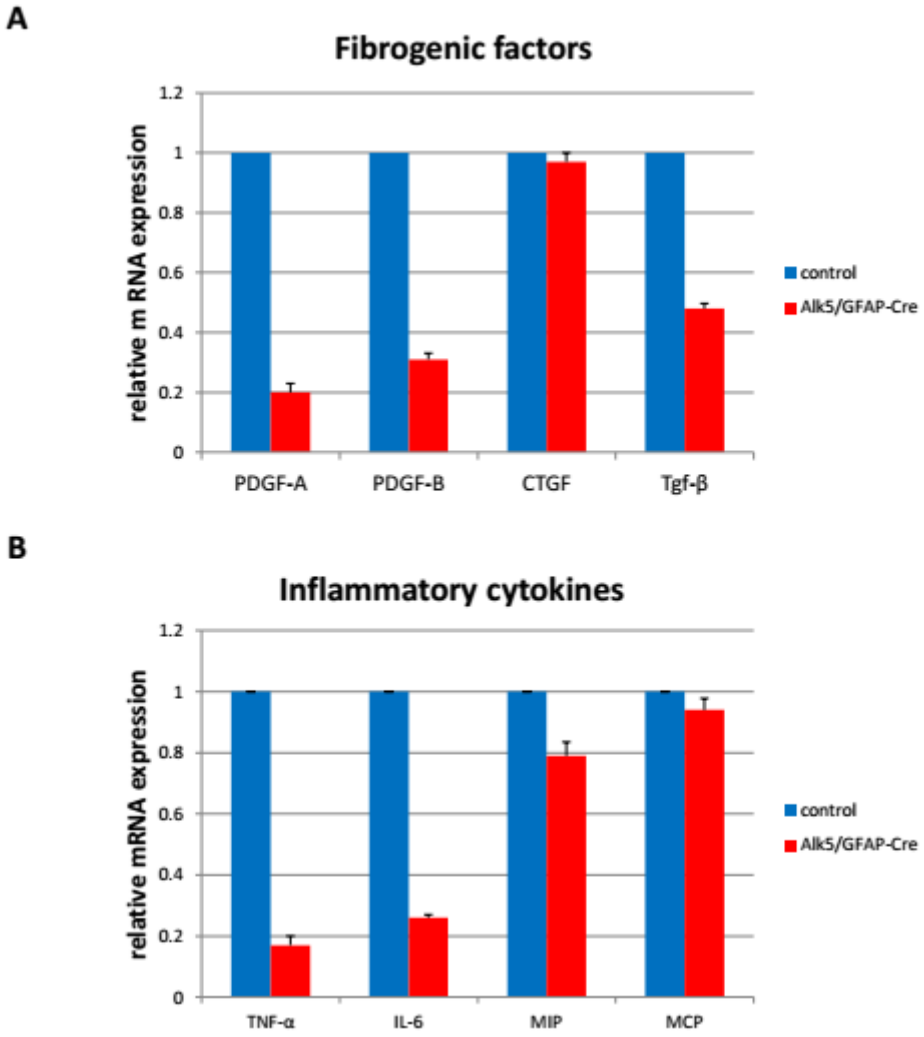


Figure 4. Decreased expression of pro-fibrogenic factors and inflammatory cytokines in livers of TAA-treated mutant mice. **A**. Hepatic mRNA expression of pro-fibrogenic factors (PDGF-A, PDGF-B, CTGF and Tgf-β) were quantified in the TAA-treated control and mutant mice. Reduced PDGF-A, PDGF-B and Tgf-β were observed in mutant livers. **B**. qRT-PCR analysis of pro-inflammatory cytokines (Tnf-α, IL-6, MIP and MCP) in livers of TAA-treated controls and mutants. Strong reduction of Tnf-α and IL-6 mRNA expression in mutant livers. qRT-PCR data are mean valves from three independent experiments, normalized to TAA-treated control mice.

4.4) *Alk5/GFAP-Cre* mice have reduced immune infiltration in their livers.

