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The contribution of toll-like receptor signaling to the development of liver fibrosis and cancer in hepatocyte-specific TAK1-deleted mice

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Abstract

Hepatocyte death is associated with liver inflammation, fibrosis and hepatocellular carcinoma (HCC). Damaged cells trigger inflammation through activation of Toll-like receptors (TLRs). Although the role of TLR4 in HCC development has been reported, the role of TLR9 in the development of HCC remains elusive. To investigate the role of TLR4 and TLR9 signaling in liver inflammation-fibrosis-cancer axis, we took advantage of mice with hepatic deletion of transforming growth factor-β- activated kinase 1 (*Tak1*Δ*Hep*) that develop spontaneous liver injury, inflammation, fibrosis, and HCC, recapitulating the pathology of human HCC. We generated double knockout mice lacking genes of our interest with hepatic *Tak1*. *Tak1*Δ*Hep* mice and *Tlr4*-deficient *Tak1*Δ*Hep* mice had similar serum ALT levels, but *Tlr4*-deficient *Tak1*Δ*Hep* mice exhibited significantly reduced macrophage infiltration, myofibroblast activation and tumor formation. Ablation of TLR9 reduced spontaneous liver injury, inflammation, fibrosis, and cancer

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development in *Tak1*Δ*Hep* mice. In addition, the common adaptor, myeloid differentiation factor 88 (MyD88)-deficient *Tak1*Δ*Hep* mice also attenuated liver injury, macrophage recruitment, collagen deposition, and tumor growth compared with control *Tak1*Δ*Hep* mice. Genetic ablation of TNF receptor type I (TNFR) in *Tak1*Δ*Hep* mice remarkably reduced liver inflammation-fibrosis-cancer axis. Surprisingly, disruption of interleukin-1 receptor (IL-1R) had no effect on liver injury and tumor formation, although *Il1r*-deficient *Tak1*Δ*Hep* showed attenuated macrophage infiltration and collagen deposition. In conclusion, TLR4- and TLR9-MyD88 are driving forces of progression to HCC accompanied by liver inflammation and fibrosis in *Tak1*Δ*Hep* mice. Importantly, TLR4 and TLR9 downstream TNFR, but not IL-1R signaling is crucial for the development of HCC in *Tak1*Δ*Hep* mice.

Keywords

TAK1; toll-like receptors; TNF receptor type I; liver fibrosis; HCC

INTRODUCTION

Transforming growth factor-β activated kinase 1 (TAK1) is a member of mitogen-activated protein kinase kinase kinases (MAP3Ks), which orchestrates various downstream effectors involved in immune system, cell death, cell proliferation, and carcinogenesis.[1] TAK1 is activated by numerous receptors that mediate inflammatory signal transduction pathways in response to ligands, such as transforming growth factor-β (TGF-β), Toll-like receptor (TLR) ligands, tumor necrosis factor α (TNFα), and interleukin-1β (IL-1β).[2] TAK1 in turn activates its downstream signaling pathways such as nuclear factor-κB (NF-κB) and MAPKs that control cell survival, proliferation, death, and carcinogenesis.[1] Therefore, TAK1 is indispensable for cell viability and tissue homeostasis. *Tak1*-deficient mice showed an embryonic lethality at E9.5–10.5 before the initiation of fetal liver development.[2,3] Inducible *Tak1* knockout mice showed massive apoptotic death of hematopoietic cells and hepatocytes, which leads to bone marrow and liver failure.[4] Studies of tissue- specific *Tak1* gene deletion suggest that loss of TAK1 activity results in apoptosis in a variety of tissues including liver, intestine and epidermis.[5–8]

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death worldwide, which accounts for 600,000 deaths each year.[9] HCC development is closely linked to chronic liver inflammation and fibrosis/cirrhosis.[10] Since TAK1 is considered as a gatekeeper for tumorigenesis in hepatocytes, we generated hepatocyte-specific *Tak1*-deleted (*Tak1*Δ*Hep*) mice that displays hepatic injury, inflammation, fibrosis, and carcinogenesis.[5] Loss of TAK1 in hepatocytes causes spontaneous liver cell death and subsequent inflammation may promote liver fibrosis. Furthermore, the compensatory liver cell proliferation drives hepatocarcinogenesis.[5] Therefore, *Tak1*Δ*Hep* mouse line that we and others have established is a good animal model for studying liver inflammation-fibrosis-cancer axis that mimics the pathology of human HCC.[5,6] Using this *in vivo* system, we previously have found that TGF-β signaling in hepatocytes promotes hepatic fibrosis and carcinogenesis.[11]

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TLRs are pattern recognition receptors, which may bridge the gap between inflammation and HCC. Due to the anatomical and functional relationship between gut and liver, liver is continuously exposed to gut-derived bacterial products, such as lipopolysaccharide (LPS) and bacterial DNA, which stimulate TLRs. Activation of TLRs have shown to be involved in the progression of the inflammation-fibrosis-HCC axis.[12–14] TLR4 promotes TGF-β signaling and hepatic fibrosis, which are associated with HCC development.[15,16] TLR9 signaling promotes steatohepatitis and fibrosis through induction of IL- 1β in mice.[17] In addition, TLR9 is also involved in cell survival and proliferation in HCC.[18] In contrast, anti-tumorigenic effects of TLR4 and TLR9 have also been reported.[19,20] It is unclear whether TLR4 and TLR9 signaling promotes or suppresses inflammation-fibrosis-HCC axis in *Tak1ΔHep* mice. In the present study, we investigated the role of TLR4 and TLR9 and its related signal molecules (e.g., myeloid differentiation factor 88 [MyD88], TNF receptor type I [TNFRI], and interleukin-1 receptor [IL-1R]) in liver injury, inflammation, fibrosis, and HCC spontaneously developed in *Tak1ΔHep* mice. We show that both TLR4 and TLR9, and their downstream adaptor molecule, MyD88, played important roles in the development of spontaneous liver inflammation, fibrosis, and HCC in mice with hepatic deletion of TAK1. In addition, we found that TNFR signaling, but not IL-1R signaling, promoted HCC development in *Tak1ΔHep* mice.

MATERIALS AND METHODS

Mouse colonies

Tlr4$^{-/-}$, *Tlr9*$^{-/-}$ and *Myd88*$^{-/-}$ mice backcrossed to C57BL/6 mice for at least ten generations were kindly provided by Dr. Akira (Osaka University, Japan).[21–23] *Albumin-Cre* (*Alb-Cre*) recombinase transgenic mice, TNFRI-deficient mice (*Tnfr*$^{-/-}$), and IL-1 R-deficient mice (*Il1r*$^{-/-}$) were purchased from the Jackson Laboratory (Bar Harbor, MA). Mice carrying the floxed allele of *Tak1* (*Tak1*$^{f/f}$)[5] were crossed with *Alb-Cre* transgenic mice to generate *Tak1*$^{f/f}$; *Alb-Cre* (*Tak1*$^{\Delta Hep}$). *Tak1*$^{\Delta Hep}$ mice were crossed with *Tlr4*$^{-/-}$, *Tlr9*$^{-/-}$, *Myd88*$^{-/-}$, *Tnfr*$^{-/-}$, and *Il1r*$^{-/-}$ mice to generate *Tak1*$^{\Delta Hep}$/*Tlr4*$^{-/-}$, *Tak1*$^{\Delta Hep}$/*Tlr9*$^{-/-}$, *Tak1*$^{\Delta Hep}$/*Myd88*$^{-/-}$, *Tak1*$^{\Delta Hep}$/*Tnfr*$^{-/-}$, and *Tak1*$^{\Delta Hep}$/*Il1r*$^{-/-}$ mice, respectively. All studies were performed in accordance with National Institutes of Health recommendations outlined in the Guide for the Care and Use of Laboratory Animals. All animal experiment protocols were approved by the University of California San Diego and Cedars-Sinai Medical Center Institutional Animal Care and Use Committee.

Alanine aminotransferase (ALT) measurement

Blood samples were collected via cardiac puncture and centrifuged at 5,000 rpm for 15 min to obtain serum. Serum ALT levels were determined by Infinity ALT (GPT) liquid stable reagent (Thermo Scientific, Middletown, VA).

Quantitative real-time polymerase chain reaction (qPCR)

The total RNAs from snap-frozen mouse liver tissues were extracted using NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany), and reverse-transcribed with High- Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed with the use of CFX96 real-time PCR system (Biorad, Hercules,

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CA). 18S rRNA, a housekeeping gene, was used as an internal control for the normalization. The following mouse PCR primer sequences were used: 18S, 5′-AGTCCCTGCCCTTTGTACACA-3′ and 5′-CGATCCGAGGGCCTCACTA-3′; C-C motif chemokine ligand 2 (Ccl2), 5′-ATTGGGATCATCTTGCTGGT-3′ and 5′-CCTGCTGTTCACAGTTGCC-3′; Tnf, 5′-AGGGTCTGGGCCATAGAACT-3′ and 5′-CCACCACGCTCTTCTGTCTAC-3′; tissue inhibitor of metalloproteinase-1 (Timp1), 5′-AGGTGGTCTCGTTGATTTCT-3′ and 5′-GTAAGGCCTGTAGCTGTGCC-3′; Tgfb1, 5′-GTGGAAATCAACGGGATCAG-3′ and 5′-ACTTCCAACCCAGGTCCTTC-3′.

