Divergent effect of Birinapant, and BV6 SMAC mimetic on TNFα induced NF-κB signaling and cell viability in activated hepatic stellate cells

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Abstract

Background Tumor necrosis factor-α (TNFα) is a pleiotropic cytokine involved in nuclear factor kappa B (NF-κB) mediated cell survival as well as cell death. High serum TNFα levels correlate with liver fibrosis and enhance hepatic stellate cell (HSC) viability. However, the regulatory role of cellular inhibitor of apoptosis-1/2 (cIAP1/2) during TNFα induced NF-κB signaling in activated HSCs is largely unknown.

Method and Results Activated HSCs were treated with cIAP1/2 inhibitors i.e., SMAC mimic BV6, and Birinapant in the presence of TNFα and macrophage conditioned media. TNFα cytokine increased cIAP2 expression and enhanced cell viability through the canonical NF-κB signaling in activated HSCs. cIAP2 inhibition via BV6 decreased the TNFα induced canonical NF-κB signaling, and reduced cell viability in activated HSCs. SMAC mimetic, Birinapant alone did not affect the cell viability but treatment of TNFα sensitized HSCs with Birinapant induced cell death. While BV6 mediated cIAP2 ablation was able to decrease the TNFα induced canonical NF-κB signaling, this effect was not observed with Birinapant treatment. Secreted TNFα from M1 polarized macrophages sensitized activated HSCs to BV6 or Birinapant mediated cytotoxicity. However, M2 polarized macrophage conditioned medium rescued the activated HSCs from BV6 mediated cytotoxicity.

Conclusion In this study, we describe the regulatory role of cIAP2 in TNFα induced NF-κB signaling in activated HSCs. Targeting cIAP2 may be a promising approach for liver fibrosis treatment via modulating NF-κB signaling in activated HSCs.

Keywords Hepatic stellate cells · Tumor necrosis factor-α (TNFα) · Inhibitor of apoptosis (IAP) · Cellular inhibitor of apoptosis-1/2 (cIAP1/2) · Cell viability · Macrophage subtype

Abbreviations

TNFα Tumor necrosis factor-α
cIAP1/2 Cellular inhibitor of apoptosis-1/2
NF-κB Nuclear factor kappa B

HSC Hepatic stellate cell
αSMA α-Smooth muscle actin
qHSC Quiescent HSC
SMAC Second mitochondria-derived activator of caspase
NASH Non-alcholic steatohepatitis

Introduction

Tumor necrosis factor-α (TNFα) is a pleiotropic cytokine predominantly produced by macrophages that trigger multiple signaling pathways involved in inflammation, proliferation, and apoptosis [1]. TNFα levels are elevated in many liver diseases and is known to promote the pathogenesis of chronic liver inflammation and liver fibrosis [2]. In NASH patients, high serum TNFα levels correlate with insulin resistance and fibrosis stage [3]. TNFα cytokine activates the quiescent hepatic stellate cell (HSC) into fibrogenic...
myofibroblasts whereas knockout of the TNF receptor 1 (TNFR1) for TNFα showed reduced liver fibrosis [4]. Interestingly, TNFα alone is incompetent in inducing cell death in hepatocytes and requires prior sensitization by N-galactosamine (GalN)/lipopolysaccharide (LPS) during liver injury [5]. TNFα and IL-1 contribute to NF-κB-dependent HSC survival and promote liver fibrosis through their pro-survival effect [6]. HSCs are the prominent players of the fibrotic response, located in the space of disse as non-fibrogenic, quiescent cells (qHSC) in a healthy liver [7]. HSCs become activated and fibrogenic (activated HSCs) during liver injury and deposit collagen I in their extracellular environment [8]. In the injured liver, cytokine response triggers macrophage infiltration, and enhances the viability of HSCs [9] whereas in the resolution phase of fibrosis, activated HSCs are cleared by apoptosis [10, 11].

