Divergent Effect of Birinapant, BV6 SMAC Mimetic on TNF α induced NF- κ B Signaling and Cell Viability in Activated Hepatic Stellate Cell

Sachin Sharma

Amity University

Shaikh Maryam Ghufra

Amity University

Sampa Ghose

All India Institute of Medical Sciences

Subhrajit Biswas (✉ sbiswas2@amity.edu)

Amity University <https://orcid.org/0000-0001-5649-364X>

Research Article

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

Posted Date: November 12th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1060020/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

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

Methods and Results: Activated HSCs treated with cIAP1/2 inhibitor SMAC mimetic BV6 and Birinapant in presence of TNF α and macrophage secretome. TNF α cytokine increased cIAP2 expression in activated HSCs and enhanced cell viability through canonical NF- κ B signaling in activated HSCs. cIAP2 inhibition via BV6 decreased the TNF α induced canonical NF- κ B signaling and reduced cell viability in activated HSCs. SMAC mimetic, Birinapant alone did not affect the cell viability and was incompetent in inducing cell death. However, pre-treatment of TNF α sensitized HSCs and Birinapant was capable in inducing cell death. Birinapant mediated cIAP2 ablation was unable to decrease the TNF α induced canonical NF- κ B signaling in comparison to BV6. Secreted TNF α from M1 polarized macrophages sensitized activated HSCs to BV6 or Birinapant mediated cell death. However, M2 polarized macrophages rescued the activated HSCs from BV6 and Birinapant mediated cytotoxicity. **Conclusions:** In this study, we describe regulatory role of cIAP2 in TNF α induced NF- κ B signaling in activated HSCs. Targeting cIAP2 may be a promising approach for liver fibrosis treatment via modulating NF- κ B signaling in activated HSCs.

Introduction

Tumor necrosis factor- α (TNF α) is a pleiotropic cytokine predominantly produced by macrophages that trigger multiple signaling pathways involved in inflammation, proliferation, and apoptosis (1). TNF α 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 TNF α levels correlated with insulin resistance and fibrosis stage (3). TNF α cytokine activates the quiescent hepatic stellate cell (HSC) into fibrogenic myofibroblasts. And knockout of the receptor TNF receptor 1 (TNFR1) for TNF α showed reduced liver fibrosis (4). Interestingly, TNF α alone is incompetent to induce cell death in hepatocytes and requires sensitization by D-galactosamine (GalN)/lipopolysaccharide (LPS) during liver injury (5). TNF α and IL-1 contribute to NF- κ B-dependent HSC survival and promote liver fibrosis through its pro-survival effect (6). HSCs are prominent players of the fibrotic response, located in the space of Disse as non-fibrogenic, quiescent cells (qHSC) in 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 viability of HSCs (9). However, in the resolution phase of fibrosis, activated HSCs are cleared by apoptosis (10, 11).

Mechanistically, the binding of TNF α to its receptor, TNFR1 leads to protein complex formation with inhibitor of apoptosis 1/2 (cIAP1 and cIAP2) that induces the NF κ B pathway activation (12). Various cancers overexpress cIAP1/2 and their targeting induces TNF α -driven apoptosis implying potential anti-

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

The regulatory role of cIAP1/2 in the modulation of NF- κ B signaling in activated HSCs, and consequently liver fibrosis progression is largely unknown. In this study, we found that TNF α induces NF- κ B 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 effect on TNF α induced NF- κ B signaling in activated HSCs. These results support that modulation of TNF α induced NF- κ B signaling through targeting cIAP2 may provide a novel anti-fibrotic therapeutic approach in liver disease.

Materials And Methods

Chemical reagents and recombinant protein

Reagents used in the study are as follows: MTT (3-(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 TNF α , (#300-01A, peprotech, USA).