Histologic examination

Formalin-fixed, paraffin-embedded mouse liver tissues were cut into 5 lm thickness using a microtome (Thermo Scientific, Waltham, MA). For immunostaining, liver sections were incubated with monoclonal antibody to F4/80 (clone BM8; eBioscience, San Diego, CA) or mouse anti-α-smooth muscle actin (α-SMA) monoclonal antibody (DakoCytomation, Glostrup, Denmark), as previously reported.[15] For α-SMA staining, the MOM kit (Vector Laboratories, Burlingame, CA) was applied to reduce endogenous mouse Ig staining. For Sirius Red staining, mouse liver sections were incubated with a solution of saturated picric acid containing 0.1% Fast Green FCF (Sigma- Aldrich, St Louis, MO) and 0.1% Direct Red 80 (Sirius Red R3B; Sigma-Aldrich, St Louis, MO) for 1 hrs. F4/80- or α- SMA-positive area was evaluated from randomly selected 5 fields of ×200 magnification per slide. Sirius red-positive area was obtained in a similar manner at ×100 magnification. Each result was quantified with NIH ImageJ software.

Tumor

All male *Tak1*$^{\Delta Hep}$/*Tlr4*$^{-/-}$, *Tak1*$^{\Delta Hep}$/*Tlr9*$^{-/-}$, *Tak1*$^{\Delta Hep}$/*Myd88*$^{-/-}$, *Tak1*$^{\Delta Hep}$/*Tnfr*$^{-/-}$, and *Tak1*$^{\Delta Hep}$/*Il1r*$^{-/-}$ mice at the age of 9 months were sacrificed and the number of externally visible tumors (>1 mm) were counted and maximal tumor size was measured.

Statistical analysis

Differences between the two groups were compared using the Mann Whitney *U* test or two-tailed unpaired Student's *t* test. Differences between multiple groups were compared using one-way ANOVA. Statistical significance was assessed by using SPSS software (SPSS Inc, Chicago, IL). *p*-values <0.05 were considered significant.

RESULTS

Genetic ablation of TLR4 or TLR9 attenuates hepatic inflammation in Tak1Δhep mice

We have previously reported that the *Tak1*Δ*Hep* mice developed spontaneous hepatocyte death, hepatic inflammation, and fibrosis.[5] TLR4 is known to promote the development of chronic liver diseases, such as hepatic fibrosis and HCC, through activation of immune cells and hepatic stellate cells (HSCs).[15,16] Apoptosis of hepatocytes also promotes liver fibrosis through activation of immune cells and HSCs. Host denatured DNA or mitochondrial DNA released from apoptotic cells are reported to activate immune cells and HSCs through TLR9.[24] To address the role of TLR4 and TLR9 in hepatocyte injury and hepatic inflammation by *Tak1* deficiency, we generated *Tlr4*-deficient *Tak1*Δ*Hep* mice and *Tlr9*-

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deficient *Tak1*ΔHep mice. The presence of hepatocyte death and injury is marked by increased levels of serum ALT. Serum ALT levels were elevated in 1-month- old *Tak1*ΔHep mice. *Tlr4*-deficient *Tak1*ΔHep mice had the similar ALT levels to that of *Tak1*ΔHep mice, while *Tlr9*-deficient *Tak1*ΔHep mice showed lower ALT levels than *Tak1*ΔHep mice (Fig. 1*a*). Subsequently, we investigated hepatic inflammation. Both *Tlr4*-deficient *Tak1*ΔHep and *Tlr9*-deficient *Tak1*ΔHep mice showed significantly lower *Ccl2* and *Tnf* mRNA levels than *Tak1*ΔHep mice (Fig. 1*b* and 1*c*). Hepatic macrophage infiltration was also decreased in *Tlr4*-deficient *Tak1*ΔHep and *Tlr9*- deficient *Tak1*ΔHep mice compared with control *Tak1*ΔHep mice (Fig. 1*d* and 1*e*). These results suggest that genetic ablation of hepatic *Tak1* causes liver injury in a TLR9-dependent but TLR4-independent mechanism. However, both TLR4 and TLR9 signaling is associated with the spontaneous liver inflammation in *Tak1*ΔHep mice.

TLR4 or TLR9 deficiency ameliorates liver fibrosis in Tak1Δhep mice

Loss of TAK1 in hepatocytes resulted in severe hepatocyte death, inflammation, and HSC activation. *Tak1*ΔHep mice developed liver fibrosis by 1 month of age.[5] We have previously shown that TLR4 signaling promotes liver fibrosis by sensitizing HSCs to TGF-β, and that TLR9 signaling enhances steatohepatitis and fibrosis mediated by IL-1β production.[15,17] Here, we investigated the effect of TLR4 or TLR9 deficiency on liver fibrosis in *Tak1*ΔHep mice. The number of activated HSCs was significantly reduced in *Tlr4*-deficient *Tak1*ΔHep and *Tlr9*-deficient *Tak1*ΔHep mice at the age of 1 month, as assessed by immunohistochemistry for α-SMA, a marker of HSC activation (Fig. 2*a* and 2*b*). Sirius Red staining showed that fibrillar collagen deposition was decreased in *Tlr4*-deficient *Tak1*ΔHep and *Tlr9*-deficient *Tak1*ΔHep mice compared with *Tak1*ΔHep mice at the age of 9 months (Fig. 2*a* and 2*c*). *Tlr4*-deficient *Tak1*ΔHep and *Tlr9*-deficient *Tak1*ΔHep mice showed reduction in fibrogenic gene expression, including *Timp1* and *Tgfb1*, compared with *Tak1*ΔHep mice (Fig. 2*d* and 2*e*). These results demonstrate that TLR4 and TLR9 drive HSC activation and fibrosis in *Tak1*ΔHep mice.

Loss of MyD88 protects from liver injury and inflammation in Tak1Δhep mice

We have shown that TLR4 and TLR9 signaling is involved in liver phenotypes in *Tak1*ΔHep mice. Since both TLR4 and TLR9 share a common adaptor molecule MyD88 to activate downstream signaling,[25] we investigated the role of MyD88- dependent pathway in *Tak1*ΔHep mice. *Myd88*-deficient *Tak1*ΔHep mice had significantly less ALT levels than *Tak1*ΔHep mice (Fig. 3*a*). In addition, *Myd88*-deficient *Tak1*ΔHep mice had reduced mRNA levels of *Ccl2* and *Tnf* compared with *Tak1*ΔHep mice (Fig. 3*b* and 3*c*). Moreover, *Myd88*-deficient *Tak1*ΔHep mice showed less hepatic macrophage infiltration than *Tak1*ΔHep mice, as assessed by immunohistochemistry for F4/80 (Fig. 3*d* and 3*e*). These results suggest that MyD88 promotes liver injury and inflammation in *Tak1*ΔHep mice.

MyD88 signaling is required for spontaneous liver fibrosis in Tak1Δhep mice

Next, we investigated whether MyD88 signaling contributes to spontaneous liver fibrosis in *Tak1*ΔHep mice. According to immunohistochemical staining for α-SMA in 1-month-old mice and Sirius Red staining in 9-month-old mice, HSC activation and collagen deposition were attenuated in the livers of *Myd88*-deficient *Tak1*ΔHep mice (Fig. 4*a*-*c*). *Myd88*-

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deficient *Tak1ΔHep* livers had decreased expression of profibrogenic genes, including *Timp1* and *Tgfb1* compared with *Tak1ΔHep* livers (Fig. 4*d* and 4*e*), providing evidence that MyD88- mediated signaling pathway plays an important role in the development of spontaneous liver fibrosis in *Tak1ΔHep* mice.

TNFR and IL-1R signaling is associated with spontaneous liver fibrosis in Tak1Δhep mice

TLR-mediated NF-κB pathway facilitates pro-inflammatory cytokine (e.g, TNFα and IL-1β) production.[26] In response to TNF and IL-1, TAK1 is also activated.[1] We have previously reported that spontaneously developed hepatocyte injury, hepatic inflammation, and fibrosis in *Tak1ΔHep* mice were reduced by additional deletion of *Tnfr1*.[5] In addition to TLR4 and TLR9, IL-1R also shares MyD88 as an adaptor molecule.[27] We showed that TLR4, TLR9, and MyD88 are involved in liver pathology of *Tak1ΔHep* mice. These findings prompt us to examine the functional importance of IL-1R in *Tak1ΔHep* mice. We investigated whether IL-1R deficiency affects liver injury, inflammation, and fibrosis in *Tak1ΔHep* mice. Interestingly, unlike how TNFR deficiency decreased serum ALT levels in *Tak1ΔHep* mice, IL-1R deficiency did not alter the serum ALT levels (Fig. 5*a*), demonstrating that liver injury is sustained in *Il1r*-deficient *Tak1ΔHep* mice. TNFR deficiency significantly downregulated gene expression of proinflammatory and profibrogenic mediators, such as *Ccl2* and *Timp1* in *Tak1ΔHep* mice, but levels of these factors were equivalent between *Tak1ΔHep* and *Il1r*-deficient *Tak1ΔHep* mice (Fig. 5*b* and 5*c*). However, decreased macrophage infiltration was observed in both *Tnfr*-deficient *Tak1ΔHep* and *IL1r*-deficient *Tak1ΔHep* mice (Fig. 5*d* and 5*e*). Furthermore, both *Tnfr*-deficient *Tak1ΔHep* and *IL1r*-deficient *Tak1ΔHep* mice had significant reduction in fibrosis as demonstrated by expression of α-SMA and Sirius Red staining (Fig. 5*d* and 5*e*). These results suggest that TNFR and IL-1R promote macrophage infiltration and liver fibrosis, but IL-1R is not associated with liver injury in *Tak1ΔHep* mice.