Mechanistically, the binding of TNFα to its receptor, TNFR1 leads to protein complex formation with inhibitor of apoptosis 1/2 (cIAP1 and cIAP2) proteins which further activates the NFκB pathway [12]. Various cancer cells overexpress cIAP1/2 and their targeting induces the TNFα-driven apoptosis implying potential anti-cancer therapy [13]. cIAP1 and cIAP2 consist of RING-domain E3 ligases that can inhibit caspase- 8 and activate the pro-survival NF-κB signaling [12]. However, ablation of cIAP1 or cIAP2 causes activation of caspase 8 induced apoptosis [14, 15]. Hepatitis B virus manipulates the cIAP1 and cIAP2 regulated NF-κB signaling in infected hepatocytes that in turn promotes viral infection. Pharmacological targeting of cIAP1/2 promotes the clearance of HBV-infected hepatocytes in the liver [16]. NF-κB plays a crucial role in HSC activation and fibrosis through mediators such as LPS, TNFα, IL-1β, angiotensin II and CD40L. Phosphorylated p65 (Ser536) responsible for NF-κB targeted gene expression is found exclusively in activated HSCs whereas little to no NF-κB activation occurs in the hepatocytes of injured liver [17]. NF-κB inhibitors such as sulfasalazine (angiotensin II inhibitors) and NF-κB inhibitory NEMO-binding peptides promote HSC apoptosis and resolution of fibrosis [17, 18]. NF-κB also promotes the secretion of various chemokines such as CCL2, CCL3, CXCL2, and CXCL5 that lead to an influx of inflammatory cells including macrophages, suggesting that targeting of NF-κB has the potential to modulate macrophage plasticity [19].

The regulatory role of cIAP1/2 in the modulation of NF-κB signaling in activated HSCs, and consequently liver fibrosis progression is largely unknown. In this study, we found that TNFα induces NF-κB signaling in hepatic stellate cells, and enhances cell viability. Inhibition of cIAP2 by both BV6 and Birinapant SMAC mimetic decreases the viability of HSCs. However, BV6 and Birinapant showed divergent behaviour on the TNFα induced NF-κB signaling in activated HSCs. These results support that modulation of TNFα induced NF-κB signaling through targeting cIAP2 exhibits the potential for the development of novel anti-fibrotic therapeutic approach in liver disease.

Materials and methods

Chemical reagents and recombinant protein

Reagents used in the study are mentioned as follows: MTT (34-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, (#TC191, himedia, India), BV6 (#S7597, selleckchem, USA), Birinapant (#S7015, selleckchem, USA), dimethyl sulfoxide (DMSO) (#D8418, sigma-aldrich, USA), human TNFα (#300-01A, peprotech, USA).

Human HSC cell line culture

Activated human hepatic stellate cell line LX2 was a gift from Dr. Scott L. Friedman which was cultured in DMEM supplemented with 4 mM L-glutamine, 100 IU/ml Penicillin/100 µg/ml Streptomycin, and 2% (v/v) FBS (#RM1112, himedia, India) at 37 °C with 5% CO2 in a CO2 incubator. Cells were regularly passaged by Trypsin–EDTA solution (#TCL007, himedia, India) at 70–80% confluency.

MTT cell viability assay

Cell viability assay was performed using MTT reagent (#TC191, himedia, India). Briefly 5 × 10^4 cells were seeded into 96-well plates containing 100 µL DMEM with 2% FBS. After 24 h, the medium was carefully removed. Cells were treated according to experimental conditions and incubated for 24 h. After 24 h, 0.5 mg/ml of MTT was added to each well and incubated further for 4 h. Equal volume of DMSO was added to solubilize the formazan crystals. After 15 min of incubation at 37 °C, absorbance was measured using multiskan multifunction microplate reader (#51119100, thermo scientific, USA) at 570 nm wavelength. All experiments were performed in triplicate.