Human HSC cell line culture

Activated human hepatic stellate cell line LX2 was a gift from Dr. Scott L Friedman; cultured in DMEM supplemented with 4 mM/L L-glutamine, 100 IU/ml Penicillin/100 μ g/ml Streptomycin, and 2 % (v/v) FBS (#RM1112, himedia, India) at 37 $^{\circ}$ C with 5% CO $_2$ in a CO $_2$ incubator. Cells were 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 μ L DMEM with 2% FBS. After 24 h, the medium was carefully removed. Cells were treated according to experimental conditions and incubated for 24 hours. After incubation for 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 minutes 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 monocytic 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 hours followed by incubation in fresh PMA free complete medium for another 48 hours. These differentiated macrophages were called M0 macrophages. For M1-like polarization, we incubated M0 macrophages with lipopolysaccharide (LPS; #L4391, sigma-aldrich, USA) and INF γ , (#300-02, peprotech, USA) 20 ng/ml each for 24 hours; 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 cytokine gene expression using RT-qPCR. Macrophages were cultured in RMPI with 1% (v/v) FBS for 24 hours to collect conditioned media.

TNF α cytokine ELISA

Macrophage conditioned media was collected and the concentration of TNF α cytokine in the conditioned media was measured using TNF α 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 Stand cDNA synthesis kit (#K1622, thermo scientific, USA) using random primers. Quantitative PCR was performed using syber fluorochrome master mix (#A25742, applied biosystems, USA) on StepOnePlus PCR System (#4376600, applied biosystems, USA) using gene specific primers (Suppl. 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 and protein samples were prepared with lamelli buffer and electrophoresed on SDS-PAGE gels followed by transfer onto PVDF membrane. The membranes were incubated in primary and secondary antibodies according to manufacturer instructions (Suppl. 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 $\pm$ 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 ANOVA using Prism-GraphPad. P values < 0.05 were considered significant for all the tests..

Results

TNF α cytokine induces cIAP2 expression in activated HSCs

The pro-inflammatory cytokine TNF α is elevated in hepatic injury and contributes to liver fibrosis progression. It participates in NF- κ B signaling and cell death in a context-dependent manner (12). To determine the regulatory role of cIAP1/2 in TNF α induced NF- κ B signaling, we stimulated human activated HSC cell line i.e., LX2 cells with TNF α (20 ng/ml) and analyzed gene expression alterations. TNF α (20 ng/ml) stimulation increased the mRNA expression of cIAP2, fibrogenic marker, α SMA, vimentin (VIM), and collagen I (COL1A1) genes in activated HSCs. However, cIAP1 expression did not increase with TNF α stimulation (Fig 1). Our results support the evidence that cIAP1 or cIAP2 compensate for expression and function (20, 21). These results suggest that pro-inflammatory TNF α cytokine increases cIAP2 expression along-with activation of HSCs.

TNF α sensitizes activated HSCs towards BV6 and Birinapant mediated cell death

To determine the regulatory role of cIAP2 in TNF α induced NF- κ B signaling in activated HSCs and the effect of their ablation on cell viability, we treated activated human HSC cell line LX2 with TNF α and various dose of BV6 or Birinapant SMAC mimetics. Both BV6 and Birinapant were capable in inhibiting cIAP2 expression in activated HSCs (22). Pre-stimulation of HSCs with 20 ng/ml of TNF α for 1 hour followed by treatment with different dose of BV6 or Birinapant for 24 hours. In results, TNF α alone

stimulation did not alter cell viability and showed $89.7 \pm 5\%$ viable cells in comparison to non-stimulated control cells. BV6 treatment decreased viability of HSCs in a dose-dependent manner and $2.876 \pm 1.268 \mu\text{M}$ was observed as the IC50 value of BV6 in LX2 cells at 24 hours of treatment. However, TNF α pre-stimulation increased sensitization of HSCs towards BV6 mediated cell death and decreased IC50 value to $1.410 \pm 0.923 \mu\text{M}$ dose at 24 hours of treatment (Fig 2A).