Ablation of TLR4, TLR9, MyD88, or TNFRI, but not IL-1R inhibits HCC growth in Tak1Δhep mice

To investigate the contribution of TLR4, TLR9, MyD88, TNFRI, and IL-1R in spontaneous hepatocarcinogenesis in *Tak1ΔHep* mice, we examined the number and maximum size of tumors in 9-month-old mice. As previously reported,[5] 9-month-old *Tak1ΔHep* mice developed spontaneous HCC. *Tlr4*-deficient *Tak1ΔHep*, *Tlr9*-deficient *Tak1ΔHep*, *Myd88*-deficient *Tak1ΔHep*, and *Tnfr1*-deficient *Tak1ΔHep* mice displayed less macroscopic tumor nodules in the liver (Fig. 6*a*). The total number of tumors and maximal tumor size were significantly lower in combined ablation of TLR4, TLR9, MyD88 or TNFR and TAK1 (Fig. 6*b* and 6*c*). Surprisingly, the whole-body knockout of IL-1R did not inhibit HCC (Fig. 6*a*–*c*). In conclusion, tumorigenesis is affected by signaling through TLR4, TLR9, MyD88, or TNFR in *Tak1ΔHep* mice. However, IL-1R signaling does not affect tumorigenesis in *Tak1ΔHep* mice.

DISCUSSION

The progression of chronic liver disease is characterized by hepatocyte death, inflammation, compensatory proliferation, and fibrosis, which eventually lead to HCC.[28] Hepatocyte death

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is a trigger of immune cell activation, recruitment, and liver inflammation, thereby driving HSC activation and hepatic fibrosis, which ultimately develops HCC.[28] The transition from inflammation to cancer has been termed as “inflammation-fibrosis-cancer axis”.[10] However, the molecular mechanism of disease progression process has been poorly understood. We and other group established a genetically engineered mouse model (*Tak1*Δ*Hep*) that recapitulates the progression of human HCC.[5,6] In this study, we investigated the role of TLR4 and TLR9 in inflammation-fibrosis-cancer axis using this excellent mouse model.

Increased translocation of microbial products is often observed in chronic liver diseases, and it promotes inflammation and fibrosis in the liver.[29] TLRs recognize pathogen- associated molecular patterns and propagate inflammatory responses. The accumulating evidences suggest that gut microbiota/TLR4 signaling is not only crucial for innate immunity and inflammation, but is also actively involved in the pathophysiology of liver diseases.[15,16] LPS, a cell-wall component of Gram-negative bacteria, activates TLR4 in multiple liver cell types. In the context of massive hepatocyte death induced by hepatocyte-specific deletion of *Tak1*, ablation of *Tlr4* did not affect serum ALT levels (Fig. 1*a*). This finding suggests that TLR4 signaling may not be crucial for the hepatocyte damage, presumably due to the low expression of TLR4 in hepatocytes (The Human Protein Atlas, http://www.proteinatlas.org/). The TLR4 bone marrow chimeric mouse study indicates that TLR4 mediates hepatocyte injury indirectly through Kupffer cell-derived TNF production in non-alcoholic steatohepatitis.[30] Among hepatic cells, HSCs express relatively high levels of TLR4. Activation of TLR4 in HSCs induces multiple chemokines and adhesion molecules, and thereby facilitates the recruitment of macrophages.[15] TLR4 is a receptor for LPS that is a strong inflammation inducer. Upon liver injury, LPS levels are often elevated in the portal and systemic circulation due to increased intestinal permeability.[31] Kupffer cells respond to circulating LPS through TLR4. Genetic ablation of TLR4 blocked TLR4- dependent inflammatory responses in macrophages. Thus, *Tlr4*-deficient *Tak1*Δ*Hep* livers had attenuated expression of inflammatory cytokines as well as reduced macrophage infiltration compared with *Tak1*Δ*Hep* livers (Fig. 1*be*), even though the degree of cell injury was similar between in *Tak1*Δ*Hep* mice and *Tlr4*-deficient *Tak1*Δ*Hep* mice. The previous study from our group showed that TLR4 signaling suppresses the expression of TGF-β decoy receptor, BMP and activin membrane bound inhibitor (BAMBI) in HSCs.[15,32] Thus, TLR4 signaling enhances TGF-β-induced HSC activation. Indeed, we observed that TLR4 deficiency attenuated liver fibrosis in *Tak1*Δ*Hep* mice (Fig. 2), which corroborates our previous findings. Furthermore, we observed tumor suppression in *Tlr4*-deficient *Tak1*Δ*Hep* mice. These results are consistent with previous findings that the intestinal microbiota and TLR4 promote chemically-induced HCC.[16] This study strongly suggested that TLR4 signaling in HSCs promotes hepatocarcinogenesis. HSC-producing TLR4-dependent growth factors, such as epiregulin, promote an early HCC development.[16] Interestingly, hepatocytes tend to be highly sensitive to LPS during the late stages of hepatocarcinogenesis, in which hepatocytes and premalignant cells become resistant to apoptosis.[16] Moreover, bone marrow-derived cells including hepatic macrophages may not be involved in HCC growth.[16] In contrast, in alcoholic and non-alcoholic steatohepatitis, TLR4 on Kupffer cells plays a dominant role in the development of liver injury, inflammation, and fibrosis through production of proinflammatory cytokines, such as TNFα, IL-1β, CCL2, and CCL20.[33–35]

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TLR9 is localized in intracellular compartments and recognizes unmethylated cytosine phosphate guanine-containing DNA.[26] Functional TLR9 is expressed in Kupffer cells, HSCs, hepatocytes, dendritic cells, and sinusoidal hepatic endothelial cells.[36–39] In chronic liver diseases, the liver is exposed to environment enriched in exogenous (e.g., bacterial DNA) or endogenous (e.g., host-derived denatured DNA or mitochondrial DNA from dead cells) TLR9 ligands.[24,40] We found that *Tlr9*-deficient *Tak1*Δ*Hep* mice showed lower ALT levels than *Tak1*Δ*Hep* mice (Fig. 1*a*). We performed TUNEL assay to detect apoptotic hepatocytes in *Tlr9*-deficient *Tak1*Δ*Hep* tissue sections. However, TLR9 deficiency unaltered the degree of apoptosis in *Tak1*Δ*Hep* mice (Supplementary Information Fig. 1a). TLR9 may be activated by DNA from apoptotic hepatocytes, thereby enhancing hepatocyte injury other than apoptosis. Since hepatocyte injury is closely associated with inflammation, less degree of hepatocyte injury in Tlr9- deficient *Tak1*Δ*Hep* mice resulted in attenuation of hepatic inflammation. *Tlr9*-deficient mice are protected from liver injury and fibrosis induced by carbon tetrachloride, acetaminophen, bile duct ligation and choline-deficient L-amino acid defined diet.[17,24,37,41] This notion is consistent with our present study showing that *Tlr9*-deficiency protected livers from fibrosis developed in *Tak1*Δ*Hep* mice. Hepatocyte apoptosis and compensatory proliferation are key drivers for HCC development. Although liver injury levels are different between *Tlr4*-deficient *Tak1*Δ*Hep* and *Tlr9*-deficient *Tak1*Δ*Hep* mice, HCC development was inhibited in both mouse strains. Through immunohistochemical staining of proliferating cell nuclear antigen (PCNA), both *Tlr4*-deficient *Tak1*Δ*Hep* and *Tlr9*-deficient *Tak1*Δ*Hep* livers showed the decreased number of PCNA-positive cells compared with *Tak1*Δ*Hep* livers (Supplementary Information Fig. 1a). This suggests that compensatory hepatocyte proliferation is more important for tumorigenesis than liver injury.

All TLRs, except for TLR3, activate MyD88-dependent signaling pathways. TLR3 and TLR4 can also activate Toll/IL-1 receptor domain containing adaptor inducing interferon-β (TRIF)-dependent signaling pathways.[25] Upon binding to TLR ligands, the receptors including TLR4 and TLR9 recruit a signal adaptor protein MyD88, which then forms MyD88-IRAK4- IRAK1/2 complex. This complex engages the ubiquitin ligase TRAF6, along with Ubc13, and activates NF-κB signaling pathways.[25] The extensive studies uncovered the TLR7 and TLR9 signaling pathways in plasmacytoid dendritic cells; these endosomal TLRs can uniquely utilize IRF7 to produce type I interferon in a MyD88-dependent manner.[25] Since TLR9 signals exclusively through MyD88, but not TRIF, *Tlr9*-deficient *Tak1*Δ*Hep* mice exhibited similar phenotype to *Myd88*-deficient *Tak1*Δ*Hep* mice. MyD88 is required for diethylnitrosamine, a chemical carcinogen, induced HCC development.[42] MyD88 controls HCC cell growth and metastasis through both TLR/1L- 1R-dependent and -independent signaling.[43,44] MyD88 is overexpressed in HCC tumors and patients with high expression of MyD88 had poor prognosis.[43] Therefore, MyD88 is considered as a biomarker for prognosis of patients with HCC. TLR9 plays important roles in immune signaling and cell survival. HCC patients who had both TLR4 and TLR9 positive immunostaining in tumor showed poor prognosis.[45] Interestingly, TLR9 expression in fibroblast-like cells was associated with shorter overall survival.[45] Hypoxia induces release of HMGB1 from nucleus and mitochondrial DNA to the cytosol, where they bind to each other. This complex stimulates TLR9 and activates growth signaling pathways, which in turn

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facilitate tumor growth.[46] In contrast, TLR9 agonist unmethylated cytosine phosphate guanine oligodeoxynucleotide treatment led to anti-tumor effect on HCC *in vivo*.[20] Effect of TLR9 on hepatocarcinogenesis has not been fully elucidated. Our present study clearly demonstrated that genetic ablation of TLR9 or its downstream molecule MyD88 significantly ameliorated liver cancer development in *Tak1*Δ*Hep* mice (Fig. 6). We postulated that TLR9 signaling contributes to tumor promotion.

