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Inhibition of HSP90 and activation of HSF1 diminishes macrophage NLRP3 inflammasome activity in alcoholic liver injury

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Abstract

Background: Activation of NLRP3 in liver macrophages contributes to alcoholic liver disease (ALD). Molecular chaperone heat shock protein (HSP) 90 facilitates NLRP3 inflammasome activity during infections and inflammatory diseases. We previously reported that HSP90 is induced in ALD and regulates pro-inflammatory cytokines, tumor necrosis factor alpha and IL-6. Whether HSP90 affects IL-1β and IL-18 regulated by NLRP3 inflammasome in ALD is unknown. Here, we hypothesize that HSP90 modulated NLRP3 inflammasome activity and affects IL-1β and IL-18 secretion in ALD.

Methods: Expression of HSP90AA1 and NLRP3 inflammasome genes was evaluated in human alcoholic livers and in mouse model of ALD. The importance of HSP90 on NLRP3 inflammasome activation in ALD was evaluated by administering HSP90 inhibitor, 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) to mice subjected to ALD and *in vitro* to bone marrow-derived macrophages (BMDM) stimulated with LPS and ATP. The effect of activation of HSF1/HSPA1A axis during HSP90 inhibition or direct activation during heat shock of BMDMs on NLRP3 activity and secretion of downstream cytokines was evaluated.

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Author contributions

AC, DB, and PM: designing research studies. AC, DB, AL, PO, EL: mouse and human liver experiments and data analysis. JA and RB: Human patient recruitment, RNA Sequencing and data analysis. AC, DB, and PM: manuscript writing. PM: project administration and funding acquisition.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Results: We found positive correlation between induction of HSP90 and NLRP3 inflammasome genes in human alcoholic cirrhotic livers. Administration of 17-DMAG in mouse model of ALD significantly downregulated NLRP3 inflammasome-mediated caspase-1 (CASP-1) activity and cytokine secretion, with reduction in ALD. 17-DMAG mediated decrease in NLRP3 was restricted to liver macrophages. Using BMDMs we show that inhibition of HSP90 prevented CASP-1 activity, and Gasdermin D (GSDMD) cleavage, important in release of active IL-1β and IL-18. Interestingly, activation of the heat shock factor 1 (HSF1)/HSPA1A axis, either during HSP90 inhibition or by heat shock, decreased NLRP3 inflammasome activity and reduced secretion of cytokines.

Conclusion: Our studies indicate that inhibition of HSP90 and activation of HSF1/HSPA1A reduces IL-1β and IL-18 via decrease in NLRP3/CASP-1 and GSDMD activity in ALD.

Keywords

Alcoholic liver disease; 17-DMAG; HSPA1A; Caspase-1; Gasdermin D

Introduction

Excessive alcohol consumption leads to a spectrum of liver injury such as fatty liver, steatohepatitis, fibrosis, and cirrhosis. Progression of alcoholic liver disease (ALD) to cirrhosis can lead to liver failure associated with mortality. A better understanding of ALD pathogenesis is important to develop effective therapies. Activation of innate immune pathways and elevated levels of pro-inflammatory chemokines and cytokines are characteristic features of ALD (Mandrekar et al., 2016). Activation of nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome in liver immune cells is crucial to the development of ALD (Petrasek et al., 2012). NLRP3 forms a complex with the adaptor molecule apoptosis-associated speck-like protein containing a CARD /PYCARD and permits the autocatalytic processing of pro-caspase (CASP) −1 to CASP-1, which then triggers the maturation of pro-interleukin (IL)-1β and pro-IL-18 to active IL-1β and IL-18, respectively. CASP-1 also cleaves Gasdermin D (GSDMD) to N-terminal product that triggers extracellular release of active IL-1β and IL-18 (He et al., 2016).

Heat shock protein (HSP) 90 is a molecular chaperone that maintains protein homeostasis by folding, activation, and stabilization of various client proteins involved in signal transduction pathways (Pearl, 2016). Studies from our group have shown the significance of proteostasis chaperones, HSP90 and HSP70/HSPA1A and transcription factor, heat shock factor 1 (HSF1) in alcohol-mediated effects on immune cells and liver injury (Muralidharan et al., 2014, Ambade et al., 2014). Recent studies have identified a link between HSP90 and NLRP3 in macular degeneration (Piippo et al., 2018), cerebrovascular disease (Zuo et al., 2018), and HSV infection (Li et al., 2019). Previous studies showed that the NLRP3-SGT1 (suppressor of the G2 allele of skp1) complex interacts with HSP90 and is important for inflammasome activation (Mayor et al., 2007). Whether inhibition of HSP90 in ALD has any impact on NLRP3 and downstream cytokines, IL-1β and IL-18 is not known. Here we investigate the effect of HSP90 inhibition on cytokines IL-1β and IL-18, regulated by NLRP3 inflammasome activity. We hypothesize that HSP90 is required for alcohol mediated

NLRP3 inflammasome activity and its inhibition results in reduced IL-1β and IL-18 secretion in ALD. We also investigated the contribution of HSF1/HSPA1A axis, induced during HSP90 inhibition on NLRP3 activity.