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

cIAP2 regulates TNF α induced canonical NF- κ B signaling and its ablation induces non-canonical NF- κ B signaling in activated HSCs

cIAP2 has a modulatory role in TNF α induced NF- κ B signaling pathway and affects cell survival (16). To determine the modulation of TNF α induced NF- κ B signaling with BV6 or Birinapant treatment in HSCs, we performed immunoblot analysis. TNF α stimulation increased cIAP2 expression in activated HSCs. TNF α induces canonical NF- κ B signaling through phosphorylated I κ B α and NF- κ B p65 mediator proteins. BV6 (5 μM) mediated cIAP2 ablation decreased phosphorylation of I κ B α and NF- κ B p65 protein expression, that indicate suppression of canonical NF- κ B signaling. Interestingly, cIAP2 ablation induces NIK accumulation which is responsible for the activation of non-canonical NF- κ B signaling (Fig 3A). However, Birinapant (50 nM) mediated cIAP2 ablation was unable to decrease the TNF α induced NF- κ B signaling in HSCs. However, Birinapant upregulated the NIK accumulation dependent non-canonical NF- κ B signaling in HSCs (Fig 3B). These results suggest that BV6 and Birinapant are both inhibitors of cIAP2 but have different modes of action. While BV6 has the potential to modulate TNF α -induced NF- κ B signaling and induce cell death in HSCs, Birinapant induces cell death without affecting the TNF α -induced NF- κ B signaling.

M1 polarized macrophages secrete higher TNF α than M2 polarized macrophage

In injured liver, pro-inflammatory microenvironment leads to the accumulation of immune cells especially macrophages that play a vital role in HSC survivability and fibrosis progression. M1 polarized macrophages secrete pro-inflammatory cytokines that induced NF- κ B signaling promoting the survival of HSCs and infiltration of other immune cells from bone marrow (1). However, M2 polarized

macrophages secreted anti-inflammatory cytokines that induced the fibrogenic activation of HSCs (23). To investigate the effect of polarized macrophage secretome on BV6 or Birinapant mediated cell death in HSCs, we cultured human monocyte cell line THP1 and differentiated them into macrophages. We incubated THP1 cells with 50 ng/ml of Phorbol 12-myristate 13-acetate (PMA) for 24 hours, followed by 48 hours of PMA free incubation. It induces differentiation of human monocyte cells into macrophages termed as naive or M0 macrophage. Next, we incubated naive macrophages with 20 ng/ml of lipopolysaccharide (LPS)/interferon-gamma (IFN γ) and 20 ng/ml of interleukin-4 (IL4)/ interleukin-13 (IL13) for 24 hours 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 qPCR by analyzing the expression of different cytokines and surface marker genes. M0 macrophages showed higher expression of CD14, CD68 surface markers than monocytes. M1 polarized macrophages expressed high CD80, CD86 as well as pro-inflammatory cytokines such as TNF α , IL-1 β , and IL-6, whereas, M2 polarized macrophages exhibited high CD206, CD209, and anti-inflammatory cytokines, IL-10, FN1, and TGF- β 1 cytokines (Fig 4B, and 4C).

M1 macrophages expressed higher TNF α mRNA expression than M2 polarized macrophages. To further confirm, we quantified secreted TNF α cytokine in macrophage secretome through ELISA. It was found that M1 polarized macrophages secreted maximum TNF α cytokines compared to M0 and M2 macrophages (Fig 4D). This suggested M1 polarized macrophages secreted TNF α cytokine in their secretome.

TNF α secreted by M1 polarized macrophages sensitize HSCs to BV6/Birinapant mediated cell death

To determine the effect of polarized macrophage secretome on BV6 and Birinapant mediated cell death in HSCs, we cultured human activated HSC cells i.e, LX2 in presence of M0, M1, and M2 polarized macrophage conditioned media (CM) and further treated them with different dose of BV6 or Birinapant for 24 hours. We found that M1 polarized macrophage secreted TNF α sensitized HSCs to BV6 and Birinapant mediated cell death. The IC₅₀ of BV6 was elevated to $3.639 \pm 0.894 \mu\text{M}$ in the presence of M1 secretome compared to BV6 alone treated cells ($2.876 \pm 1.268 \mu\text{M}$). However, M0 secretome sensitizes HSCs to BV6 to a lesser extent with an IC₅₀ value at $10.58 \pm 2.234 \mu\text{M}$. Interestingly, M2 macrophages rescued HSCs from BV6 mediated cell death and showing a high IC₅₀ value ($76.55 \pm 5.34 \mu\text{M}$) of BV6 compared to BV6 alone treated cells (Fig 5A and 2A). Similar results were found with Birinapant treatment; TNF α secreted by M1 polarized macrophages sensitized HSCs towards Birinapant mediated cell death. Birinapant exhibited an IC₅₀ value of $90.939 \pm 1.964 \text{ nM}$ in presence of M1 polarized macrophage secretome in comparison to Birinapant alone treated cells (Fig 5B and 2B). However, M0 and M2 secretome completely abolished clAP2 ablation mediated cell death in HSCs and was unable to sensitize HSCs to Birinapant induced cytotoxicity. (Fig 5B). This suggested that M1 (pro-inflammatory) polarized macrophages increase HSC sensitization towards both BV6 and Birinapant SMAC mimetic mediated cell death. Contrarily, non-polarized or naive M0 macrophages, and M2 (anti-inflammatory)