Macrophage polarization and characterization

Human monocyte THP-1 cells were cultured in RPMI 1640 medium (#AT162, himedia, India) containing 10% FBS, and maintained at 37 °C in a 5% CO2 incubator. For macrophage differentiation, THP-1 cells were seeded at a density of 0.5 × 10^6 cells/ml and treated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA; #79346, sigma-aldrich, USA) for 24 h followed by incubation in fresh PMA free complete medium for another 48 h. These differentiated macrophages were called as M0 macrophages. For M1-like polarization, we incubated M0 macrophages
with lipopolysaccharide (LPS; #L4391, sigma-aldrich, USA) and INFγ (#300–02, peprotech, USA), 20 ng/ml each for 24 h; for M2-like polarization M0 macrophages were treated with IL-4 (#200–04, peprotech, USA) and IL13 (#200–13, peprotech, USA), 20 ng/ml each. Macrophages were characterized by analyzing surface markers, and gene expression of cytokines using RT-qPCR. Macrophages were cultured in RMPI with 1% (v/v) FBS for 24 h to collect conditioned media.

**TNFα cytokine ELISA**

Macrophage conditioned media was collected and the concentration of TNFα cytokine in the conditioned media was measured using TNFα cytokine ELISA kit (#KHC3011, thermo scientific, USA).

**RNA isolation and quantitative polymerase chain reaction (qPCR)**

Total RNA was isolated from cells using trizol reagent (#15596026, invitrogen, USA), and cDNA synthesis was performed with RevertAid First Strand cDNA synthesis kit (#K1622, thermo scientific, USA) using random primers. Quantitative PCR was performed using syber fluorochromes master mix (#A25742, applied biosystems, USA) on StepOnePlus PCR System (#4376600, applied biosystems, USA) using gene specific primers (Supplementary. Table S1).

**Western blot**

Cells were homogenized in cold RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% SDS, with protease inhibitor cocktail (04693132001, sigma-aldrich, USA) and phosphatase inhibitor (PHOSS-RO, sigma-aldrich, USA). Cell lysates were collected, protein samples were prepared with lamelli buffer and electrophoresed on SDS-PAGE gel followed by transfer onto PVDF membrane. The membranes were incubated in primary and secondary antibodies according to manufacturer instructions (Supplementary. Table S2). The protein bands on membrane blot were visualized with ECL detection reagent (#37074, thermo scientific, USA) using ImageQuant LAS 500 chemiluminescence. The intensity of individual bands was quantified using ImageJ densitometry software and normalized with loading control.

**Statistical analysis**

All data were presented as mean ± S.D. (standard deviation) from at least three separate independent experiments. Differences among groups were tested for statistical significance by the Student’s t-test; when comparing 2 groups, by one-way ANOVA; when comparing multiple conditions with repeated measures by two-way AVOVA using Prism-GraphPad. P values < 0.05 were considered significant for all the tests..

**Results**

**TNFα cytokine induces cIAP2 expression in activated HSCs**

The pro-inflammatory cytokine TNFα is elevated in hepatic injury and contributes to liver fibrosis progression. It participates in NF-κB signaling and cell death in a context-dependent manner [12]. To determine the regulatory role of cIAP1/2 in TNFα induced NF-κB signaling, we stimulated human activated HSC cell line i.e., LX2 cells with TNFα (20 ng/ml), and analyzed gene expression alterations. TNFα (20 ng/ml) stimulation increased the mRNA expression of cIAP2, fibrogenic marker, αSMA, vimentin (VIM), and collagen I (COL1A1) in activated HSCs. However, cIAP1 expression did not increase with TNFα stimulation (Fig. 1).

Our results support the evidence that cIAP1 or cIAP2 compensate for expression and function [20, 21]. These results suggest that the pro-inflammatory TNFα cytokine increases cIAP2 gene expression along-with the expression of HSC activation markers.