In this study, we show that expression of *HSP90AA1* (referred as HSP90 throughout the text) and NLRP3 inflammasome genes are upregulated in livers of human alcoholic hepatitis (AH) and alcoholic cirrhosis that positively correlated. Further, *in vivo* administration of specific HSP90 inhibitor, 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) decreased NLRP3 inflammasome activation in alcoholic liver macrophages. Our mechanistic studies using bone marrow derived macrophages (BMDM) as surrogate for liver macrophages, show that HSP90 inhibition contributes to inactivation of NLRP3 inflammasome, reduces CASP-1 activity, GSDMD cleavage and subsequent cytokine production. Interestingly, activation of HSF1/HSPA1A also contributes to decreased NLRP3 inflammasome activity and reduces cytokine production suggesting an anti-inflammatory effect. Overall our results suggest that inhibition of HSP90 and/or activation of HSF1 is an attractive strategy to reduce NLRP3 inflammasome activity and downstream IL-1β and IL-18 production in ALD.

Material and methods

Patients

The protocol was conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the University of North Carolina at Chapel Hill Internal Review Board. Only patients with signed informed consent were included. For RNA-sequencing experiments in AH livers, patients from the InTeam Human Biorepository Core with clinical, analytical, and histologic features of AH were included (Argemi et al., 2019). Patients with asymptomatic early stages of Alcoholic Steatohepatitis (ASH), patients with severe AH, and explants from AH patients who underwent early liver transplantation (Mathurin et al., 2011) were included, as previously described (Argemi et al., 2019). Liver biopsies were obtained using a transjugular approach. Patients with other liver diseases or malignancies were excluded from the study. These groups were compared with fragments of non-diseased human livers (Argemi et al., 2019). Biochemical profiles of these patients are described by Argemi et. al., 2019 and are summarized in Table 1. RNA extraction, RNA sequencing, and bioinformatic analysis was carried out as described before (Argemi et al., 2019).

Real time PCR was carried out on human alcoholic cirrhotic liver samples obtained from the Liver Tissue Cell Distribution System, Division of Pediatric Gastroenterology and Nutrition, University of Minnesota, Minneapolis, MN. Normal liver tissue was the non-involved surrounding tissue, obtained from patients undergoing partial hepatectomy for liver cancer. Biochemical profiles of these patients are described in Table 2.

RNA-sequencing and bioinformatic analysis

As described before (Argemi et al., 2019), high quality total RNA was sequenced using the Illumina HiSeq2000 platform. Libraries were built using Illumina TruSeq Stranded Total

RNA Ribo-Zero GOLD. Sequencing was paired-end (2×100bp) and multiplexed. Ninety-eight paired-end sequenced samples obtained an average of 36.9 million total reads with 32.5 million (88%) mapped to GRCh37/hg19 human reference. Short read alignment was performed using the STAR alignment algorithm with default parameters (Dobin et al., 2013). Normalization of gene expression level across samples was computed and illustrated as transcripts per million (TPM) mapping read. Sequencing read counts were processed by the voom function in limma (Ritchie et al., 2015) to convert them into log 2 counts per million (logCPM), with associated precision weights. Additional normalization methods using cyclic loess were performed in increasing order of normalization strength. Linear model fitting and differential expression analyses were carried out using fit linear models to genes, and the eBayes moderated *t*-statistic was used to assess differential expression.

Murine model of early alcoholic steatohepatitis

All mice received appropriate care in agreement with animal protocols approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. Animals received human care per the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health (NIH publication 86–23 revised 1985). NIAAA-Gao binge model was used to induce ALD (Bertola et al., 2013). Induction of ALD and administration of 17-DMAG was carried as described previously (Ambade et al., 2014). Eight to ten-week-old C57BL/6 female mice were fed liquid Lieber-DeCarli control diet (Bioserv, Frenchtown, NJ) for two days, before randomly being divided into ethanol-fed or pair-fed diet groups. Ethanol-fed mice were provided the Lieber-DeCarli ethanol diet (Bioserv), increasing from 1% to 5% (vol/vol) ethanol over the first five days of feeding. This group was maintained at 5% ethanol for ten additional days. Pair-fed mice were maintained on the liquid Lieber-DeCarli control diet throughout the feeding, matching the daily caloric intake of the ethanol group. The ethanol-fed mice were randomly subdivided into saline control and 17-DMAG treatment group. On day 11 of 5% alcohol, the treatment group of mice were intraperitoneally injected with 50mg/kg body weight of 17-DMAG, and the control group of mice were injected with saline. Three hours later, all ethanol-fed mice were gavaged with a single dose of ethanol (5g/kg body weight), and the pair-fed mice were gavaged with a calorically equivalent dextrin maltose. All mice were sacrificed 9 hours after ethanol gavage.