Activation of NF-κB by TLRs triggers the production of pro-inflammatory cytokines, such as TNFα and IL-1β.[26] Previously, we have shown that *Tak1*-deficient hepatocytes conferred susceptible to TNF-mediated cell death and *Tnfr*- deficient *Tak1*Δ*Hep* mice showed reduced liver injury, apoptosis and fibrosis.[5] Here, we show that *Tnfr*-deficient *Tak1*Δ*Hep* mice displayed fewer and smaller tumors compared with *Tak1*Δ*Hep* mice (by numbers, 4 vs. 18 in *Tak1*Δ*Hep*; by max sizes 1.2 vs 7.1 mm in *Tak1*Δ*Hep*) at 9 months of age (Fig. 6). These findings serve as additional evidence that hepatocyte-specific deletion of TAK1 in mice causes hepatocarcinogenesis that is partially mediated by TNFR signaling.

IL-1β is a known potent proinflammatory cytokine. Pro- IL-1β is upregulated by TLR-mediated MyD88/NF-κB pathway. Serum IL-1β levels are elevated in patients with HBV-induced acute hepatitis and primary HCC.[47] Previously, we have shown that Kupffer cells, but not hepatocytes and HSCs, produce IL-1β through TLR9 activation.[17] Liver endothelial cells also produce pro-IL-1β via TLR9.[41] In a mouse model of non-alcoholic steatohepatitis, TLR9 promotes steatosis, inflammation, and fibrosis by induction of IL-1β. Hepatocyte death and lipid accumulation was increased by IL-1β treatment. Also, Kupffer cell-derived IL-1β is involved in HSC activation.[17] In a mouse model of acetaminophen- induced hepatotoxicity, DNA from apoptotic hepatocytes increased TLR9-mediated pro-IL-1β mRNA levels in liver endothelial cells. Mice received IL-1β neutralization antibody exhibited reduced mortality in response to acetaminophen.[41] Moreover, administration of IL-1R antagonist (IL-1ra) ameliorated TLR9-associated liver injury.[48] Activation of TLR4 also drives Kupffer cells to secrete IL-1β.[49] TAK1 is essential for IL-1R signal transduction. In TAK1-deficient cells, IL-1β-induced activation of NF-κB and MAPKs is impaired.[3] In addition, we have shown that hepatocyte death in hepatocyte-specific *Tak1*-deleted mice induced IL-1β production in Kupffer cells.[5] Importantly, the previous study has clearly demonstrated IL-1R plays a major role in the development of diethylnitrosamine-induced HCC.[50] Therefore, we expected IL-1R signaling to have significant roles in the development of liver phenotypes in *Tak1*Δ*Hep* mice. To verify the role of IL-1R signaling for the regulation of inflammation-fibrosis-cancer axis in *Tak1*Δ*Hep* mice, we have generated *Il1r*-deficient *Tak1*Δ*Hep* mice. Surprisingly, in contrast to our expectation, additional deletion of IL-1R in *Tak1*Δ*Hep* mice resulted in no difference in ALT levels, hepatocyte apoptosis, and compensatory proliferation compared with *Tak1*Δ*Hep* mice at the 1 month of age (Fig. 5*a*, Supplementary Information Fig. 1b). Our results suggested that IL-1R signaling is less important in hepatocyte injury and compensatory proliferation caused in *Tak1*Δ*Hep* mice. *Il1r*-deficient *Tak1*Δ*Hep* mice showed delayed progression of liver fibrosis. HSC activation is crucial for the development of liver fibrosis. We have previously demonstrated that IL-1 R signaling is crucial for stellate cell activation.[17] Therefore, *IL1r*-deficient *Tak1*Δ*Hep* mice showed reduced liver fibrosis. Although liver injury, inflammation, and carcinogenesis seem independent of IL-1R signaling in *Tak1*Δ*Hep* mice, the HSC activation and liver fibrosis in

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*Tak1*Δ*Hep* mice require IL-1R signaling. The previous study showing the role of senescence- associated secretory phenotype in obesity-associated HCC development confirmed the pro-tumorigenic effect of IL-1β in the liver.[51] However, tumor sizes and numbers were not reduced in *IL1r*-deficient *Tak1*Δ*Hep* mice, although these mice had reduced macrophage infiltration and hepatic fibrosis (25 and 37% vs. *Tak1*Δ*Hep*) (Fig. 6).

In conclusion, ablation of TLR4, TLR9, and their downstream molecule MyD88 attenuates spontaneous liver inflammation, fibrosis, and cancer in *Tak1*Δ*Hep* mice. Thus, our findings support the indispensable role of TLR4 and TLR9 signaling in inflammation-fibrosis-cancer axis. Finally, we demonstrated that TNFR signaling, but not IL-1R signaling, contributes to carcinogenesis in *Tak1*Δ*Hep* mice.

Supplementary Material

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Acknowledgments

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Abbreviations

Alb-Cre	albumin-Cre
ALT	alanine aminotransferase
α-SMA	α-smooth muscle actin
Ccl2	C-C motif chemokine ligand 2
HCC	hepatocellular carcinoma
HSC	hepatic stellate cell
IL-1β	interleukin-1β
IL-1R	interleukin-1 receptor
LPS	lipopolysaccharide
MAP3K	mitogen-activated protein kinase kinase kinase
MyD88	myeloid differentiation factor 88
NF-κB	nuclear factor-κB
PCNA	proliferating cell nuclear antigen

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qPCR	quantitative polymerase chain reaction
TAK1	TGF-β-activated kinase 1
***Tak1*$^{\Delta Hep}$**	hepatocyte-specific *Tak1*-deleted mice
TGF-β	transforming growth factor-β
Timp1	tissue inhibitor of metalloproteinase-1
TLR	Toll- like receptor
TNFα	tumor necrosis factor α
TNFRI	TNF receptor type I

References

1. Roh YS, Song J, Seki E. TAK1 regulates hepatic cell survival and carcinogenesis. J Gastroenterol. 2014; 49:185–94. [PubMed: 24443058]
2. Shim JH, Xiao C, Paschal AE, et al. TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. Genes Dev. 2005; 19:2668–81. [PubMed: 16260493]
3. Sato S, Sanjo H, Takeda K, et al. Essential function for the kinase TAK1 in innate and adaptive immune responses. Nat Immunol. 2005; 6:1087–95. [PubMed: 16186825]
4. Tang M, Wei X, Guo Y, et al. TAK1 is required for the survival of hematopoietic cells and hepatocytes in mice. J Exp Med. 2008; 205:1611–9. [PubMed: 18573910]
5. Inokuchi S, Aoyama T, Miura K, et al. Disruption of TAK1 in hepatocytes causes hepatic injury, inflammation, fibrosis, and carcinogenesis. Proc Natl Acad Sci U S A. 2010; 107:844–9. [PubMed: 20080763]
6. Bettermann K, Vucur M, Haybaeck J, et al. TAK1 suppresses a NEMO-dependent but NF- kappaB-independent pathway to liver cancer. Cancer Cell. 2010; 17:481–96. [PubMed: 20478530]
7. Kajino-Sakamoto R, Inagaki M, Lippert E, et al. Enterocyte-derived TAK1 signaling prevents epithelium apoptosis and the development of ileitis and colitis. J Immunol. 2008; 181:1143–52. [PubMed: 18606667]
8. Omori E, Matsumoto K, Sanjo H, et al. TAK1 is a master regulator of epidermal homeostasis involving skin inflammation and apoptosis. J Biol Chem. 2006; 281:19610–7. [PubMed: 16675448]
9. Arzumanyan A, Reis HM, Feitelson MA. Pathogenic mechanisms in HBV- and HCV-associated hepatocellular carcinoma. Nat Rev Cancer. 2013; 13:123–35. [PubMed: 23344543]
10. Elsharkawy AM, Mann DA. Nuclear factor- kappaB and the hepatic inflammation-fibrosis-cancer axis. Hepatology. 2007; 46:590–7. [PubMed: 17661407]
11. Yang L, Inokuchi S, Roh YS, et al. Transforming growth factor-beta signaling in hepatocytes promotes hepatic fibrosis and carcinogenesis in mice with hepatocyte-specific deletion of TAK1. Gastroenterology. 2013; 144:1042–54 e4. [PubMed: 23391818]
12. Mencin A, Kluwe J, Schwabe RF. Toll-like receptors as targets in chronic liver diseases. Gut. 2009; 58:704–20. [PubMed: 19359436]
13. Roh YS, Seki E. Toll-like receptors in alcoholic liver disease, non-alcoholic steatohepatitis and carcinogenesis. J Gastroenterol Hepatol. 2013; 28(Suppl 1):38–42.
14. Lopes JA, Borges-Canha M, Pimentel-Nunes P. Innate immunity and hepatocarcinoma: Can toll-like receptors open the door to oncogenesis? WJH. 2016; 8:162–82. [PubMed: 26839640]
15. Seki E, De Minicis S, Osterreicher CH, et al. TLR4 enhances TGF-beta signaling and hepatic fibrosis. Nat Med. 2007; 13:1324–32. [PubMed: 17952090]
16. Dapito DH, Mencin A, Gwak GY, et al. Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. Cancer Cell. 2012; 21:504–16. [PubMed: 22516259]