**TNFα sensitizes activated HSCs towards BV6 and Birinapant mediated cell death**

To determine the regulatory role of cIAP2 in TNFα induced NF-κB signaling and the effect of cIAP2 ablation on HSC viability, we treated activated human HSC cell line LX2 with TNFα and various dose of BV6 or Birinapant SMAC mimetics. Both BV6 and Birinapant are capable of inhibiting cIAP2 expression in activated HSCs [22]. Pre-stimulation of HSCs with 20 ng/ml of TNFα for 1 h was followed by treatment with different dose of BV6 or Birinapant for 24 h. TNFα stimulation alone did not alter the cell viability with 89.7 ± 5% viable cells in comparison to non-stimulated HSCs. BV6 treatment decreased the viability of HSCs in a dose-dependent manner with an IC50 value of 2.876 ± 1.268 μM in LX2 cells at 24 h of treatment. However, TNFα pre-stimulation increased the sensitization of HSCs towards BV6 mediated cell death and decreased the IC50 value to 1.410 ± 0.923 μM at 24 h of treatment (Fig. 2A).

Interestingly, Birinapant alone was incompetent in decreasing HSC’s viability, even at higher concentrations. However, pre-stimulation with 20 ng/ml TNFα followed by Birinapant treatment showed a significant (p < 0.001) reduction in cell viability. It was observed that 29.910 ± 2.5 nM
Birinapant was sufficient to suppress 50% cell viability in HSCs that were pretreated with 20 ng/ml of TNFα (Fig. 2B). This suggested that TNFα pre-treatment sensitizes the activated HSCs towards cell death mediated by cIAP2 suppression in the presence of BV6 or Birinapant. Based on these results, we conclude that cIAP2 regulates TNFα induced cell survival and its suppression switches off the TNFα pro-survival signal, resulting in decreased cell viability of activated HSCs.

**cIAP2 regulates TNFα induced canonical NF-κB signaling and its ablation induces non-canonical NF-κB signaling in activated HSCs**

cIAP2 has a modulatory role in TNFα induced NF-κB signaling pathway that affects cell survival [16]. To investigate the modulation of TNFα induced NF-κB signaling with BV6 or Birinapant treatment in HSCs, we performed immunoblotting. It was found that TNFα stimulation increased cIAP2 expression in activated HSCs. TNFα induces canonical NF-κB signaling through phosphorylated IκBα and NF-κB p65 mediator proteins. BV6 (5 μM) mediated cIAP2 ablation decreased the expression of phosphorylated IκBα and NF-κB p65 proteins, that indicate the suppression of canonical NF-κB signaling. Interestingly, cIAP2 ablation induces NIK accumulation which is responsible for the activation of non-canonical NF-κB signaling (Fig. 3A). Birinapant (50 nM) mediated cIAP2 ablation was unable to decrease the TNFα induced NF-κB signaling in HSCs. However, Birinapant upregulated the NIK accumulation-dependent non-canonical NF-κB signaling in HSCs (Fig. 3B). We further inactivated cIAP2 protein expression in LX2 cells using 60 nM siRNA specific to cIAP2 gene (IDT, CAT# 106080097), and observed that cIAP2 gene silencing...
Fig. 3 cIAP2 regulates TNFα induced canonical NF-κB signaling and its ablation induces non-canonical NF-κB signaling in HSCs. A Western blot representing the expression of cIAP2, NF-κB p65, pIkBα, and NIK proteins in LX2 cells after 24 h of BV6 (5 μM) treatment pre-stimulated with TNFα (20 ng/ml). B Western blot representing the expression of cIAP2, NF-κB p65, pIkBα, and NIK proteins in LX2 cells after 24 h of Birinapant (50 nM) treatment and pre-stimulated with TNFα (20 ng/ml). Protein expression was quantified by analysis of band intensity and normalized with α-tubulin expression. nsP > 0.05, *P < 0.05 **P < 0.01, ***P < 0.001 [two-way ANOVA for (A and B)]
suppresses the NF-κB p65 protein expression (Supplementary Fig S1A) and NF-κB activity measured by reporter assay (Supplementary Fig S1B) in TNFα treated LX2 cells. However, inactivation of cIAP2 protein by siRNA silencing also led to the accumulation of NIK protein (Supplementary Fig S1A). These results suggest that BV6 and Birinapant...
are both capable of inhibiting cIAP2 expression but have different modes of action. While BV6 has the potential to modulate TNFα-induced NF-κB signaling and induce cell death in HSCs, Birinapant induces cell death in the presence of the TNFα without affecting NF-κBp65 expression.