The reduced expression of pro-inflammatory cytokines and chemokines in mutant liver (Fig.3c) suggested that Tgf-β signaling in HSCs might be required for liver inflammation. To test whether HSC-specific abrogation of Tgf-β signaling could attenuate hepatic immune response triggered by TAA-induced damage, we perform CD3 and myeloperoxidase immunostaining to evaluate infiltration of T-lymphocytes and neutrophils, respectively. CD3 immunostaining demonstrates a large amount of T-cells infiltrating into periportal area of livers in control mice (Fig.5a). We found that mutant livers have fewer T-cells, compared to control liver (Fig.5b). Similarly, neutrophil accumulation is significantly decreased in mutant livers, as indicated by myeloperoxidass immunostaining (Fig.5c-d).

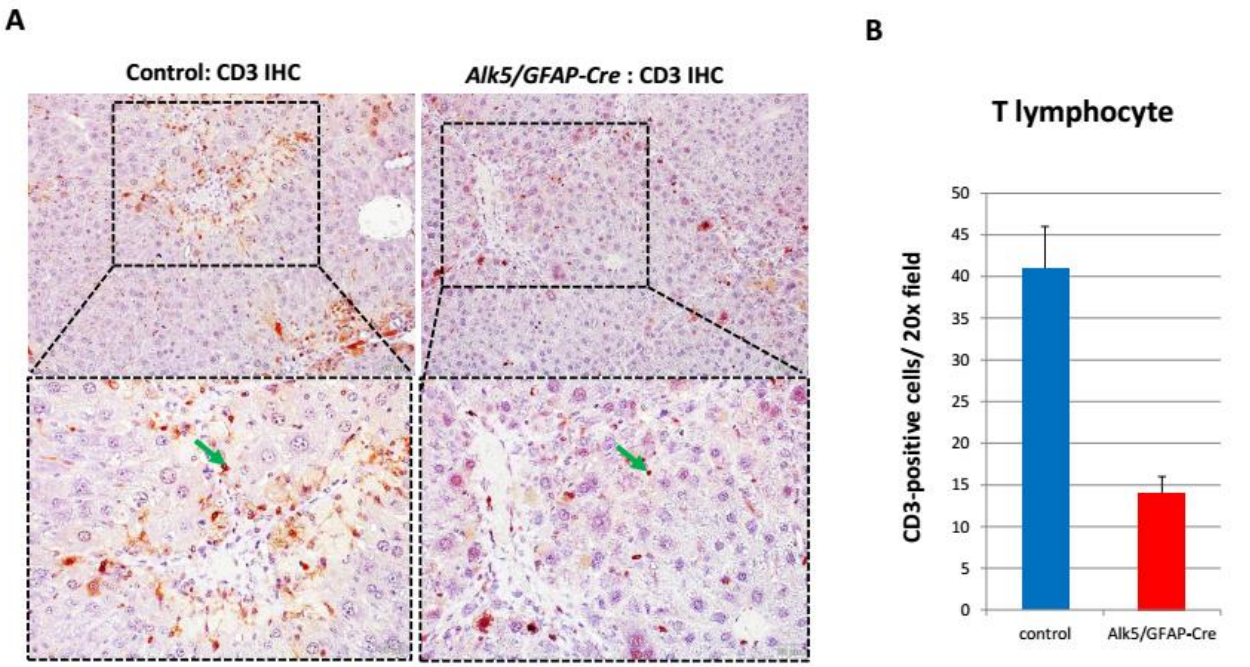


Figure 5. A reduced immune cell infiltration in liver of TAA-treated Alk5/GFAP-Cre mice.

A.CD3 antibody staining of control livers shows a number of T lymphocytes (arrows) surrounding interlobular blood vessel. Immune infiltration was greatly reduced in the periportal area of *Alk5/GFAP-Cre* mutant liver comparing to those of control. **B**. Quantification of CD3-positive cells after TAA treatment shows a decrease in mutant livers compared with those of control livers.