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17. Miura K, Kodama Y, Inokuchi S, et al. Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1beta in mice. Gastroenterology. 2010; 139:323–34 e7. [PubMed: 20347818]
18. Tanaka J, Sugimoto K, Shiraki K, et al. Functional cell surface expression of toll-like receptor 9 promotes cell proliferation and survival in human hepatocellular carcinomas. Int J Oncol. 2010; 37:805–14. [PubMed: 20811701]
19. Xu D, Wang X, Yan S, et al. Interaction of PTPRO and TLR4 signaling in hepatocellular carcinoma. Tumor Biol. 2014; 35:10267–73.
20. Zhang Y, Lin A, Sui Q, et al. Phosphorothioate modification of the TLR9 ligand CpG ODN inhibits poly(I:C)-induced apoptosis of hepatocellular carcinoma by entry blockade. Cancer Lett. 2014; 355:76–84. [PubMed: 25224571]
21. Hemmi H, Takeuchi O, Kawai T, et al. A Toll- like receptor recognizes bacterial DNA. Nature. 2000; 408:740–5. [PubMed: 11130078]
22. Adachi O, Kawai T, Takeda K, et al. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity. 1998; 9:143–50. [PubMed: 9697844]
23. Hoshino K, Takeuchi O, Kawai T, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol. 1999; 162:3749–52. [PubMed: 10201887]
24. Watanabe A, Hashmi A, Gomes DA, et al. Apoptotic hepatocyte DNA inhibits hepatic stellate cell chemotaxis via toll-like receptor 9. Hepatology. 2007; 46:1509–18. [PubMed: 17705260]
25. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol. 2010; 11:373–84. [PubMed: 20404851]
26. Seki E, Brenner DA. Toll-like receptors and adaptor molecules in liver disease: update. Hepatology. 2008; 48:322–35. [PubMed: 18506843]
27. Muzio M, Ni J, Feng P, et al. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science. 1997; 278:1612–5. [PubMed: 9374458]
28. Luedde T, Kaplowitz N, Schwabe RF. Cell death and cell death responses in liver disease: mechanisms and clinical relevance. Gastroenterology. 2014; 147:765–83 e4. [PubMed: 25046161]
29. Wiest R, Garcia-Tsao G. Bacterial translocation (BT) in cirrhosis. Hepatology. 2005; 41:422–33. [PubMed: 15723320]
30. Yang L, Miura J, Zhang B, et al. TRIF differentially regulates steatosis and inflammation/fibrosis in mice. Cell Mol Gastroenterol Hepatol. 2017; 3:469–83. [PubMed: 28462384]
31. Schwabe RF, Seki E, Brenner DA. Toll-like receptor signaling in the liver. Gastroenterology. 2006; 130:1886–900. [PubMed: 16697751]
32. Liu C, Chen X, Yang L, et al. Transcriptional repression of the transforming growth factor beta (TGF-beta) Pseudoreceptor BMP and activin membrane-bound inhibitor (BAMBI) by Nuclear Factor kappaB (NF-kappaB) p50 enhances TGF-beta signaling in hepatic stellate cells. J Biol Chem. 2014; 289:7082–91. [PubMed: 24448807]
33. Csak T, Velayudham A, Hritz I, et al. Deficiency in myeloid differentiation factor-2 and toll-like receptor 4 expression attenuates nonalcoholic steatohepatitis and fibrosis in mice. Am J Physiol Gastrointest Liver Physiol. 2011; 300:G433–41. [PubMed: 21233280]
34. Inokuchi S, Tsukamoto H, Park E, et al. Toll-like receptor 4 mediates alcohol-induced steatohepatitis through bone marrow-derived and endogenous liver cells in mice. Alcohol Clin Exp Res. 2011; 35:1509–18. [PubMed: 21463341]
35. Affo S, Morales-Ibanez O, Rodrigo-Torres D, et al. CCL20 mediates lipopolysaccharide induced liver injury and is a potential driver of inflammation and fibrosis in alcoholic hepatitis. Gut. 2014; 63:1782–92. [PubMed: 24415562]
36. Schuchmann M, Hermann F, Herkel J, et al. HSP60 and CpG-DNA-oligonucleotides differentially regulate LPS-tolerance of hepatic Kupffer cells. Immunol Lett. 2004; 93:199–204. [PubMed: 15158617]
37. Gabele E, Muhlbauer M, Dorn C, et al. Role of TLR9 in hepatic stellate cells and experimental liver fibrosis. Biochem Biophys Res Commun. 2008; 376:271–6. [PubMed: 18760996]
38. Liu S, Gallo DJ, Green AM, et al. Role of toll-like receptors in changes in gene expression and NF-kappa B activation in mouse hepatocytes stimulated with lipopolysaccharide. Infect Immun. 2002; 70:3433–42. [PubMed: 12065483]

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39. Martin-Armas M, Simon-Santamaria J, Pettersen I, et al. Toll-like receptor 9 (TLR9) is present in murine liver sinusoidal endothelial cells (LSECs) and mediates the effect of CpG-oligonucleotides. J Hepatol. 2006; 44:939–46. [PubMed: 16458386]
40. Frances R, Zapater P, Gonzalez-Navajas JM, et al. Bacterial DNA in patients with cirrhosis and noninfected ascites mimics the soluble immune response established in patients with spontaneous bacterial peritonitis. Hepatology. 2008; 47:978–85. [PubMed: 18306221]
41. Imaeda AB, Watanabe A, Sohail MA, et al. Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome. J Clin Invest. 2009; 119:305–14. [PubMed: 19164858]
42. Naugler WE, Sakurai T, Kim S, et al. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science. 2007; 317:121–4. [PubMed: 17615358]
43. Liang B, Chen R, Wang T, et al. Myeloid differentiation factor 88 promotes growth and metastasis of human hepatocellular carcinoma. Clin Cancer Res. 2013; 19:2905–16. [PubMed: 23549880]
44. Jia RJ, Cao L, Zhang L, et al. Enhanced myeloid differentiation factor 88 promotes tumor metastasis via induction of epithelial-mesenchymal transition in human hepatocellular carcinoma. Cell Death Dis. 2014; 5:e1103. [PubMed: 24603331]
45. Eiro N, Altadill A, Juarez LM, et al. Toll-like receptors 3, 4 and 9 in hepatocellular carcinoma: Relationship with clinicopathological characteristics and prognosis. Hepatol Res. 2014; 44:769–78. [PubMed: 23742263]
46. Liu Y, Yan W, Tohme S, et al. Hypoxia induced HMGB1 and mitochondrial DNA interactions mediate tumor growth in hepatocellular carcinoma through Toll-like receptor 9. J Hepatol. 2015; 63:114–21. [PubMed: 25681553]
47. Su B, Luo T, Zhu J, et al. Interleukin-1beta/Iinterleukin-1 receptor-associated kinase 1 inflammatory signaling contributes to persistent Gankyrin activation during hepatocarcinogenesis. Hepatology. 2015; 61:58597.
48. Petrasek J, Dolganiuc A, Csak T, et al. Type I interferons protect from Toll-like receptor 9-associated liver injury and regulate IL-1 receptor antagonist in mice. Gastroenterology. 2011; 140:697–708 e4. [PubMed: 20727895]
49. Seki E, Tsutsui H, Nakano H, et al. Lipopoly- saccharide-induced IL-18 secretion from murine Kupffer cells independently of myeloid differentiation factor 88 that is critically involved in induction of production of IL-12 and IL-1beta. J Immunol. 2001; 166:2651–7. [PubMed: 11160328]
50. Sakurai T, He G, Matsuzawa A, et al. Hepatocyte necrosis induced by oxidative stress and IL-1 alpha release mediate carcinogen-induced compensatory proliferation and liver tumorigenesis. Cancer Cell. 2008; 14:156–65. [PubMed: 18691550]
51. Yoshimoto S, Loo TM, Atarashi K, et al. Obesity- induced gut microbial metabolite promotes liver cancer through senescence secretome. Nature. 2013; 499:97–101. [PubMed: 23803760]

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What's new?

Toll-like receptors (TLRs) are associated with chronic liver disease. Of particular interest are TLR4 and TLR9, which are suspected of having tumorigenic effects, though whether they promote the development of hepatocellular carcinoma (HCC) remains uncertain. Here, in a mouse model characterized by hepatic deletion of transforming growth factor-β-activated kinase 1 (Tak1ΔHep), recapitulating human HCC progression, deficiency of either TLR4 or TLR9 was found to block the liver inflammation-fibrosis-cancer axis. Mice lacking the adaptor molecule myeloid differentiation factor 88 also exhibited reduced liver injury and tumor growth. Meanwhile, TNF receptor type I signaling contributed to spontaneous HCC development.