polarized macrophages provide resistance against BV6 and Birinapant SMAC mimetic induced cell death in activated HSCs. This results suggest that macrophages modulate cIAP2 mediated NF- κ B survival signaling in HSCs that 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 (TNF α) is a prominent inflammatory cytokine involved in liver inflammation and sustained liver fibrosis. TNF α is a pleiotropic cytokine produced by immune cells including macrophages that triggers multiple signaling pathways involved in inflammation, proliferation, and apoptosis (1). Liver fibrosis is a wound healing process in liver injury induced by various aetiologies, and characterized by 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 cells that contain fibroblast-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 anti-fibrotic therapy that inhibits the progression of hepatic fibrosis.

HSC activation consists of two major phases- initiation (pre-inflammatory) and perpetuation (continuation). In the initiation phase, qHSCs respond to injury-induced paracrine cytokines (26). qHSCs start to gradually lose lipid droplets and become activated, a characteristic feature of HSC activation in injured liver. Activated HSC's begin to express fibrogenic activation makers, α -smooth muscle actin (α SMA), and collagen I (27). However, in the overlapping perpetuation phase, HSCs increase the survival signaling and become proliferative. It was reported that TNF α cytokine is responsible for the transdifferentiation of activated HSCs into myofibroblasts which persist the liver fibrosis progression (28). So, targeting novel 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 the one of the survival factors best known for involvement in cell survival, and proliferation through regulating NF- κ B pathway (12). It was found that pro-inflammatory cytokine TNF α and interleukin-1 (IL-1) activated NF- κ B signaling pathways in HSCs enhanced their survival and resulted in liver fibrosis progression (29). However, the regulatory role of cIAP1 and cIAP2 proteins in TNF α induced NF- κ B signaling pathways in HSCs is unclear.

Studies have highlighted the overexpression of cIAP1/2 in various cancers and their role in regulating the NF- κ B 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- κ B signaling in hepatocytes to enhance infected hepatocyte survival and correlate with disease progression (13, 16). In our results, we found that cIAP2 expression was increased in activated HSCs in the presence of most prominent pro-inflammatory TNF α cytokine. However, their role in HSC activation and cell survival has not been investigated. We found that TNF α cytokine increases cIAP2 expression along with fibrogenic activation marker α SMA, vimentin, and collagen I gene expression. However, cIAP1 expression was not altered with the TNF α stimulation in HSCs. This supported the previous evidence 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, BV6 SMAC (second mitochondrial-derived activator of caspase) mimetic drug (cIAP1/2 inhibitor) to investigate the regulatory role of cIAP2 in TNF α induced NF- κ B signaling in HSCs. TNF α stimulation did not induce cell death in HSCs and cells appeared normal and similar to control cells. We found that TNF α induces p65 protein-dependent canonical NF- κ B signaling in HSCs that enhances HSC's viability similar to non-stimulated control cells. BV6 SMAC mimetic treatment decreased the TNF α induced p65 expression, and canonical NF- κ B signaling in HSCs which ultimately affected the HSC viability. Surprisingly, we found that treatment with Birinapant alone did not induce cell death in HSCs, even at a higher dose. However, pre-stimulation of TNF α sensitizes HSCs towards Birinapant mediated cell death by ablating cIAP2 expression. Contrarily, cIAP2 ablation by Birinapant was unable to decrease TNF α induced NF- κ B signaling in HSCs. However, both the SMAC mimetics accumulated NIK that was responsible for activation of non-canonical NF- κ B signaling in HSCs. This suggested that BV6 and Birinapant SMAC mimetic exhibited divergent effects on HSC viability and TNF α induced NF- κ B signaling in HSCs.