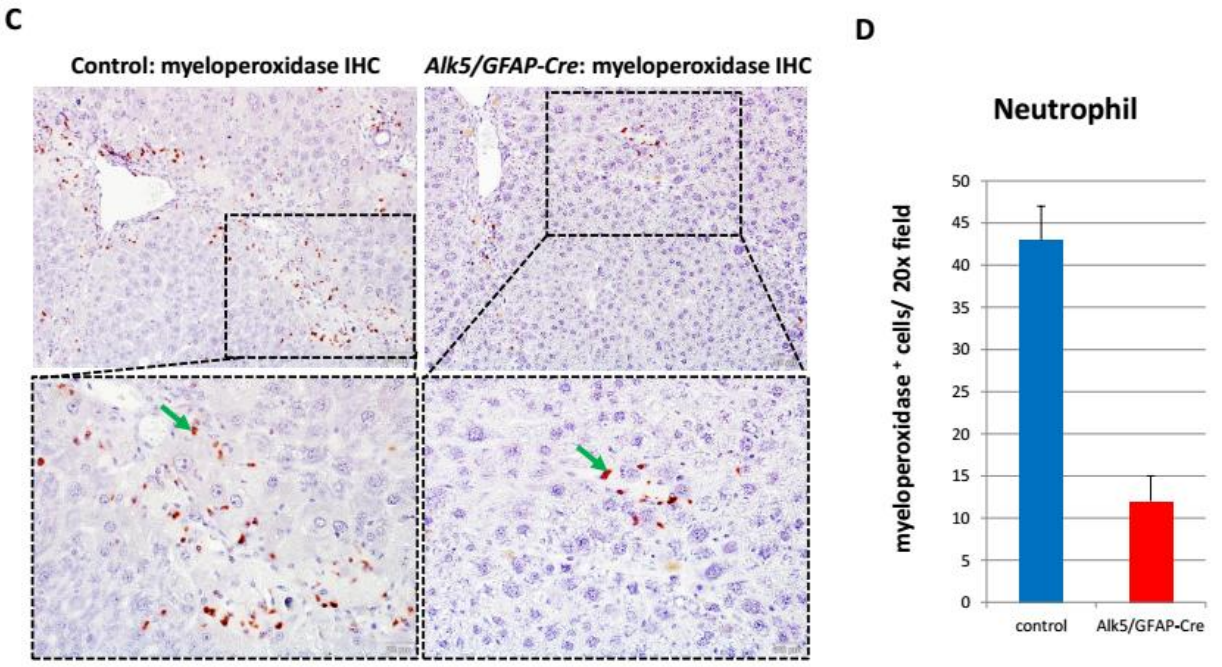


Figure 5 (continued).

C.Myeloperoxidase antibody staining of control livers shows a number of neutrophils (arrows) surrounding interlobular blood vessel. Immune infiltration was greatly reduced in the periportal area of *Alk5/GFAP-Cre* mutant liver comparing to those of control. **D**. Quantification of myeloperoxidase-positive cells after TAA treatment shows a decrease in mutant livers compared with those of control livers.

5) Amelioration of liver damage in *Alk5/GFAP-Cre* mice.

Having found a reduced liver fibrogenesis and inflammation in mutant mice, we next investigate Tgf-β–dependent function of HSCs on liver injury. The extent of hepatic injury is assessed by histology scores for apoptotic bodies in periportal and centrilobular areas. While there are abundant apoptotic bodies in control liver, the number of these degenerative cells is significantly reduced in mutants (Fig.6a-b). We also analyze the serum level of ALT/AST which is a common indicator of liver injury. The results show the amount of serum ALT/AST in mutant is less than that of controls (Fig.6c-d). In addition, we also measure expression of apoptotic factor in the liver to further demonstrate the reduced level of injury in mutants. qRT-PCR analysis of Bax and Bcl shows that mRNA expression of these apoptotic marker is decreased in liver of mutant mice (Fig.6e-f).