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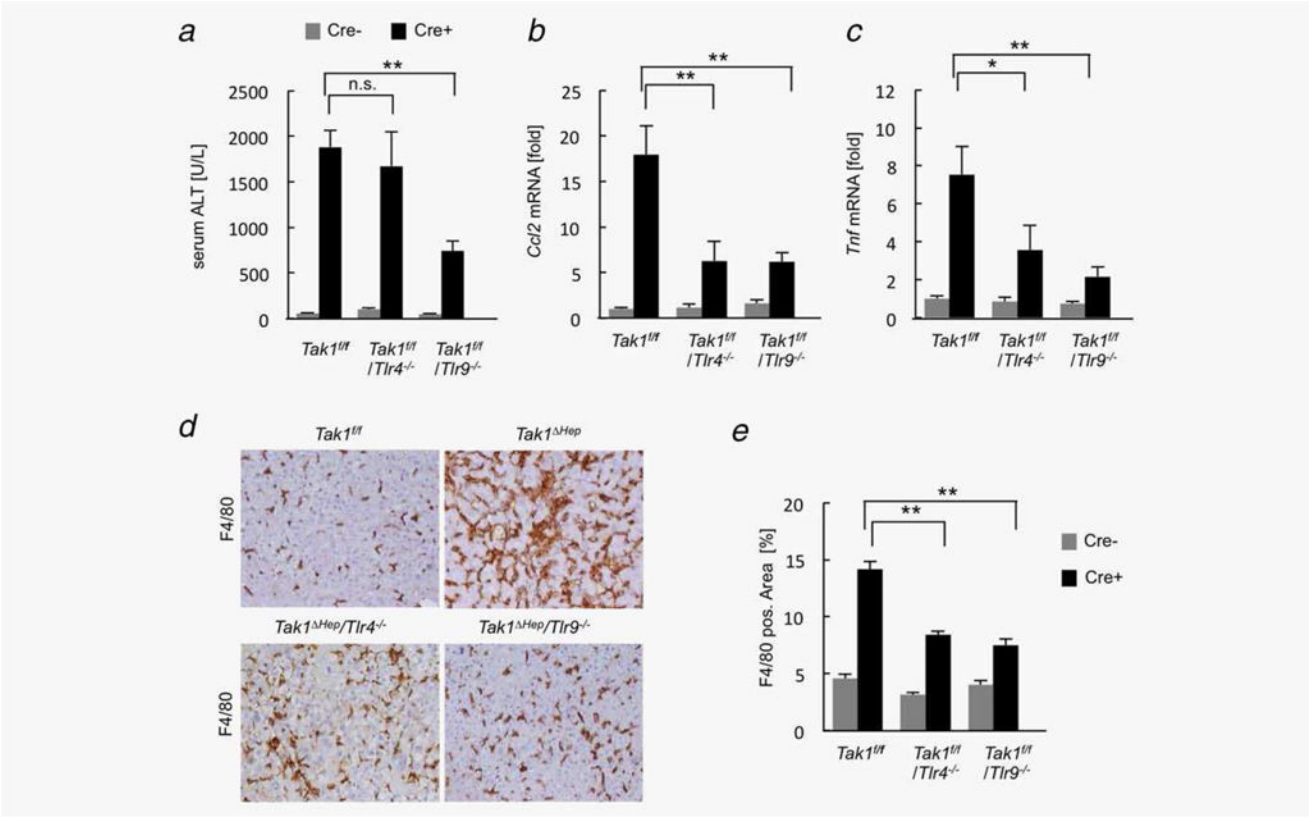

Figure 1.

Ablation of *Tlr4* or *Tlr9* in *Tak1*$^{\Delta Hep}$ mice attenuates spontaneous liver inflammation. (*a*) Serum ALT levels were measured in *Tak1*$^{fl/fl}$, *Tak1*$^{\Delta Hep}$, *Tak1*$^{fl/fl}$/*Tlr4*$^{-/-}$, *Tak1*$^{\Delta Hep}$/*Tlr4*$^{-/-}$, *Tak1*$^{fl/fl}$/*Tlr9*$^{-/-}$ and *Tak1*$^{\Delta Hep}$/*Tlr9*$^{-/-}$ mice at the age of 1 month (n = 8–18). (*b, c*) Hepatic messenger RNA expression of inflammatory genes (*Ccl2* and *Tnf*) in liver tissues from 1-month-old mice were measured by qPCR. (*d, e*) Macrophage infiltration was determined by immunohistochemistry for F4/80 (d). F4/80 positive area was quantified by ImageJ software (e). Original magnification ×200. Gray bar, Alb-Cre negative mice. Black bar, Alb-Cre positive mice. Data represent mean ± SEM; **, $p < 0.01$; *, $p < 0.05$; n.s., not significant. [Color figure can be viewed at wileyonlinelibrary.com]

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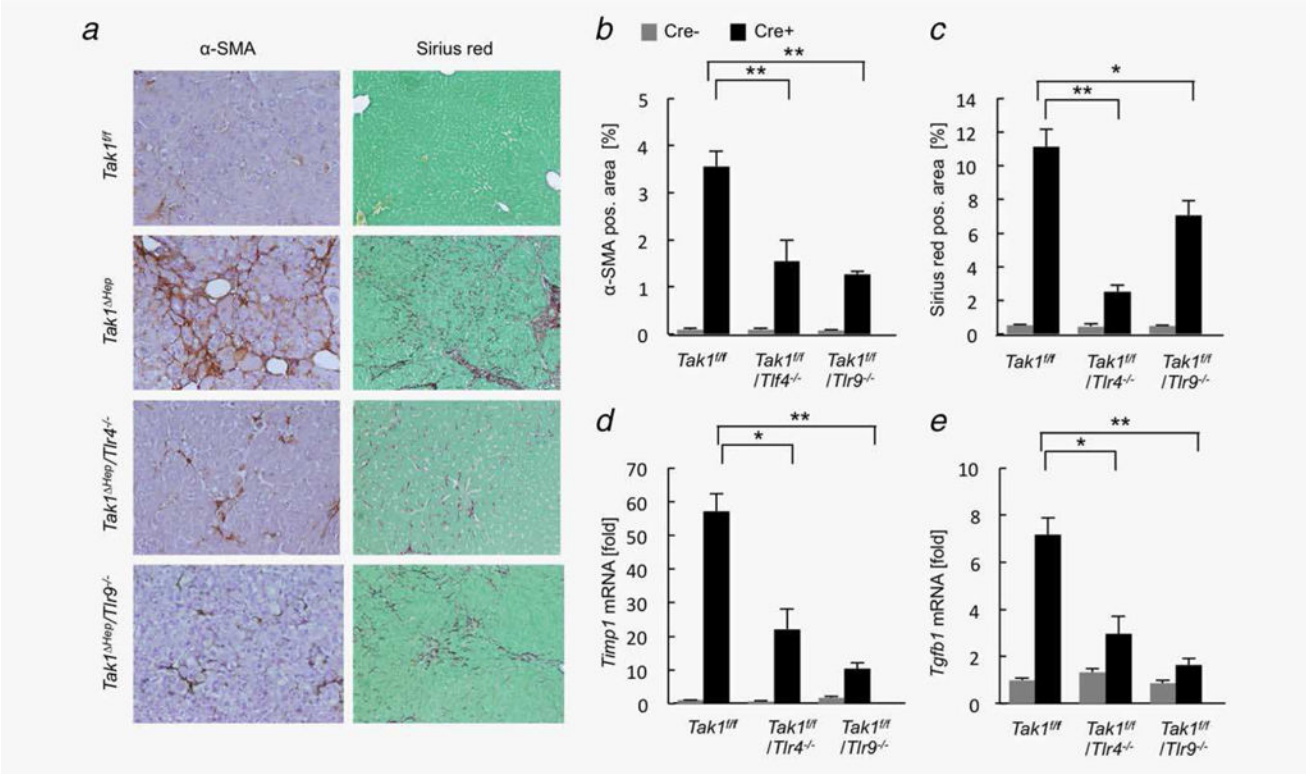

Figure 2.