Next, we also evaluated the effect of cIAP2 ablation on HSC viability after the treatment of BV6 or Birinapant in presence of macrophage secretome. The rationale of this investigation was to analyze the effect of macrophage on cIAP2 regulated NF- κ B signaling and viability of activated HSCs. Macrophages are the key players in fibrosis progression, and participate in HSC survival or fibrogenic activation via TNF α and TGF β 1 cytokine respectively. In our study, we found that naive M0 macrophages and M2 polarized macrophages rescue the HSCs from BV6 and Birinapant mediated cell death. However, TNF α secreted by M1 polarized macrophages sensitized HSC's to BV6 or Birinapant mediated cell death. This investigation highlighted that macrophages are capable in modulating the cIAP2 mediated NF- κ B signaling in HSCs, and enhanced their survival that can be targeted in the development of anti-fibrotic therapy. However, the effect of immune cells in cIAP2 mediated NF- κ B signaling needs to be extensively studied in the development of SMAC mimetic as anti-fibrotic therapy.

The regulatory role of cIAP1 and cIAP2 in liver fibrosis is less investigated, although TNF receptor 1 (TNFR1) knockout mice showed a promising effect in the reduction of liver fibrosis in a carbon tetrachloride (CCl₄) induced liver injury mice model (4) In NASH patients, higher TNF α levels correlated with fibrosis progression (3). TNF α alone also is impotent to induce cell death in hepatocytes and

requires sensitization by D-galactosamine (GalN)/lipopolysaccharide (LPS), suggesting the involvement of intestinal dysbiosis in TNF α mediated hepatocyte cell death (5). This report is similar to our own results where we found that TNF α 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 possess the ability to inhibit caspase-8-induced apoptosis and also activate the pro-survival NF- κ B signaling (12). However, their ablation causes activation of caspase 8 mediated apoptosis (14, 15). Decreased NF- κ B activity in hepatocytes causes hepatocellular injury, however, its inhibition in Kupffer cells and HSCs causes decreased liver fibrosis, suggesting the need for cell-specific targeting in the development of anti-fibrotic therapy (32). In HSCs, NF- κ B inhibitors such as sulfasalazine (angiotensin II inhibitors) and NF- κ B inhibitory NEMO-binding peptides promote resolution of fibrosis via affecting HSC viability (17, 18). Inhibition of NF- κ B also has the potential to modulate macrophage plasticity via affecting various chemokines and cytokine secretion in the fibrotic liver (19). Thus, targeting of cIAP2 in the modulation of NF- κ B in the fibrotic liver and activated HSCs could provide a novel anti-fibrotic therapeutic approach in chronic liver disease.

Abbreviations

TNF α : Tumor necrosis factor- α ; cIAP1/2: Cellular inhibitor of apoptosis-1/2; NF- κ B: Nuclear factor kappa B; HSC: Hepatic stellate cell; α SMA: α -Smooth muscle actin; qHSC: quiescent HSC; SMAC: Second mitochondria-derived activator of caspase; NASH: Non-alcoholic steatohepatitis

Declarations

Author Contributions:

SS designed the experiments, performed most of the biochemical and cellular experiments, acquired, analyzed, and interpreted the data with statistical validation. SMG supported with experimentation especially cell line work. 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.

Acknowledgments

The authors would like to appreciate to their laboratory members, Bornika Roy, Basundhra Das, and Umesh Gautam for helpful and constructive discussion. The authors grateful to Dr. W. Selvamurthy, Prof BC Das for encouraging and supporting our research at Amity Institute of Molecular Medicine and Stem Cell Research (AIMMSCR), Amity University campus.