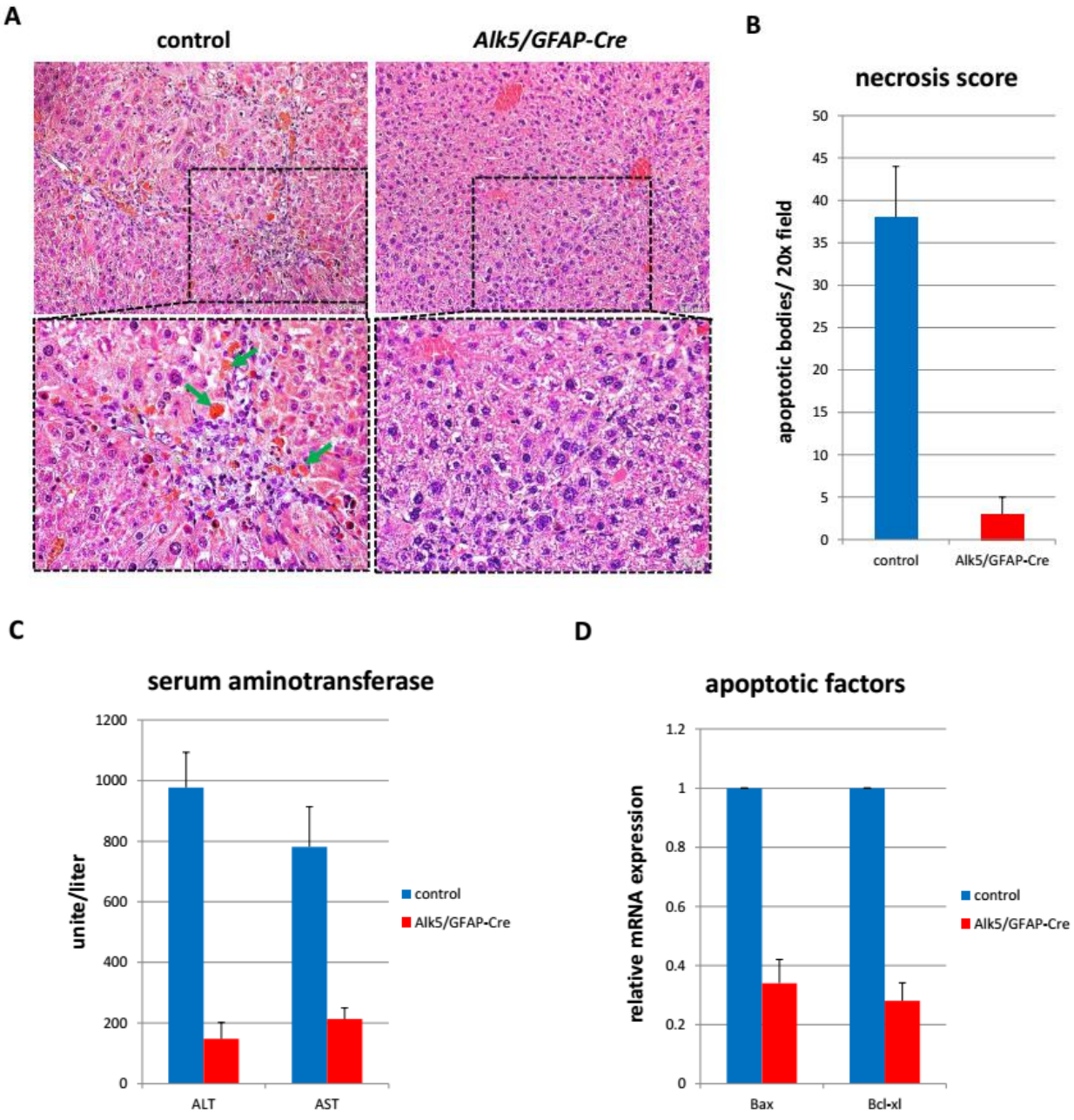


Figure 6. Reduced hepatic cell degeneration in *Alk5/GFAP-Cre* livers.

A. Representative photomicrographs for H&E staining of livers from control and mutant after TAA treatment. There was significant hepatic injury following TAA treatment in control liver as indicated by the amount of hypereosinophilic, apoptotic bodies (green arrows in A) in periportal areas. This indication of cell degeneration was rarely detected in mutant livers. **B**. Quantification of apoptotic bodies shows a decrease in mutant livers, compared to those of control. **C**. Quantification of serum ALT and AST in control and mutant after TAA treatment. A significant reduction of liver injury was observed in serum from mutant. **D**. Hepatic mRNA expression of apoptotic factor from whole livers of control and mutants.