TLR4 and TLR9 signaling is required for spontaneous liver fibrosis in *Tak1*$^{\Delta Hep}$ mice. (*a*) α-smooth muscle actin (α-SMA) expression in the livers of *Tak1*$^{fl/fl}$, *Tak1*$^{\Delta Hep}$, *Tak1*$^{\Delta Hep}$/*Tlr4*$^{-/-}$, and *Tak1*$^{\Delta Hep}$/*Tlr9*$^{-/-}$ mice at the age of 1 month was determined by immunohistochemistry (*left*) (n = 6–8). Fibrillar collagen deposition in the livers of mice at the age of 9 months was evaluated by Sirius red staining (*right*) (n = 4–6). Original magnification, ×200 for immunohistochemistry for α-SMA and ×100 for Sirius Red Staining. (*b, c*) Quantification for staining for α-SMA (b) and Sirius Red staining (c). (*d, e*) Hepatic messenger RNA expression of fibrogenic genes (*Timp1* and *Tgfb1*) in liver tissues from 1-month-old mice was determined by qPCR. Gray bar, Alb-Cre negative mice. Black bar, Alb-Cre positive mice. Data represent mean ± SEM; $^{**}p < 0.01$; $^{*}p < 0.05$. [Color figure can be viewed at wileyonlinelibrary.com]

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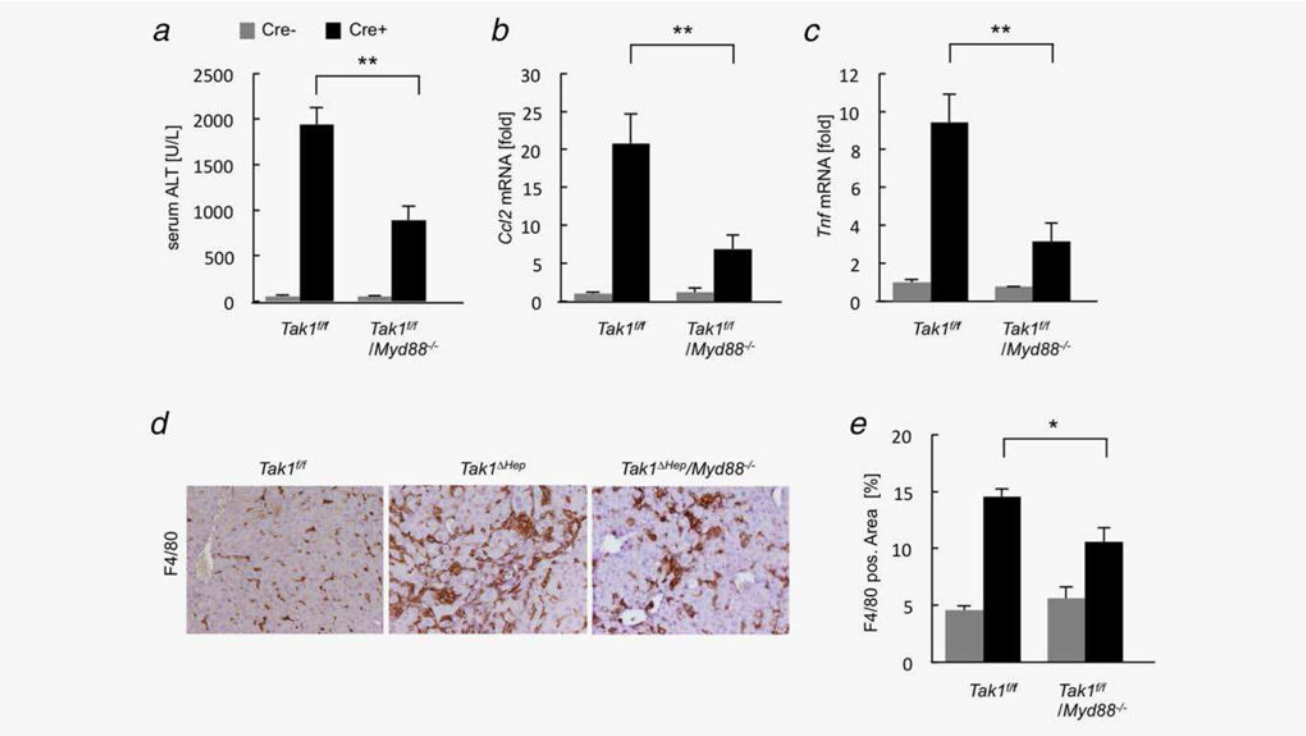

Figure 3.

Additional deletion of *Myd88* reduces spontaneous liver inflammation in the livers of *Tak1*$^{\Delta Hep}$ mice. (*a*) Serum ALT levels were measured in *Tak1*$^{fl/fl}$, *Tak1*$^{\Delta Hep}$, *Tak1*$^{fl/fl}$/*Myd88*$^{-/-}$ and *Tak1*$^{\Delta Hep}$/*Myd88*$^{-/-}$ mice at the age of 1 month (n = 4–17). (*b, c*) Hepatic messenger RNA expressions of *Ccl2* (b) and *Tnf* (c) were assessed by qPCR. (*d, e*) Representative pictures (d) and quantification (e) of immunohistochemistry for F4/80. Original magnification ×200. Gray bar, Alb-Cre negative mice. Black bar, Alb-Cre positive mice. Data represent mean ± SEM; **$p < 0.01$; *, $p < 0.05$. [Color figure can be viewed at wileyonlinelibrary.com]

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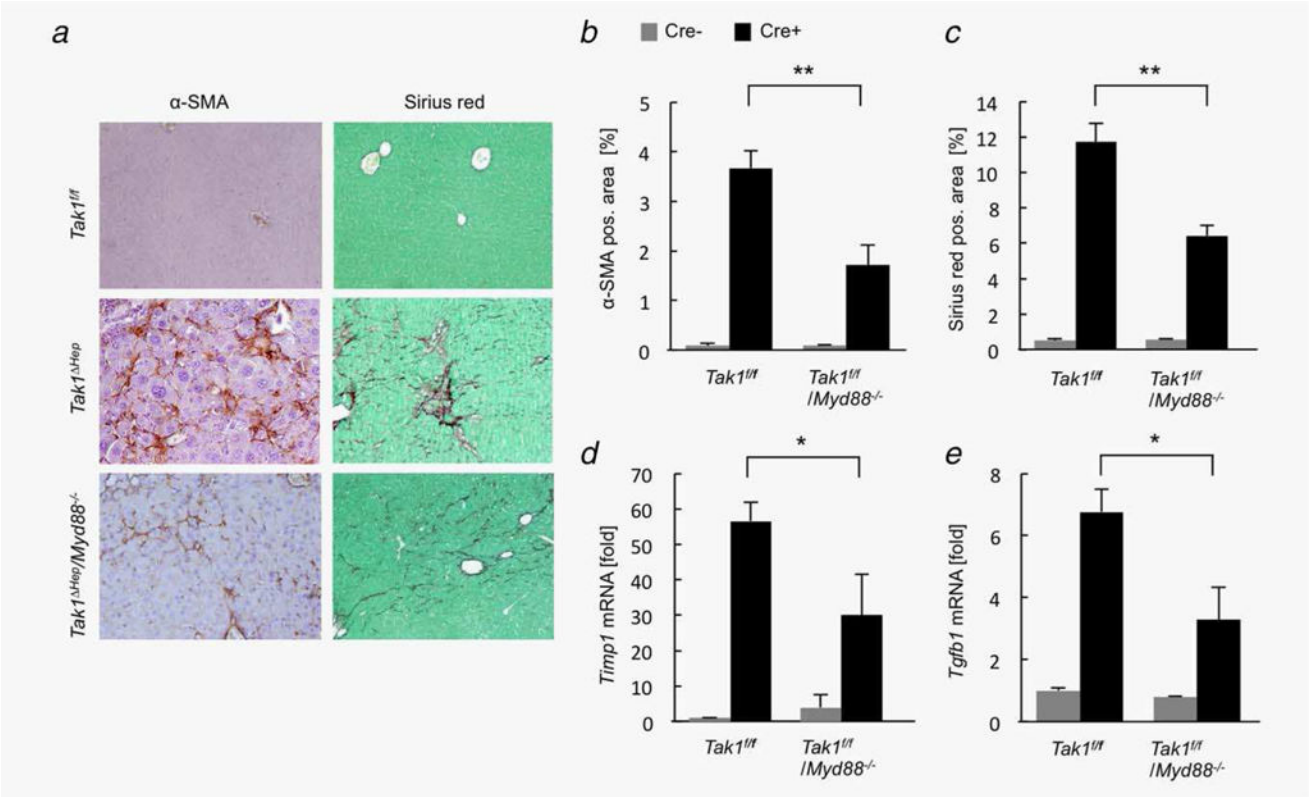

Figure 4.

Loss of *Myd88* in $Tak1^{\Delta Hep}$ mice suppresses spontaneous liver fibrosis. (*a*) Representative pictures of immunohistochemistry for α-SMA (*left*) in the livers of $Tak1^{fl/fl}$, $Tak1^{\Delta Hep}$, and $Tak1^{\Delta Hep}/Myd88^{-/-}$ mice at the age of 1 month ($n = 4–7$). Sirius Red staining (*right*) in the livers of mice at the age of 9 months ($n = 5–6$). Original magnification, ×200 for immunohistochemistry for α-SMA and ×100 for Sirius Red staining. (*b, c*) Quantification for staining for α-SMA (b) and Sirius Red staining (c). (*d, e*) Hepatic messenger RNA expressions of *Timp1* (d) and *Tgfb1* (e) in liver tissues from 1-month-old mice were assessed by qPCR. Gray bar, Alb-Cre negative mice. Black bar, Alb-Cre positive mice. Data represent mean ± SEM; $^{**}P < 0.01$; $^{*}P < 0.05$. [Color figure can be viewed at wileyonlinelibrary.com]