**M1 polarized macrophages secrete higher TNFα than M2 polarized macrophages**

In an injured liver, a pro-inflammatory microenvironment leads to the accumulation of immune cells especially macrophages, that play a vital role in HSC survival and fibrosis progression. M1 polarized macrophages are known to secrete pro-inflammatory cytokines that induce the NF-κB signaling further promoting the survival of HSCs and infiltration of other immune cells from bone marrow [1]. However, M2 polarized macrophages have been studied to secrete anti-inflammatory cytokines that induce the fibrogenic activation of HSCs [23]. To investigate the effect of polarized M1/M2 macrophage secretome on BV6 or Birinapant mediated cell death in HSCs, we cultured the human monocyte cell line THP1 and differentiated them into macrophages. We treated the THP1 cells with 50 ng/ml of Phorbol 12-myristate 13-acetate (PMA) for 24 h, followed by incubating cells in PMA free culture medium for 48 h. This induces differentiation of human monocyte cells into macrophages termed as naive or M0 macrophage. We further treated the naive macrophages with 20 ng/ml of lipopolysaccharide (LPS)/interferon-gamma (IFNγ) and 20 ng/ml of interleukin-4 (IL4)/interleukin-13 (IL13) for 24 h to polarize them into M1 (pro-inflammatory phenotypic), and M2 (anti-inflammatory phenotypic) macrophages, respectively (Fig. 4A). The phenotype of polarized macrophages was confirmed by analyzing the expression of different cytokines and surface marker genes by RT-qPCR. M0 macrophages showed higher expression of CD14, CD68 surface markers as compared to monocytes. M1 polarized macrophages expressed high CD80, CD86 as well as TNFα, IL-1β, and IL-6 (pro-inflammatory cytokines) mRNA expression whereas, M2 polarized macrophages exhibited high CD206, CD209, as well as IL-10, FN1, and TGF-β1 (anti-inflammatory cytokines) (Fig. 4B, C)

M1 macrophages were found to exhibit higher TNFα mRNA expression than M2 polarized macrophages. We further quantified the secreted TNFα cytokine in macrophage conditioned medium through ELISA. It was found that M1 polarized macrophages secreted maximum TNFα cytokines when compared in totality to M0 and M2 macrophages (Fig. 4D).