Conclusion and Discussion

The present study examined the response to long-term TAA administration in a novel mouse model lacking Tgf-β signaling in HSCs. The TAA treatment has been used in mice to study various aspects of liver injury including fibrogenesis and inflammation. Following exposure to TAA, there is continued abnormal serum biochemistry indicating chronic hepatic insult. Hepatocyte injury is followed by periportal immune infiltration and fibrosis. Hepatic fibrosis is associated with myofibroblast proliferation and overexpression of proinflammatory cytokines and profibrotic factors that perpetuate liver inflammation and fibrosis. The current work sought to explore whether abrogation of Tgf-β signaling in HSCs might reduce myofibroblast production *in vivo* and how it might influence the inflammatory response after chronic TAA exposure through examination of histology, serum biochemistry and gene expression analysis. The premise behind the work was a role of Tgf-β signaling in HSCs suggested by previous studies using cell culture experiments and rodent models. In mice, absence of Tgf-β1 reduced collagen synthesis and α-SMA protein expression following exposure to carbon tetrachloride (CCL_4)[3]. It has also been shown that hepatic overexpression of Tgf-β1 increased collagen and α-SMA protein level leading to liver fibrosis[3-7]. These studies implicated that the change in collagen synthesis and mRNA expression in their models was mostly attributed to dysregulation of HSCs, since Tgf-β1 was previously known to be a potent inducer of collagen expression and HSC differentiation into myofibroblast i*n vitro*[3]. However, it is still debatable that other cell lineages could also contribute to Tgf-β–mediated liver fibrosis in those mutant mouse models. For instance, reduced collagen and α-SMA expression in Tgf-β1 knockout mice exposed to CCL_4 may be a result of deficient hepatocyte-dependent production of profibrotic factors in mutant livers. This idea is supported by a study showing that Tgf-β signaling in hepatocytes promoted production of Connective tissue growth factor (Ctgf) which is essential mediator of liver fibrosis[8-10]. It is possible that spontaneous liver fibrosis developed in mutant mice overexpressing Tgf-β1 was derived from increased immune cell infiltration since inflammatory cells have been known to be one of major source of profibrotic factors. An essential role of Tgf-β signaling for HSC-dependent liver fibrosis has not been unambiguously demonstrated *in vivo* so far.

Following liver injury of any etiology, Tgf-β ligands are secreted from various types of cell to orchestrate hepatic regenerative processes. Tgf-β elicits their effects through interaction with Tgf-β receptor type II (TβR-II) that recruits and activates Tgf-β receptor type I (TβR-I). The ligand-reptors complex then propagates downstream signaling transduction via phosphorylation of receptor-Smad (R-Smad) proteins. These is one subclass of TβR-II and two subclass of TβR-I, i.e. Alk1 and Alk5. Binding of ligand to TβR-II mediates Alk1 activation leading to phosphorylation of R-Smad1/5/8. Tgf-β also binds to Alk5 and signals through activation of R-Smad2/3. In liver, Alk1 expression was found in sinusoidal endothelial cells, HSCs and myofibroblasts, while Alk5 was expressed in most cell types including hematopoietic cells. Although previous *in vitro* studies have shown that both type I receptors are used by HSCs[9, 11]. A requirement of these receptors in HSCs during Tgf-β–mediated liver fibrosis remains to be elucidated. Here, we report on a new HSC-specific, Alk5-deficient murine model that clarify a role of Tgf-β signaling in HSCs during liver fibrosis progression. The results from our work demonstrated that genetic ablation of Alk5 in HSCs leaded to a decrease in collagen gene expression in liver of mice exposed to TAA. This data suggested that Alk5-Smad2/3 signaling is necessary for HSC-dependent collagen synthesis during liver fibrogenesis. We also found that loss of Alk5 in HSCs inhibited hepatic α-SMA expression and TAA-induced myofibroblast production in livers. This finding delineated an essential role of Alk5 during the transformation of quiescent HSCs to activated HSCs *in vivo*.