Funding

This work was supported by fellowships and grant supports from the Council of Scientific & Industrial Research (CSIR-09/915(006)/2017-EMRI to S. Sharma), University Grants Commission (UGC Ref no-581/CSIR-UGC-NET June 2017 to S.M. Ghufra), Department of Biotechnology (DBT-102/IFD/SAN/3003/2017-2018 to S. Biswas) and Science and Engineering Research Board (SERB) (CRG/2018/003918 to S. Biswas and SERB- YSS/2015/000092 to S. Ghose) Indian Council of Medical Research (ICMR-2019-1306/5CR/ADHOC-BMS to S. Biswas), India.

Data availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Conflicts of Interest

The authors declare that they have no competing interests.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

References

1. Yang YM, Seki E. TNF α in liver fibrosis. *Curr Pathobiol Rep*. 2015;3(4):253-61.
2. Osawa Y, Hoshi M, Yasuda I, Saibara T, Moriwaki H, Kozawa O. Tumor Necrosis Factor- α Promotes Cholestasis-Induced Liver Fibrosis in the Mouse through Tissue Inhibitor of Metalloproteinase-1 Production in Hepatic Stellate Cells. *PloS one*. 2013;8(6):e65251.
3. Crespo J, Cayón A, Fernández-Gil P, Hernández-Guerra M, Mayorga M, Domínguez-Díez A, et al. Gene expression of tumor necrosis factor alpha and TNF-receptors, p55 and p75, in nonalcoholic

- steatohepatitis patients. *Hepatology*. 2001;34(6):1158-63.
4. Sudo K, Yamada Y, Moriwaki H, Saito K, Seishima M. Lack of tumor necrosis factor receptor type 1 inhibits liver fibrosis induced by carbon tetrachloride in mice. *Cytokine*. 2005;29(5):236-44.
 5. Osawa Y, Kojika E, Hayashi Y, Kimura M, Nishikawa K, Yoshio S, et al. Tumor necrosis factor- α -mediated hepatocyte apoptosis stimulates fibrosis in the steatotic liver in mice. *Hepatology Communications*. 2018;2(4):407-20.
 6. Pradere J-P, Kluwe J, De Minicis S, Jiao J-J, Gwak G-Y, Dapito DH, et al. Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. *Hepatology*. 2013;58(4):1461-73.
 7. Iwaisako K, Jiang C, Zhang M, Cong M, Moore-Morris TJ, Park TJ, et al. Origin of myofibroblasts in the fibrotic liver in mice. *Proceedings of the National Academy of Sciences*. 2014;111(32):E3297-E305.
 8. Nishio T, Hu R, Koyama Y, Liang S, Rosenthal SB, Yamamoto G, et al. Activated hepatic stellate cells and portal fibroblasts contribute to cholestatic liver fibrosis in MDR2 knockout mice. *J Hepatol*. 2019;71(3):573-85.
 9. Ramachandran P, Pellicoro A, Vernon MA, Boulter L, Aucott RL, Ali A, et al. Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proceedings of the National Academy of Sciences*. 2012;109(46):E3186-E95.
 10. Kisseleva T, Cong M, Paik Y, Scholten D, Jiang C, Benner C, et al. Myofibroblasts revert to an inactive phenotype during regression of liver fibrosis. *Proceedings of the National Academy of Sciences*. 2012;109(24):9448-53.
 11. Sharma S, Ghufuran SM, Ghose S, Biswas S. Cytoplasmic vacuolation with endoplasmic reticulum stress directs sorafenib induced non-apoptotic cell death in hepatic stellate cells. 2021;11(1):3089.
 12. Sharma S, Kaufmann T, Biswas S. Impact of inhibitor of apoptosis proteins on immune modulation and inflammation. *Immunology & Cell Biology*. 2017;95(3):236-43.
 13. Fulda S, Vucic D. Targeting IAP proteins for therapeutic intervention in cancer. *Nature reviews Drug discovery*. 2012;11(2):109-24.
 14. Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P, et al. IAP antagonists induce autoubiquitination of c-IAPs, NF- κ B activation, and TNF α -dependent apoptosis. *Cell*. 2007;131(4):669-81.
 15. Vince JE, Wong WW-L, Khan N, Feltham R, Chau D, Ahmed AU, et al. IAP antagonists target cIAP1 to induce TNF α -dependent apoptosis. *Cell*. 2007;131(4):682-93.
 16. Ebert G, Allison C, Preston S, Cooney J, Toe JG, Stutz MD, et al. Eliminating hepatitis B by antagonizing cellular inhibitors of apoptosis. *Proceedings of the National Academy of Sciences*. 2015;112(18):5803-8.
 17. Oakley F, Teoh V, Ching ASG, Bataller R, Colmenero J, Jonsson JR, et al. Angiotensin II activates I kappaB kinase phosphorylation of RelA at Ser 536 to promote myofibroblast survival and liver fibrosis. *Gastroenterology*. 2009;136(7):2334-44.e1.