Isolation of liver cells

Hepatocytes and liver macrophages were isolated as per the protocol previously described (Ambade et al., 2014). Briefly, nine hours after gavage, mice were anesthetized with 100mg/kg ketamine and 10mg/kg xylazine and livers were perfused with saline for 5 minutes. Livers were then digested with 20mg/L Liberase for 5 minutes *in vivo*, followed by a 30 minutes digestion at 37°C *in vitro*. Hepatocytes were collected after 5-minute centrifugation at 300xg. The remaining liver portion was loaded onto the top of a 50%−25% percoll gradient and the interphase layer was washed and used as the macrophage population.

Primary mouse bone marrow-derived macrophage isolation and culture

Bone marrow collected from C57BL/6J mice was pipetted up and down to bring it to a single cell suspension and passed through a 70μm cell strainer. Red blood cells were lysed in ACK lysis buffer (Gibco, Grand Island, NY) and cells were plated in DMEM/F-12 GlutaMAX™ (Gibco) supplemented with 50ng/mL M-CSF, (PeproTech, Rocky Hill, NJ) on petri dish. On day 4, media was replenished with fresh DMEM containing 50ng/mL M-CSF. On day 6, cells were lifted from dishes using 1mM EDTA (Corning Inc., Manassas, VA) in PBS, and plated in 6-well culture plates at a density of 0.8 million cells per well. The following day fresh DMEM with 5ng/mL M-CSF was added and cells were stimulated as indicated with 1μg/mL lipopolysaccharide (LPS; Sigma), 5mM ATP (Invivogen, San Diego, CA), 1μM 17-DMAG, or heat shock for the indicated times. RNA, culture supernatants, and whole cell lysates were collected for the indicated assays.

Biochemical assays and cytokine measurement

ALT levels in blood serum was determined using a liquid activity assay (Pointe Scientific, Canton, MI). The L-Type TG M kit (Wako Chemicals USA, Inc., Richmond, VA) was used to measure liver homogenate triglyceride levels. IL-1β and IL-18 protein levels were measured in whole liver and BMDM culture supernatants with the Mouse IL-1β/IL1F2 Quantikine ELISA kit (R&D Systems, Minneapolis, MD) and Mouse IL-18 ELISA kit (MBL, Nagoya, Japan) respectively. Caspase-1 activity was measured in whole liver lysates using a CASP-1/ICE Colorimetric Assay Kit (R&D Systems). All assays were performed according to manufacturer instructions.

Immunoblot Analysis

Forty micrograms of lysate were resolved on 12.5% polyacrylamide gel and electroblotted on PVDF membrane (Bio-Rad) for 1h at 100V. Membranes were blocked overnight with 5% milk, in 0.1% TBS-Tween-20 solution (Thermo Scientific, Wilmington, DE). Blots were incubated overnight with primary antibodies diluted in the blocking buffer. Antibodies used in the study are CASP-1 p10 (abcam, EPR16883), Gsdmd (abcam, EPR19828), HSP70 (Enzo, C92F3A-5), beta Actin (abcam, AC-15), and beta Tubulin (Abcam). Membranes were incubated with the corresponding horseradish peroxidase-labeled secondary antibody (Abcam). Clarity Western ECL substrate (Biorad) was used for the detection of the bands. Bands were quantified by densitometric analysis using Fiji ImageJ 1.51d software.

RNA Extraction and RT-PCR

RNA was extracted using the RNeasy Mini Kit as per the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). On column DNase digestion (RNase-Free DNase Set, Qiagen) was performed and total RNA was quantified using a Nanodrop 2000 Spectrophotometer (Thermo Scientific). Complementary DNA was transcribed from the extracted RNA using the iScript Reverse Transcription Supermix for RT-qPCR (Biorad Laboratories, Hercules, CA) on a Mastercycler™ pro PCR System (Eppendorf, Hamburg, Germany). Real-time PCR was performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). Primers used for the study are listed in Supplementary Table 1. The comparative cycle threshold (Ct) method was used to determine relative gene

expression. Target gene expression level was normalized to 18S rRNA expression for each sample.