**TNFα secreted by M1 polarized macrophages sensitize HSCs to BV6/Birinapant mediated cell death**

To determine the effect of polarized macrophage conditioned medium (CM) on BV6 and Birinapant mediated cell death in HSCs, we cultured activated HSCs i.e., LX2 cells in the presence of M0, M1, and M2 polarized macrophage CM, and further treated them with different dose of BV6 or Birinapant for 24 h. We found that M1 polarized macrophage secreted TNFα that sensitized HSCs to BV6 and Birinapant mediated cell death. The IC50 of BV6 in LX2 cells was observed to be slightly higher i.e., 3.639 ± 0.894 μM in the presence of M1 CM compared to LX2 cells treated with BV6 alone (2.876 ± 1.268 μM). The higher IC50 value of BV6 observed in HSCs inspite of the presence of TNFα in M1 CM could be attributed to the presence of other secretory proteins that may have a role in attenuating the BV6 induced cell death. However, M0 CM suppresses the BV6 induced cell death with an IC50 value at 10.58 ± 2.234 μM. Interestingly, presence of M2 CM rescued the HSCs from BV6 mediated cell death, as indicated by the very high IC50 value (76.55±5.34 μM) when compared to LX2 cells treated with BV6 alone (Fig. 5A and 2A). Similar results were found with Birinapant treatment; TNFα secreted by M1 polarized macrophages sensitized HSCs towards Birinapant mediated cell death. Birinapant exhibited an IC50 value of 90.939 ± 1.964 nM in presence of M1 polarized macrophage CM in comparison to HSCs treated with Birinapant alone (Fig. 5B and 2B). However, Birinapant showed no cytotoxicity in activated HSCs in the presence of M0 or M2 CM (Fig. 5B). This suggested that M1 (pro-inflammatory) polarized macrophages increase HSC’s sensitization towards both BV6 and Birinapant SMAC mimetic mediated cell death. Contrarily, non-polarized or naive M0 macrophages as well as M2 (anti-inflammatory) polarized macrophages did not increase the BV6 and Birinapant SMAC mimetic induced cytotoxicity in activated HSCs. Infact M2 CM provided resistance against BV6 induced cell death in activated HSCs. We further investigated the effect of M0, M1, and M2 macrophage CM on the NF-κB signaling molecules as well as cIAP2 protein expression (Supplementary Fig S2A). It was observed that M1 polarized macrophages increased cIAP2...
protein expression and activated the NF-κB signaling in activated HSCs. Increased expression of NF-κBp65 and IκBα and decreased expression of pIκBα and RelB proteins was observed in activated HSCs after M1 CM stimulation. Both M0 and M2 polarized macrophages suppressed the cIAP2 protein expression and NF-κB signaling in activated HSCs. Furthermore, HSCs pre-stimulated with TNFα, when treated with M2 CM showed reduced expression of NF-κBp65 compared to HSCs stimulated with TNFα alone (Supplementary Fig S2B). These results suggest that macrophages modulate the cIAP2 mediated NF-κB survival signaling in HSCs and can be targeted for development of anti-fibrotic therapy in chronic liver disease.

Discussion

Hepatocyte death, infiltrating immune cell-mediated inflammation, and liver fibrosis are the key characteristics of chronic liver disease. Tumor necrosis factor-α (TNFα) is a prominent pro-inflammatory cytokine involved in liver inflammation and sustained liver fibrosis. TNFα is a pleiotropic cytokine produced by the immune cells including macrophages that trigger multiple signaling pathways such as inflammation, proliferation, and apoptosis [1]. Liver fibrosis is a wound healing process in liver injury induced by various aetiologies, and characterized by the increased net deposition of extracellular matrix (ECM) proteins such as collagen I [24]. Activated HSCs are the key source of ECM deposition, and are responsible for fibrosis progression in the injured liver. In the homeostatic liver, HSCs are present in the space of disse as non-fibrogenic, quiescent cells, called as quiescent HSCs (qHSCs) [7]. During liver injury, they become fibrogenic and activated, called as activated HSCs, and ultimately transdifferentiate into myofibroblasts with fibroblast cell-like features. [8]. Persistent hepatocyte death results in the progression of liver fibrosis. Extensive fibrosis contributes to the development of end-stage liver disease, cirrhosis being the major determinant of severe complications, such as liver failure, portal hypertension, and liver cancer in chronic liver disease patients. Nevertheless, effective therapy for fibrosis or cirrhosis has not yet been established; liver transplantation (LT) is the only radical treatment for severe cases [25]. However, LT becomes challenging by the extreme disparity between the need for liver transplants and the shortage of donor organs. Therefore, there is an urgent requirement for an alternative anti-fibrotic therapy that inhibits the progression of hepatic fibrosis.