Besides being responsible for ECM accumulation in damaged organ, activated HSCs/myofibroblasts has been recognized as an important regulator in inflammation-associated fibrosis. In response to liver damage, activated HSCs produce several profibrotic factors such as Tgf-β, interleukin (IL)-6, and Platelet-derived growth factor (Pdgf) to mediate healing process at the injured site[12]. Among these cytokines released by HSCs, Pdgf and Tgf-β are the most potent inducer of proliferation and fibrogenesis, respectively[13]. In addition, several studies have demonstrated that HSCs could modulate immunological response in livers by secreting chemokines such as monocyte chemoattractant protein (MCP)-1, regulated and normal T cell expressed and secreted (Rantes) and macrophage inflammatory proteins (MIPs)[13, 14]. These chemokines are powerful chemoattractant effectors which accelerate infiltration of pro-fibrotic immune cells including macrophage, neutrophils and T lymphocytes into the liver to participate in the progression of fibrosis[15-18]. Our results displayed that there was a dramatic decrease in CD3- and myeloperoxidase-expressing cells that coincided with reduced number of activated HSCs and collagen deposition, indicating that deficient Tgf-β signaling in HSCs affected the recruitment of immune cells most critical to fibrogenesis. Of interest, expression of Tnf-α, IL-6, Pdgf and MIP-1 was decreased in liver of mutant mice exposed to TAA. The reduced immune cell infiltration in mutant livers reflect decreased gene expression of these cytokines and chemokines, since Tgf-β signaling was known to be an upstream regulator of their expressions. Further studies is needed to clarify whether Tgf-β/Alk5 signaling could promote accelerate liver inflammation via the production of these cytokines and chemokines by HSCs.

It is becoming clearer that HSCs mediates amplification of fibrogenesis and inflammation –associated liver damage, and these HSC actions are enhanced by Tgf-β signaling. In the past years, liver fibrosis has been known as a wound-healing response to chronic liver injury. Previous studies using cell culture and mouse model have shown that ECM released by HSC during fibrosis protects liver against hepatocellular injury[19]. However, works by Puche et al.[20] and Stewart et al.[21] have demonstrated that HSCs contribute to hepatocyte death and exacerbate liver injure caused by a variety of toxic stimuli. These studies suggested that HSCs mediates liver damage via production of inflammatory cytokines including IL-10, IFN-Cs mediates liver damage via production of inflammatory cytokines including IL-10, IFN-γ and TNF-α[20, 21]. Recently, additional report from Fujita et al.[22] showed that HSCs integrate cytokine-mediated immune response in hepatic sinusoid to amplify liver injury. This gives more support to the prominent role of HSCs in inflammation-associated liver injury. Results from our studies further suggested that the pivotal functions of HSCs are mediated by Tgf-β/Alk5 signaling. We have shown that inflammation-associated liver damage was reduced because Tgf-β signaling in HSCs was blocked by genetic ablation of Alk5. Although the precise mechanism of how Tgf-β mediates HSC-dependent liver damage remain to be elucidated, our findings point to Tgf-β/Alk5 signaling as a fundamental pathway directing liver damage via inflammatory cytokines, chemokines and fibrogenic mediators. Therefore, an abrogation of Alk5 signaling in HSCs by which amplify chronic liver injury has potential therapeutic benefit for inflammation-associated liver diseases.

7. Appendix

- The poster presented that TRF Meeting on 6-8 January 2016

The Requirement of Stromal Tgf-β signaling in the Pathogenesis of Chronic Liver Disease

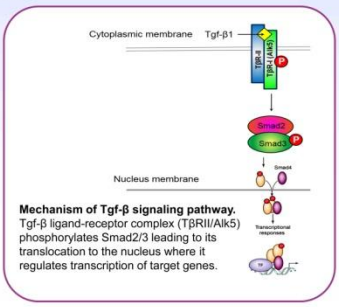
Chen Ke, Wanthita Kongphat, Somyoth Sridurongrit*

Department of Anatomy, Faculty of Science, Mahidol University, Rama VI Road, Bangkok, Thailand

***Corresponding author, e-mail: somyoth.sri@mahidol.ac.th**

Introduction

The chronic liver disease often arises from an aberrant healing response to repetitive organ damage. Following hepatic cell injury, Hepatic Stellate Cells (HSCs) are activated to initiate a series of events that are critical for liver regeneration. HSCs are being considered as key regulators of organ damage due to their diverse roles in immunomodulation and fibrogenesis. Several *in vitro* studies have shown that Transforming growth factor (Tgf)-β controls various HSC behaviors including extracellular matrix (ECM) production, cell migration and differentiation. However, it is unclear whether Tgf-β can exert these effects on HSCs during the progression of liver injury.