Statistical Analysis

Statistical significance was determined using the Student *t*-test for two groups, or nonparametric ANOVA followed by the Kruskal-Wallis test for multiple groups with GraphPad Prism 8 software.

RESULTS

HSP90 induction correlates with NLRP3 inflammasome components in human alcoholic hepatitis and alcoholic cirrhotic livers

We previously demonstrated that HSP90 is important in regulation of tumor necrosis factor alpha (TNFα) and IL-6 in alcoholic livers (Ambade et al., 2014). Here, we first evaluated whether the expression of HSP90 and NLRP3 inflammasome components are simultaneously induced in livers from patients with alcoholic hepatitis and alcoholic cirrhosis. RNA sequencing of liver was performed in a cohort of 3 patient populations i.e. early ASH, severe AH, and explants from AH patients undergoing liver transplantation and compared to that of normal livers. The biochemical and clinical characteristics of the patients are enlisted in Table 1. We observed significant increase in hepatic expression of *HSP90* in severe AH and explant AH whereas no significant induction was seen in early ASH compared to normal livers, as depicted by TPM (Figure 1A). Livers from severe AH and explant AH patients also demonstrated marked upregulation in inflammasome gene expression such as *NLRP3*, *pro-CASP-1*, and *PYCARD* as well as pro-inflammatory cytokine *pro-IL-1β* and *pro-IL-18* (Figure 1A). We found similar increase in an additional independent cohort of livers from alcoholic cirrhosis patients, that showed high levels of *HSP90, NLRP3*, *pro-CASP-1*, and *PYCARD*, and the cytokines *pro-IL-1β* and *pro-IL-18* by real-time PCR (Figure 1B). Correlation analysis using Pearson test, showed that the expression of *pro-CASP-1*, *PYCARD*, *pro-IL-1β*, and *pro-IL-18* positively correlated with *HSP90* (Figure 1C). These data suggest a possible link between human HSP90 and NLRP3 pathway genes in alcoholic liver injury.

Therapeutic targeting of HSP90 *in vivo* reduces IL-1β and IL-18 via disruption of NLRP3 inflammasome activity in alcoholic liver

Similar to our human studies, we found that murine livers also showed increase an in NLRP3 components and HSP90. Hepatic *Hsp90*, *Nlrp3*, and *pro-Il-1β* was significantly increased in alcoholic murine livers whereas *pro-Casp-1* and *Pycard* remained unchanged (Figure 2A). Next, we wanted to assess whether alcohol induced HSP90 is required for NLRP3 inflammasome activation in a murine model of ALD. For this, we employed the NIAAA-Gao binge model of ALD (Bertola et al., 2013) and administered a HSP90 inhibitor *in vivo* at the end of the alcohol feeding in a treatment regimen. Ethanol-fed mice were injected with 50mg/kg, body weight of 17-DMAG, 3h prior to the ethanol binge at the end of the chronic ethanol feeding. 17-DMAG treatment *in vivo* significantly decreased serum ALT and liver triglyceride levels without any effect on blood alcohol levels (Supplementary Figure 1A–C), similar to our previous data (Ambade et al., 2014). Bioavailability and

activity of 17-DMAG was confirmed by increased downstream gene, *Hspa1a* (Figure 2B) induced via HSF1 activation in alcoholic livers. Interestingly, treatment with 17-DMAG did not affect *pro-Il-1β* gene expression in the alcoholic livers (Figure 2C), whereas the protein level of IL-1β and IL-18 was significantly decreased in livers of these animals (Figure 2D and E). The expression of hepatic *Nlrp3* was also downregulated upon HSP90 inhibition in alcoholic livers (Figure 2F). Next, using western blotting, we observed that 17-DMAG treatment reduced cleaved CASP-1 p10 fragment (Figure 2G) as well CASP-1 activity (Figure 2H) in alcoholic livers.

We then assessed whether hepatocytes and/or liver macrophages alter NLRP3 components and downstream cytokine expression in response to 17-DMAG. While isolated alcoholic macrophages showed a significant increase in mRNA levels of *pro-Il-1β*, *pro-Il-18*, *Nlrp3*, and *pro-Casp-1* (Figure 3A–D), their expression was unchanged in alcoholic hepatocytes (data not shown). *In vivo* treatment with 17-DMAG predominantly reduced *pro-IL-1β*, *pro-IL-18*, and *Nlrp3* in liver macrophages from alcohol-fed mice (Figure 3A–C). In addition, *pro-Casp-1* and *Pycard* showed a trend to decrease in the *in vivo* 17-DMAG treated macrophages (Figure 3D and E). Bioavailability and activity of 17-DMAG in liver macrophages was confirmed by elevated expression of *Hspa1a* (Figure 3F). Taken together our data demonstrates that inhibition of HSP90 reduces NLRP3 inflammasome activity in alcoholic liver macrophages.