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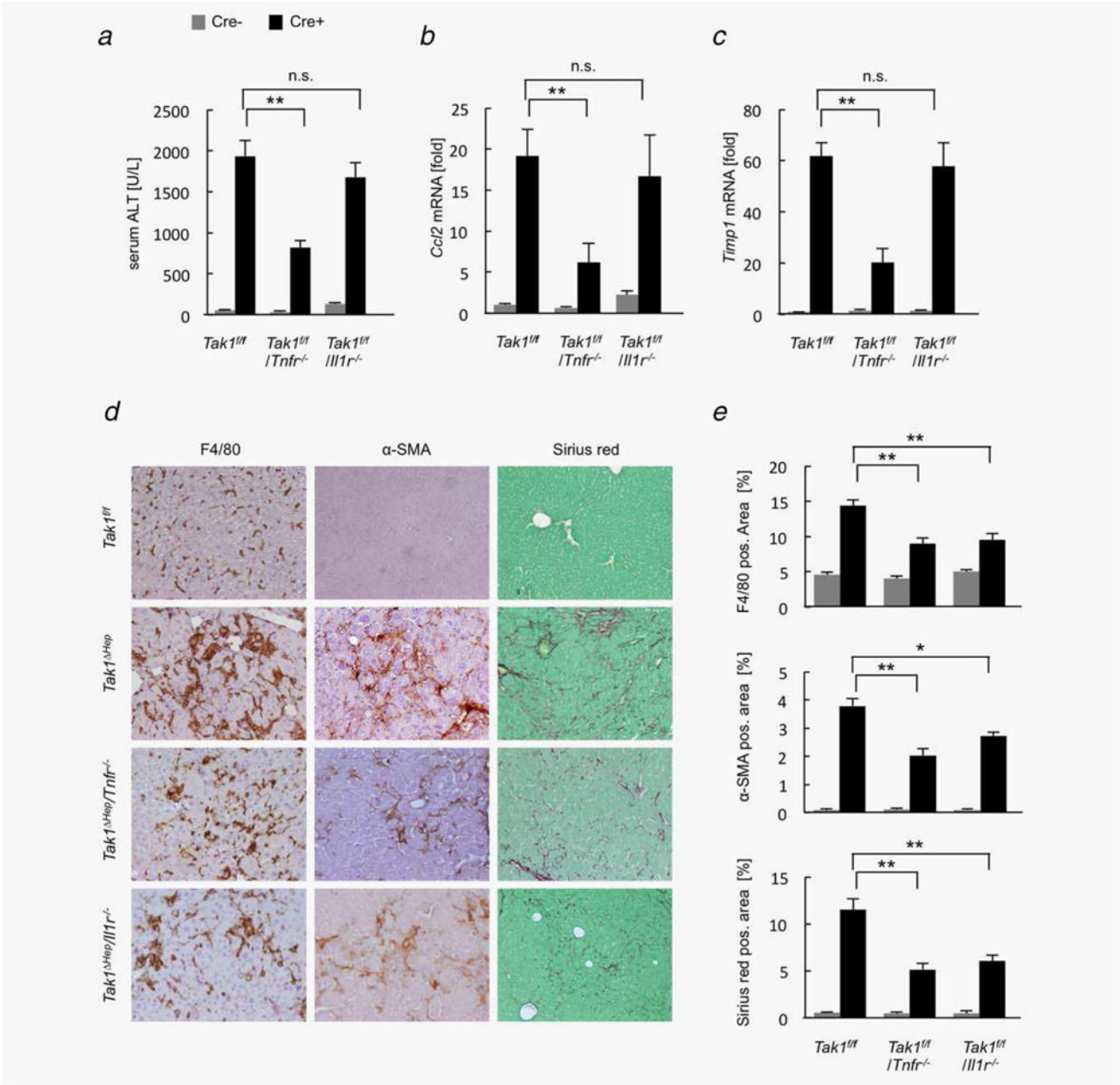

Figure 5.

TNF receptor and IL-1 receptor signaling is partially responsible for macrophage infiltration and liver fibrosis in *Tak1*$^{\Delta Hep}$ mice. (*a*) Serum ALT levels of 1-month-old *Tak1*$^{fl/fl}$, *Tak1*$^{\Delta Hep}$, *Tak1*$^{fl/fl}$/*Tnfr*$^{-/-}$, *Tak1*$^{\Delta Hep}$/*Tnfr*$^{-/-}$, *Tak1*$^{fl/fl}$/*Il1r*$^{-/-}$, and *Tak1*$^{\Delta Hep}$/*Il1r*$^{-/-}$ mice (n = 5–17). (*b, c*) Hepatic messenger RNA expression of *Ccl2* (b) and *Timp1* (c) was measured by qPCR in 1-month-old mice. (*d, e*) Representative pictures (d) and quantification (e) of immunohistochemistry for F4/80 and α-SMA (1-month-old, n = 4–10), and Sirius Red staining (9-month-old, n = 4–6). Original magnification, ×200 for immunohistochemistry for F4/80 and α-SMA, and ×100 for Sirius Red staining. Gray bar, Alb-Cre negative mice.

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Black bar, Alb-Cre positive mice. Data represent mean ± SEM; $^{**}p < 0.01$; $^{*}p < 0.05$; n.s., not significant. [Color figure can be viewed at wileyonlinelibrary.com]

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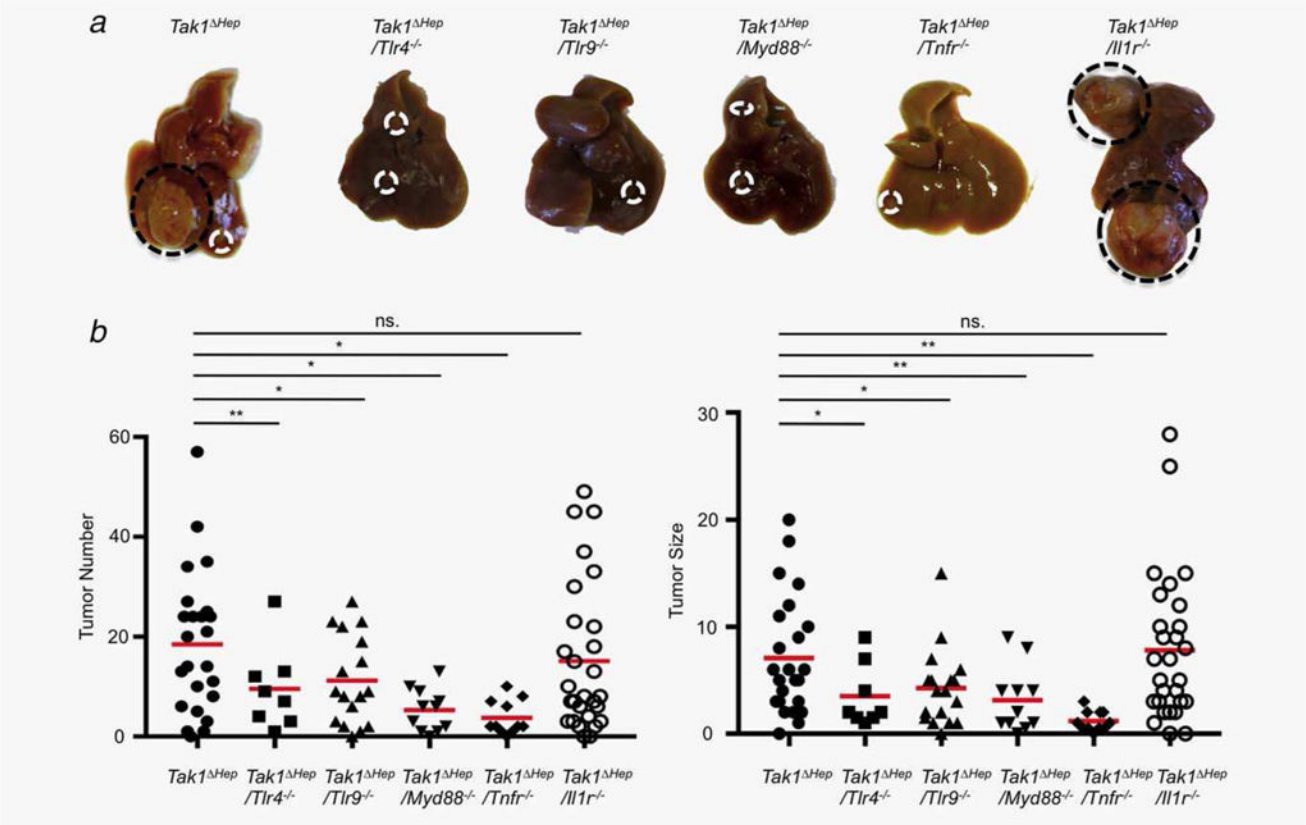

Figure 6.

Spontaneous hepatocarcinogenesis in *Tak1*$^{\Delta Hep}$ mice is suppressed by additional deletion of TLR4, TLR9, MyD88, and TNFR, but not IL-1R. (*a*) Representative macroscopic pictures of livers of 9-month-old *Tak1*$^{\Delta Hep}$, *Tak1*$^{\Delta Hep}$*/Tlr4*$^{-/-}$, *Tak1*$^{\Delta Hep}$*/Tlr9*$^{-/-}$, *Tak1*$^{\Delta Hep}$*/Myd88*$^{-/-}$, *Tak1*$^{\Delta Hep}$*/Tnfr*$^{-/-}$, and *Tak1*$^{\Delta Hep}$*/Il1r*$^{-/-}$ mice. (*b*) The number of tumor nodules per mouse was counted (left) and the maximal tumor size was measured (right). (n = 8–28). **$p < 0.01$; *$p < 0.05$; n.s., not significant. [Color figure can be viewed at wileyonlinelibrary.com]