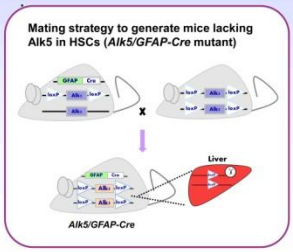
Mechanism of Tgf-β signaling pathway. Tgf-β ligand-receptor complex (TβRII/Alk5) phosphorylates Smad2/3 leading to its translocation to the nucleus where it regulates transcription of target genes.

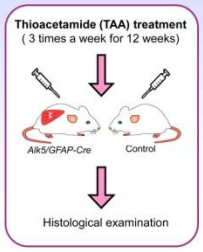
Objective

- To determine whether deletion of Alk5 in HSC reduces fibrosis and protects mice against liver damage

Methods

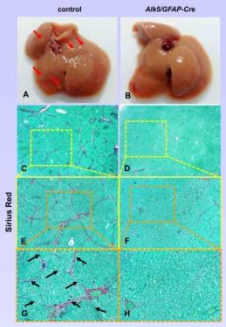
We utilize Cre/loxP technology to generate mutant mice lacking Tgf-β type I receptor, Alk5 in HSCs (*Alk5/GFAP-Cre* mutants). Liver damage is induced by intraperitoneal (IP) injection of thioacetamide (250 mg/kg weight of animal).





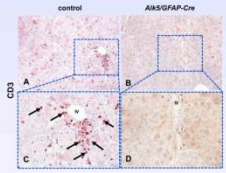
Results

1. HSC-specific deletion of Alk5 attenuates fibrogenesis.



After TAA treatment, mutant livers appear to have normal, smooth outer surface while several lobes of control livers show rough surface with multiple nodules (red arrows in A), which are common characteristics of fibrotic liver. Sirius Red staining is used to analyze an amount of collagen deposit in mutant liver (D,F,H) compared to those of control (C,E,G). Control livers display a number of fibrous septa (black arrow in G) connecting periportal areas whereas mutant livers have fewer number of fibrotic bridging that is stained positive for Sirius Red.

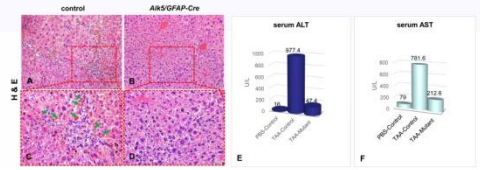
2. Liver inflammation is reduced in *Alk5/GFAP-Cre* mutants.



CD3 antibody staining of control liver (A and C) show a number of immune cells (black arrows) surrounding interlobular blood vessel (IV). Immune infiltration is greatly reduced in the periportal area of *Alk5/GFAP-Cre* mutant liver (C and D) comparing to those of control.

3. *Alk5/GFAP-Cre* mutants are protected against liver injury.

There is significant hepatic injury following TAA treatment in control liver as indicated by the amount of hypereosinophilic, apoptotic bodies (green arrows in A and C) in periportal areas. This indicator of cell degeneration is not detected in mutant liver (B and D). Reduction of mutant liver damage is confirmed by serum ALT and AST (E and F).



Conclusions

Our data indicates that Alk5 mediates HSC-dependent amplification of chemically induced liver damage. Alk5 may exert this effect in HSC by promoting of ECM production as well as enhancing synthesis of pro-inflammatory cytokines.

Bibliography

- Stewart R, Dangi A, Gandhi C, et al. A novel mouse model of depletion of stellate cells clarifies their role in ischemia/reperfusion- and endotoxin-induced acute liver injury. Journal of Hepatology 2014;Vol.60:298-305

8. Output (Acknowledge the Thailand Research Fund)

We are preparing a final draft of manuscript to be submitted to The American Journal of Pathology in July.