Inhibition of HSP90 impairs ATP/NLPR3 and Gasdermin D activity and reduces secretion of IL-1b and IL-18 in murine macrophages

Here, we assessed whether inhibition of HSP90 contributes to ATP mediated NLRP3 activity and downstream cytokines in murine macrophages. Since liver macrophage numbers are limited, we employed BMDMs from C57BL/6 mice and stimulated them with LPS, which acts as the priming signal to induce mRNA expression of NLRP3 components and cytokines, *pro-Il-1β* and *pro-Il-18*. Macrophages were treated with 17-DMAG at 1μM concentration *in vitro* for 6h in the presence or absence of LPS, based on our previous studies (Ambade et al., 2014). This concentration of 17-DMAG did not affect the viability of the cells as measured by the MTT assay (Supplementary Figure 2). We observed that 17-DMAG reduced LPS-induced expression of *Nlrp3, pro-Il-1β, and pro-Il-18 in vitro* in BMDMs (Figure 4A–C), in agreement with our *in vivo* 17-DMAG treatment studies. Next, we sought to delineate the importance of HSP90 in regulation of the second step of the NLRP3 signaling model of inflammasome activation and cytokine secretion. For this, we primed the BMDMs with LPS followed by stimulation with ATP as the second danger signal. ATP can serve as a danger associated molecular pattern (DAMP) in ALD (Petrasek et al., 2015). BMDMs were treated with 17-DMAG only during the activation step with ATP for 1h to assess its effect on NLRP3 activity and cytokines. Exposure to ATP significantly enhanced secretion of ATP mediated IL-1β and IL-18, whereas 17-DMAG markedly repressed the ATP-mediated release of IL-1β and IL-18 (Figure 4D and E). Next to assess NLRP3 inflammasome mediated proteolytic cleavage of pro-CASP-1 to active CASP-1, we performed western blot analysis of BMDM cell lysates and found that 17-DMAG significantly diminished ATP/NLRP3 mediated cleavage of pro-CASP-1 to CASP-1 p10 fragment in BMDMs (Figure 4F). These data were in accordance with the decreased

CASP-1 p10 fragment and CASP-1 activity in alcoholic livers treated with 17-DMAG *in vivo* (Figure 2G and H).

GSDMD, which is a substrate of CASP-1, is cleaved during NLRP3 inflammasome activation facilitating the secretion of IL-1β, independent of its role in pyroptotic cell death (Evavold et al., 2018, Heilig et al., 2018). We investigated whether HSP90 affects cleavage of GSDMD in macrophages. 17-DMAG treatment decreased proteolytic cleavage of the full length GSDMD to 32kDa pore-forming fragment (Figure 4G) suggesting that HSP90 is crucial in GSDMD mediated secretion of IL-1β. Taken together our data suggests that HSP90 is important in promoting ATP/NLRP3 function and pro-CASP-1 as well as GSDMD cleavage, leading to release of active inflammatory cytokines, IL-1β and IL-18 during ALD.

HSF1/HSPA1A axis contributes to inactivation of NLRP3 mediated inflammatory cytokines

Inhibition of HSP90 induces HSF1-mediated HSPA1A through a compensatory mechanism (Kim et al., 1999). HSF1 is a transcription factor that induces HSPA1A and can exert anti-inflammatory responses (Muralidharan et al., 2014). To elucidate whether the HSF1/HSPA1A axis induced during HSP90 inhibition contributes in reduction of NLRP3 inflammasome activity, we employed two different approaches. In the first approach, we isolated BMDMs from *Hsf1*$^{-/-}$ and wild-type (WT) littermates and stimulated with 17-DMAG. Induction of *Hspa1a* was observed in WT-BMDMs, whereas no inducible *Hspa1a* was observed in the *Hsf1*$^{-/-}$ BMDMs (Figure 5A), confirming lack of HSF1 induced HSPA1A expression. Having confirmed this, we challenged BMDMs with LPS and ATP in the presence or absence of 17-DMAG. *Hsf1*$^{-/-}$ macrophages showed slightly higher LPS-mediated IL-1β secretion compared to WT macrophages (Figure 5B). While 17-DMAG treatment led to a significant 71% reduction in ATP mediated IL-1β secretion in WT BMDMs, the *Hsf1*$^{-/-}$ BMDMs exhibited only 26% decrease (Figure 5B) suggesting significant contribution of HSF1/HSPA1A axis in DMAG-mediated inhibition of IL-1β.