HSC activation consists of two major phases i.e., initiation (pre-inflammatory) and perpetuation (continuation) phase. In the initiation phase, qHSCs respond to injury-induced paracrine cytokines [26]. qHSCs start to gradually lose lipid droplets a characteristic feature of HSC activation in injured liver. Activated HSCs begin to express fibrogenic activation makers, α-smooth muscle actin (αSMA), and collagen I [27]. However, in the overlapping perpetuation phase, HSCs increase the survival signaling and become proliferative. It was reported that TNFα cytokine is responsible for the transdifferentiation of activated HSCs into myofibroblasts that persist the liver fibrosis progression [28]. Therefore targeting the mediator proteins that participate in HSC transdifferentiation or maintaining cell viability can...
be an effective strategy in the development of anti-fibrotic therapy in liver fibrosis. cIAP1/2 proteins are best known for their involvement in cell survival, and proliferation by regulating the NF-κB pathway [12]. It was found that pro-inflammatory cytokines TNFα and interleukin-1 (IL-1) activated the NF-κB signaling pathways in HSCs and enhanced their survival and liver fibrosis progression [29]. However, the regulatory role of cIAP1 and cIAP2 proteins in TNFα induced NF-κB signaling pathways in HSCs is unclear.

Studies have highlighted the overexpression of cIAP1/2 in various cancers and their role in regulating the NF-κB pathway that promotes cancer cell survival. A similar mechanism of exploitation is found in Hepatitis B virus (HBV)-infected hepatocytes. HBV hijack the cIAP1/2 regulated NF-κB signaling in hepatocytes to enhance infected hepatocyte survival and disease progression [13, 16]. In our results, we found that cIAP2 expression was increased in activated HSCs in the presence of the most prominent pro-inflammatory TNFα cytokine. However, it’s role in HSC activation and survival has not been investigated. We found that TNFα cytokine increases the mRNA expression of cIAP2 along with fibrogenic activation markers, αSMA, vimentin, and collagen I. However, cIAP1 expression was not altered with the TNFα stimulation in HSCs. This observation supports the notion that cIAP1 and cIAP2 work in a cooperative manner and compensate for their function given that one’s expression is ablated [20, 21]. We used Birinapant, and BV6 SMAC (second mitochondrial-derived activator of caspase) mimetic drugs (cIAP1/2 inhibitor) to investigate the regulatory role of cIAP2 in TNFα induced NF-κB signaling in HSCs. TNFα stimulation did not induce cell death in HSCs and cells appeared normal and similar to uninduced control cells. We found that TNFα induced the p65 protein-dependent canonical NF-κB signaling in HSCs with no change in viability or proliferation of HSCs compared to non-stimulated control cells. BV6 SMAC mimetic treatment decreased the TNFα induced p65 protein expression, and canonical NF-κB signaling in HSCs which affected the HSC viability as well. Surprisingly, we found that treatment with Birinapant alone did not induce cell death in HSCs, even at high dose. Pre-stimulation with TNFα also sensitized HSCs towards Birinapant mediated cell death but contrary to BV6, cIAP2 ablation by Birinapant was unable to decrease TNFα induced NF-κB signaling in HSCs. However, both the SMAC mimetics induced the accumulation of NIK which is responsible for the activation of non-canonical NF-κB signaling in HSCs. This suggested that BV6 and Birinapant SMAC mimetics exhibit divergent effects on cell survival and TNFα induced NF-κB signaling in HSCs.