18. Oakley F, Meso M, Iredale JP, Green K, Marek CJ, Zhou X, et al. Inhibition of inhibitor of kappaB kinases stimulates hepatic stellate cell apoptosis and accelerated recovery from rat liver fibrosis. *Gastroenterology*. 2005;128(1):108-20.
19. De Minicis S, Seki E, Uchinami H, Kluwe J, Zhang Y, Brenner DA, et al. Gene Expression Profiles During Hepatic Stellate Cell Activation in Culture and In Vivo. *Gastroenterology*. 2007;132(5):1937-46.
20. Graber TE, Holcik M. Distinct roles for the cellular inhibitors of apoptosis proteins 1 and 2. *Cell Death & Disease*. 2011;2(3):e135-e.
21. Jin HS, Lee DH, Kim DH, Chung JH, Lee SJ, Lee TH. cIAP1, cIAP2, and XIAP act cooperatively via nonredundant pathways to regulate genotoxic stress-induced nuclear factor-kappaB activation. *Cancer Res*. 2009;69(5):1782-91.
22. Benetatos CA, Mitsuuchi Y, Burns JM, Neiman EM, Condon SM, Yu G, et al. Birinapant (TL32711), a bivalent SMAC mimetic, targets TRAF2-associated cIAPs, abrogates TNF-induced NF- κ B activation, and is active in patient-derived xenograft models. *Mol Cancer Ther*. 2014;13(4):867-79.
23. van der Heide D, Weiskirchen R, Bansal R. Therapeutic Targeting of Hepatic Macrophages for the Treatment of Liver Diseases. *Frontiers in immunology*. 2019;10(2852).
24. Parola M, Pinzani M. Liver fibrosis: Pathophysiology, pathogenetic targets and clinical issues. *Mol Aspects Med*. 2019;65:37-55.
25. Rudnick DA. Antifibrotic therapies in liver disease: Ready for primetime? *Clinical liver disease*. 2017;9(6):138-40.
26. An P, Wei L-L, Zhao S, Sverdlov DY, Vaid KA, Miyamoto M, et al. Hepatocyte mitochondria-derived danger signals directly activate hepatic stellate cells and drive progression of liver fibrosis. *Nature Communications*. 2020;11(1):2362.
27. Tsuchida T, Friedman SL. Mechanisms of hepatic stellate cell activation. *Nature Reviews Gastroenterology & Hepatology*. 2017;14(7):397-411.
28. Chang W-J, Song L-J, Yi T, Shen K-T, Wang H-S, Gao X-D, et al. Early activated hepatic stellate cell-derived molecules reverse acute hepatic injury. *World J Gastroenterol*. 2015;21(14):4184-94.
29. Pradere JP, Kluwe J, De Minicis S, Jiao JJ, Gwak GY, Dapito DH, et al. Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. *Hepatology*. 2013;58(4):1461-73.
30. Conte D, Holcik M, Lefebvre CA, LaCasse E, Picketts DJ, Wright KE, et al. Inhibitor of apoptosis protein cIAP2 is essential for lipopolysaccharide-induced macrophage survival. *Molecular and cellular biology*. 2006;26(2):699-708.
31. Moulin M, Anderton H, Voss AK, Thomas T, Wong WW-L, Bankovacki A, et al. IAPs limit activation of RIP kinases by TNF receptor 1 during development. *The EMBO Journal*. 2012;31(7):1679-91.
32. Son G, Iimuro Y, Seki E, Hirano T, Kaneda Y, Fujimoto J. Selective inactivation of NF- κ B in the liver using NF- κ B decoy suppresses CCl₄-induced liver injury and fibrosis. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 2007;293(3):G631-G9.