Next, we induced direct activation of HSF1/HSPA1A axis by heat shocking BMDMs, independent of 17-DMAG, followed by LPS challenge. Heat shocking BMDMs induced HSF1-mediated HSPA1A (Supplementary Figure 3A) and dramatically blunted the LPS-mediated upregulation of *pro-Il-1β* and *Nlrp3* (Figure 5C and D). Subsequent challenge of BMDMs with LPS, followed by heat shocking at 42°C for 1h just prior to ATP addition and recovery for 1h (recovery period ensures induction of HSF1/HSPA1A axis; Supplementary Fig 3B) showed significant decrease in secretion of IL-1β (Figure 5E). Likewise, CASP-1 cleavage was diminished in heat shocked cells compared to controls (Figure 5F). Together, our data suggest that inhibitory effects of 17-DMAG on NLRP3 inflammasome signaling are also mediated by HSF1/HSPA1A axis.

Discussion

Activation of the NLRP3 inflammasome-mediated Caspase-1 cleavage and IL-1β production is well established in ALD (He et al., 2016). The role of stress-mediated protein homeostasis pathways and molecular chaperones including HSP90 and HSPA1A in alcohol-mediated TNFα and IL-6 has been reported by our group (Muralidharan et al., 2014, Ambade et al.,

2014). Here we report for the first time that molecular chaperone HSP90 plays an important role in regulation of NLRP3 inflammasome activity and downstream IL-1β and IL-18 secretion in ALD. Recent reports have identified various strategies to target NLRP3 including small molecule inhibitors and anti-oxidants to impair IL-1β production and inhibit liver injury (Mangan et al., 2018). Here we show that targeting HSP90 inhibits NLRP3, reduces CASP-1 and GSDMD cleavage in macrophages and prevents cellular secretion of IL-1β, relevant to ALD. Our data also uncover the importance of HSF1/HSPA1A axis in alleviation of NLRP3 activity and IL-1β and IL-18 production. Overall, we establish that pharmacological inhibition of HSP90 and/or activation of HSF1/HSPA1A reverses NLRP3 inflammasome activity and downstream cytokine secretion alleviating liver injury. Here we suggest another plausible novel treatment strategy to target NLRP3 activity in the liver in ALD (Figure 6).

Despite considerable efforts by NIAAA Alcoholic Hepatitis consortia and various investigators to test new or repurposed drugs in clinical trials, the current outcomes do not show significant clinical benefit (Lieber et al., 2018). HSP90 inhibitors have been in clinical development as anti-cancer agents for several decades. However, limitations in terms of predictive markers of anti-cancer activity and in some cases non-clinical reasons, have impeded their progress to FDA approval (Yuno et al., 2018). Currently, five N-terminal HSP90 inhibitors remain in clinical development to determine its impact on oncogenic growth (Yuno et al., 2018). Pre-clinical models using HSP90 inhibitors in neurodegenerative diseases (Pratt et al., 2015) and viral infections (Shatzer et al., 2017), show beneficial disease outcomes. Our studies suggest the potential for repurposing N-terminal HSP90 inhibitors such as 17-DMAG in ALD.

The induction of stress-mediated protein homeostasis chaperones in human alcoholic cirrhotic livers has been previously reported by our group (Ambade et al., 2014). Here we observe high levels of inducible form of *HSP90* in severe AH as well as explants from AH compared to normal liver samples. Interestingly, key inflammasome genes, *pro-CASP-1*, *PYCARD*, as well as *pro-IL-1β*, and *pro-IL-18* were upregulated in human alcoholic cirrhotic livers and correlated with *HSP90*, suggesting an intrinsic link between proteostasis chaperones and inflammasome activity. The HSP90 family of molecular chaperones regulates a wide range of clientele including protein kinases, transcription factors, and components of signal transduction pathways to maintain protein homeostasis (Pearl, 2016). In plant and mammalian cells, HSP90 is shown to associate and stabilize the NLR proteins through its interaction with SGT1, a ubiquitin ligase associated protein (Mayor et al., 2007). In addition, our group has previously demonstrated that HSP90 can promote pro-inflammatory cytokines and its inhibition *in vivo* can attenuate LPS- and alcohol-mediated inflammation and liver injury (Ambade et al., 2014). Data presented here further support that clinical targeting of HSP90 can diminish NLRP3 inflammasome activation and downstream cytokine secretion ameliorating ALD. Our data here show clinical relevance of HSP90 human alcoholic hepatitis and alcoholic cirrhosis livers.