References

1. Postic, C*., et al.* Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. *J Biol Chem* **274**, 305-315 (1999).
2. Larsson, J*., et al.* Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. *EMBO J* **20**, 1663-1673 (2001).
3. Hellerbrand, C., Stefanovic, B., Giordano, F., Burchardt, E.R. & Brenner, D.A. The role of TGFbeta1 in initiating hepatic stellate cell activation in vivo. *J Hepatol* **30**, 77-87 (1999).
4. Sanderson, N*., et al.* Hepatic expression of mature transforming growth factor beta 1 in transgenic mice results in multiple tissue lesions. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 2572-2576 (1995).
5. Clouthier, D.E., Comerford, S.A. & Hammer, R.E. Hepatic fibrosis, glomerulosclerosis, and a lipodystrophy-like syndrome in PEPCK-TGF-beta1 transgenic mice. *The Journal of clinical investigation* **100**, 2697-2713 (1997).
6. Kanzler, S*., et al.* TGF-beta1 in liver fibrosis: an inducible transgenic mouse model to study liver fibrogenesis. *The American journal of physiology* **276**, G1059-1068 (1999).
7. Ueberham, E*., et al.* Conditional tetracycline-regulated expression of TGF-beta1 in liver of transgenic mice leads to reversible intermediary fibrosis. *Hepatology* **37**, 1067-1078 (2003).
8. Paradis, V*., et al.* Effects and regulation of connective tissue growth factor on hepatic stellate cells. *Laboratory investigation; a journal of technical methods and pathology* **82**, 767-774 (2002).
9. Weng, H.L*., et al.* Profibrogenic transforming growth factor-beta/activin receptor-like kinase 5 signaling via connective tissue growth factor expression in hepatocytes. *Hepatology* **46**, 1257-1270 (2007).
10. Li, G*., et al.* Inhibition of connective tissue growth factor by siRNA prevents liver fibrosis in rats. *The journal of gene medicine* **8**, 889-900 (2006).
11. Wiercinska, E*., et al.* Id1 is a critical mediator in TGF-beta-induced transdifferentiation of rat hepatic stellate cells. *Hepatology* **43**, 1032-1041 (2006).
12. Yi, H.S. & Jeong, W.I. Interaction of hepatic stellate cells with diverse types of immune cells: foe or friend? *Journal of gastroenterology and hepatology* **28 Suppl 1**, 99-104 (2013).
13. Friedman, S.L. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev* **88**, 125-172 (2008).
14. Hernandez-Gea, V. & Friedman, S.L. Pathogenesis of liver fibrosis. *Annual review of pathology* **6**, 425-456 (2011).
15. Bataller, R. & Brenner, D.A. Liver fibrosis. *The Journal of clinical investigation* **115**, 209-218 (2005).
16. Maher, J.J., Lozier, J.S. & Scott, M.K. Rat hepatic stellate cells produce cytokine-induced neutrophil chemoattractant in culture and in vivo. *The American journal of physiology* **275**, G847-853 (1998).

17. Sprenger, H., *et al.* Induction of neutrophil-attracting chemokines in transforming rat hepatic stellate cells. *Gastroenterology* **113**, 277-285 (1997).

18. Casini, A., *et al.* Neutrophil-derived superoxide anion induces lipid peroxidation and stimulates collagen synthesis in human hepatic stellate cells: role of nitric oxide. *Hepatology* **25**, 361-367 (1997).

19. Bourbonnais, E., *et al.* Liver fibrosis protects mice from acute hepatocellular injury. *Gastroenterology* **142**, 130-139 e134 (2012).

20. Puche, J.E., *et al.* A novel murine model to deplete hepatic stellate cells uncovers their role in amplifying liver damage in mice. *Hepatology* **57**, 339-350 (2013).

21. Stewart, R.K., *et al.* A novel mouse model of depletion of stellate cells clarifies their role in ischemia/reperfusion- and endotoxin-induced acute liver injury. *Journal of hepatology* **60**, 298-305 (2014).

22. Fujita, T., *et al.* Hepatic stellate cells relay inflammation signaling from sinusoids to parenchyma in mouse models of immune-mediated hepatitis. *Hepatology* **63**, 1325-1339 (2016).