Figures

Fig. 1.

Figure 1

TNFα induces cIAP2 expression in activated hepatic stellate cells. (A) mRNA expression of cIAP1 and cIAP2 gene in TNFα (20 ng/ml) stimulated human HSC LX2 cells for 24 hours. (B) mRNA expression of fibrogenic activation marker αSMA, vimentin (VIM), and collagen I (COL1A1) gene in TNFα (20 ng/ml) stimulated human HSC LX2 cells for 24 hours. nsP>0.05, *P<0.05, **P<0.01, ***P<0.001 [two-way ANOVA for (A and B)].

Fig. 2.

Figure 2

TNF α pre-stimulation sensitizes HSCs to BV6 or Birinapant mediated cell death. (A) Human HSC LX2 cells were pre-stimulated with TNF α (20 ng/ml) HSC and treated with various BV6 concentrations for 24 hours. Cell viability examined through MTT viability assay. IC50 value calculated using non-linear regression. (B) Human HSC LX2 cells were pre-stimulated with TNF α (20 ng/ml) HSC and treated with various Birinapant concentrations for 24 hours. Cell viability examined through MTT viability assay. IC50 value calculated using non-linear regression. nsP>0.05, *P<0.05, **P<0.01, ***P<0.001 [one-way ANOVA for (A and B)].

Fig. 3.

Figure 3

clAP2 regulates TNF α induced canonical NF- κ B signaling and its ablation induces non-canonical NF- κ B signaling in HSCs. (A) Western blot expression analysis of clAP2, NF- κ B p65, plkB, and NIK after 24 hours of BV6 (5 μ M) treatment and pre-stimulated with TNF α (20 ng/ml) HSCs LX2 cells. (B) Western blot expression analysis of clAP2, NF- κ B p65, plkB, and NIK after 24 hours of Birinapant (50 nM) treatment and pre-stimulated with TNF α (20 ng/ml) HSCs LX2 cells. 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 [one-way ANOVA for (A and B)].

Fig. 4.

Figure 4

Polarization and characterization of macrophage. (A) Microscopic images and protocol of macrophage differentiation and polarization using human monocyte THP1 cell line (scale bar: 100 μ m, 10X magnification, with zoom image of 20X image). (B) Relative mRNA expression of macrophage surface markers using CD14, CD68 for M0 non-polarized macrophages. (C) Relative mRNA expression of CD80 and CD86 for M1 polarized -macrophages and CD206, CD209 for M2 polarized macrophages. Characterization of non-polarized and polarized macrophage by analysis of relative mRNA expression of TNF α , IL-1 β , IL-6, IL-10, FN1, and TGF- β 1 cytokine gene expression through quantitative PCR. (D) ELISA a of secreted TGF β 1 cytokine in CM of polarized macrophage at 24 hours. nsP>0.05, *P<0.05**P<0.01, ***P<0.001 [one-way ANOVA for (B, C, and D)].

Fig. 5.

Figure 5

M1 polarized macrophages secreted TNF α sensitize HSC to BV6/Birinapant mediated cell death. (A) Human HSC LX2 cells were cultured in M0, M1-polarized and M2-polarized macrophages conditional media and treated with various BV6 concentrations for 24 hours. Cell viability was examined through MTT viability assay. IC50 value calculated using non-linear regression. (B) Human HSC LX2 cells were cultured in M0, M1-polarized and M2-polarized macrophages conditional media and treated with various Birinapant concentrations for 24 hours. Cell viability was examined through MTT viability assay. IC50 value calculated using non-linear regression. nsP>0.05, *P<0.05, **P<0.01, ***P<0.001 [one-way ANOVA for (A and B)].

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplimentrymaterialsSMACcIAP2NFkb.pdf](#)