Chronic alcohol exposure sensitizes liver macrophages to LPS via oxidative and cellular stress mediated mechanisms (Cohen et al., 2011, Muralidharan et al., 2014). Several studies have identified the link between cellular stress and NLRP3 activation in inflammatory

diseases (Abderrazak et al., 2015). Pathophysiological activation of NLRP3 and release of IL-1β involves a two-signal model (He et al., 2016). The first step involves priming of toll-like receptor (TLR) by pathogen-associated molecular patterns such as LPS, followed by a second danger signal commonly mediated by stress induced DAMPs such as ATP (Carta et al., 2015) that leads to inflammasome assembly and secretion of IL-1β and IL-18. Exposure of BMDM to 17-DMAG significantly decreased *pro-Il-1β*, *pro-Il-18*, and *Nlrp3* mRNA indicating that inhibition of HSP90 affects the first step mediated by TLR4 signaling. Interestingly *pro-IL-1β* mRNA level was not affected in 17-DMAG treated alcoholic livers whereas IL-1β protein was significantly reduced suggesting that HSP90 regulates NLRP3 activation to reduce IL-1β and IL-18 secretion in ALD. Using ATP as a second signal, we found that inhibition of HSP90 significantly decreased the secretion of IL-1β and IL-18 in BMDMs. The precursors pro-IL-1β and pro-IL-18 are converted to their respective biologically active forms by active serine protease CASP-1 (He et al., 2016). Activated CASP-1 mediates proteolytic cleavage of GSDMD, releasing the N-terminal pore-forming fragment and contributing to IL-1β release (Evavold et al., 2018). Inhibiting HSP90 prevented ATP mediated proteolytic cleavage of CASP-1 and GSDMD, indicating decreased formation of oligomeric GSDMD cell membrane pores and reduced IL-1β release.

Inhibition of HSP90 induces HSPA1A via activation of HSF1 (Kim et al., 1999). *In vivo* administration of 17-DMAG led to the induction of liver HSF1/HSPA1A as reported in our current and previous studies (Ambade et al., 2014). Direct activation of HSF1/HSPA1A axis in BMDMs by heat shock prior to priming with LPS, resulted in HSP90 independent ablation of *pro-Il-1β* transcript levels, indicating an inhibitory role for HSF1/HSPA1A in LPS induced cytokine induction, similar to our previous reports (Ambade et al., 2012). Interestingly, activation of the HSF1/HSPA1A axis prior to addition of ATP resulted in reduced NLRP3 activation and diminished CASP-1 cleavage and IL-1β secretion, suggesting direct impact of HSF1/HSPA1A on both priming and activation steps of IL-1β production. These data strongly supports the notion that HSF1/HSPA1A induced during HSP90 inhibition likely contributes to reduced NLRP3 activation and subsequent decrease in IL-1β. Studies have shown that HSF1 can repress transcription of *pro-Il-1β* through physical interaction with NF-IL6, an essential regulator of IL-1β transcription (Xie et al., 2002). In addition, heat shocking cells can prevent activation of pro-CASP-1 in BMDMs thereby decreasing IL-1β secretion (Levin et al., 2008). Our studies confirm an important contribution of the HSF1/HSPA1A axis during HSP90 inhibition in modulating NLRP3 inflammasome activity and IL-1β production. In fact, deficiency of HSF1, prevented HSP90 mediated inhibition in IL-1β secretion in LPS stimulated $Hsf1^{-/-}$ BMDMs compared to WT. Previous studies have also suggested a role for HSF1 in NLRP3 inactivation using knockout mice in a model of ischemia/reperfusion liver injury (Yue et al., 2016). Our data identifies the importance of HSF1/HSPA1A axis in inhibition of NLRP3 inflammasome activity during therapeutic treatment in ALD, suggesting its clinical potential in liver disease.

Elevated NLRP3 inflammasome activity and downstream , IL-1β and IL-18, are implicated in the pathogenesis of liver diseases including ALD, suggesting their potential as therapeutic targets (Tilg et al., 2016). Use of inhibitors to explicitly target NLRP3 activity, thereby allowing the modulation of secretion of these cytokines is attractive. Given the diversity of NLRP3 upstream activators, it is important to identify common pathways or molecules that

can directly inactivate/inhibit NLRP3 and downstream events, allowing the ease to modulate NLRP3 activity. Direct inhibition of HSP90 affects NLRP3 by reducing CASP-1 activation and subsequent IL-1β and IL-18 maturation and hence is an attractive target. Additionally, the impact of 17-DMAG to effectively ameliorate alcoholic liver injury, further demonstrates its clinical potential in ALD. Our current study strengthens our hypothesis that HSP90 is an attractive therapeutic target in ALD and its inhibition can attenuate NLRP3 inflammasome activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