We also evaluated the effect of cIAP2 ablation on HSC viability after the treatment of BV6 or Birinapant in the presence of macrophage secretome. The rationale behind this investigation was to analyze the effect of macrophage on the modulation of cIAP2 regulated NF-κB signaling and survival of activated HSCs. Macrophages are the key players in fibrosis progression, and participate in HSC survival and fibrogenic activation via TNFα and TGFβ1 cytokines respectively. Our results showed that M1 polarized macrophages enhanced the NF-κB signaling whereas, both M0 and M2 polarized macrophages downregulated the NF-κB signaling pathway in activated HSCs. Furthermore, naive M0 macrophages and M2 polarized macrophages rescued the HSCs from BV6 mediated cell death. As Birinapant does not exhibit any cytotoxicity in LX2 cells in the absence of TNFα, therefore the effect of M2 secretome alone in increasing HSC’s resistance against Birinapant mediated cell death in-vitro was not validated. Further experiments in-vivo would reveal the potential of M2 macrophages in rescuing activated HSCs against Birinapant mediated cell death. However, TNFα secreted by the M1 polarized macrophages sensitized HSCs to both BV6 and Birinapant mediated cell death. These results highlight that macrophages are capable of modulating the cIAP2 mediated NF-κB signaling and survival in HSCs, and can be targeted in the development of anti-fibrotic therapy. However, the cumulative effect of other immune cells in cIAP2 mediated NF-κB signaling needs to be extensively studied for the development of SMAC mimetic based anti-fibrotic therapy.

The regulatory role of cIAP1 and cIAP2 in liver fibrosis is less investigated, although studies with TNF receptor 1 (TNFR1) knockout mice showed reduced liver fibrosis in carbon tetrachloride (CCL4) induced liver injury mice model [4]. In NASH patients, higher TNFα levels correlated with fibrosis progression [3]. TNFα alone is impotent in inducing cell death in hepatocytes and requires sensitization by α-galactosamine (GalN)/lipopolysaccharide (LPS), suggesting the involvement of intestinal dysbiosis in TNFα mediated hepatocyte cell death [5]. Similarly, in this study we found that TNFα alone was incompetent in inducing cell death in activated HSCs. Liver injury occurs predominantly through TNFR1 mediated signaling, whereas TNFR2 is involved in protective pathways in non-alcoholic fatty liver disease (NAFLD). It was found that loss of both cIAP1 and cIAP2 is embryonic lethal; however, mice lacking either cIAP1 or cIAP2 are normal [30, 31]. cIAP1 and cIAP2 consist of E3 ligase RING domain that possesses the ability to inhibit caspase-8 depended apoptosis and also activate the pro-survival NF-κB signaling [12]. Ablation of cIAP1 and/or cIAP2 by SMAC mimetics attenuates TNFα-mediated phosphorylation of IKKβ and IκBα, which blocks the degradation of IκBα that inhibits the NF-κB/p65 activation [32, 33]. Consequently, cells missing cIAP1 and/or cIAP2 are sensitized to caspase 8 dependent apoptosis [14, 15]. Decreased NF-κB activity in hepatocytes causes hepatocellular injury, however, its inhibition in Kupffer cells and HSCs decrease liver fibrosis, suggesting the need for cell-specific targeting
in the development of anti-fibrotic therapy [34]. In HSCs, NF-κB inhibitors such as sulfasalazine (angiotensin II inhibitors) and NF-κB inhibitory NEMO-binding peptides promote resolution of fibrosis via affecting HSC viability [17, 18]. Inhibition of NF-κB also has the potential to modulate macrophage plasticity via affecting various chemokines and cytokine secretion in the fibrotic liver [19]. Further studies based on targeting cIAP2 and the modulation of NF-κB signaling in activated HSCs in the fibrotic liver could provide a mechanistic base for the development of novel anti-fibrotic therapeutic approach in chronic liver disease.

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Author contributions SMG and SS together designed the experiments, performed most of the biochemical and cellular experiments, acquired, analyzed, and interpreted the data with statistical validation. SG carried out ELISA, and critical revision of the manuscript. SB was involved in study concept and design, acquisition, analysis, and interpretation of data; drafting of the manuscript, critical revision of the manuscript for intellectual content.

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Data availability The data acquired and analyzed in the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical approval Not applicable.

Consent to participate Not applicable.

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