17-DMAG	17-dimethylaminoethylamino-17-demethoxygeldanamycin
ALD	alcoholic liver disease
AH	alcoholic hepatitis
ASH	alcoholic steatohepatitis
BMDM	bone marrow-derived macrophages; Casp-1, Caspase-1
DAMP	danger associated molecular pattern
GSDMD	Gasdermin D
HSP	heat shock protein
HSF1	heat shock factor 1
IL	interleukin
LPS	lipopolysaccharide
NLRP3	nucleotide-binding domain leucine-rich-containing family pyrin domain-containing-3
TLR	toll-like receptor
TPM	transcripts per million

TNFα	tumor necrosis factor alpha
WT	wild type

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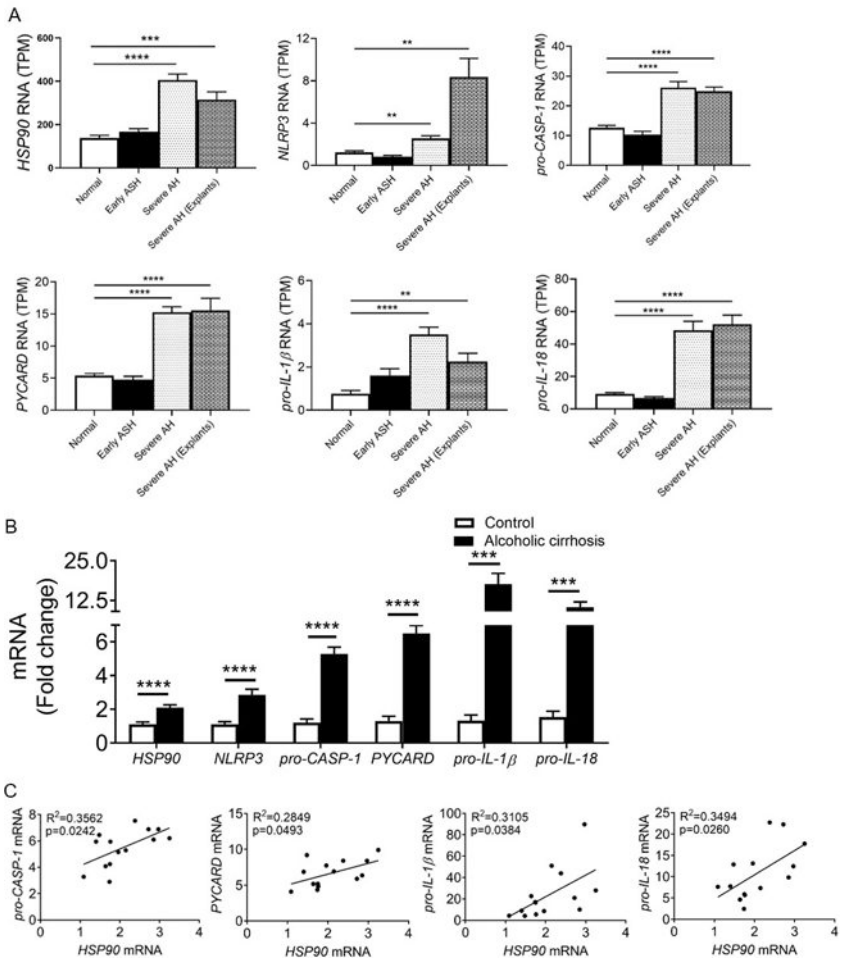

Figure 1. Induction and correlation between hepatic HSP90 and inflammasome components in patients with alcoholic hepatitis and alcoholic cirrhosis

A) Transcript levels of *HSP90*, *NLRP3*, *pro-CASP-1*, *PYCARD*, *pro-IL-1β*, and *pro-IL-18* evaluated by RNA sequencing in patients with normal livers (n=10), early ASH (n=12), severe AH (n=11), and severe AH (explant, n=18). (B) mRNA expression of *HSP90*, *NLRP3*, *pro-CASP-1*, *PYCARD*, *pro-IL-1β*, and *pro-IL-18* in patients with normal livers (controls; n=15) or alcoholic cirrhosis (n=18). (C) Correlation between the mRNA expression of *HSP90* with *pro-CASP-1*, *PYCARD*, *pro-IL-1β*, and *pro-IL-18* evaluated by the Pearsons test. Data are represented as mean ± SEM, ** p <0.01, ***p<0.001, **** p<0.0001. ASH, alcoholic steatohepatitis; AH, alcoholic hepatitis; TPM, transcripts per million.

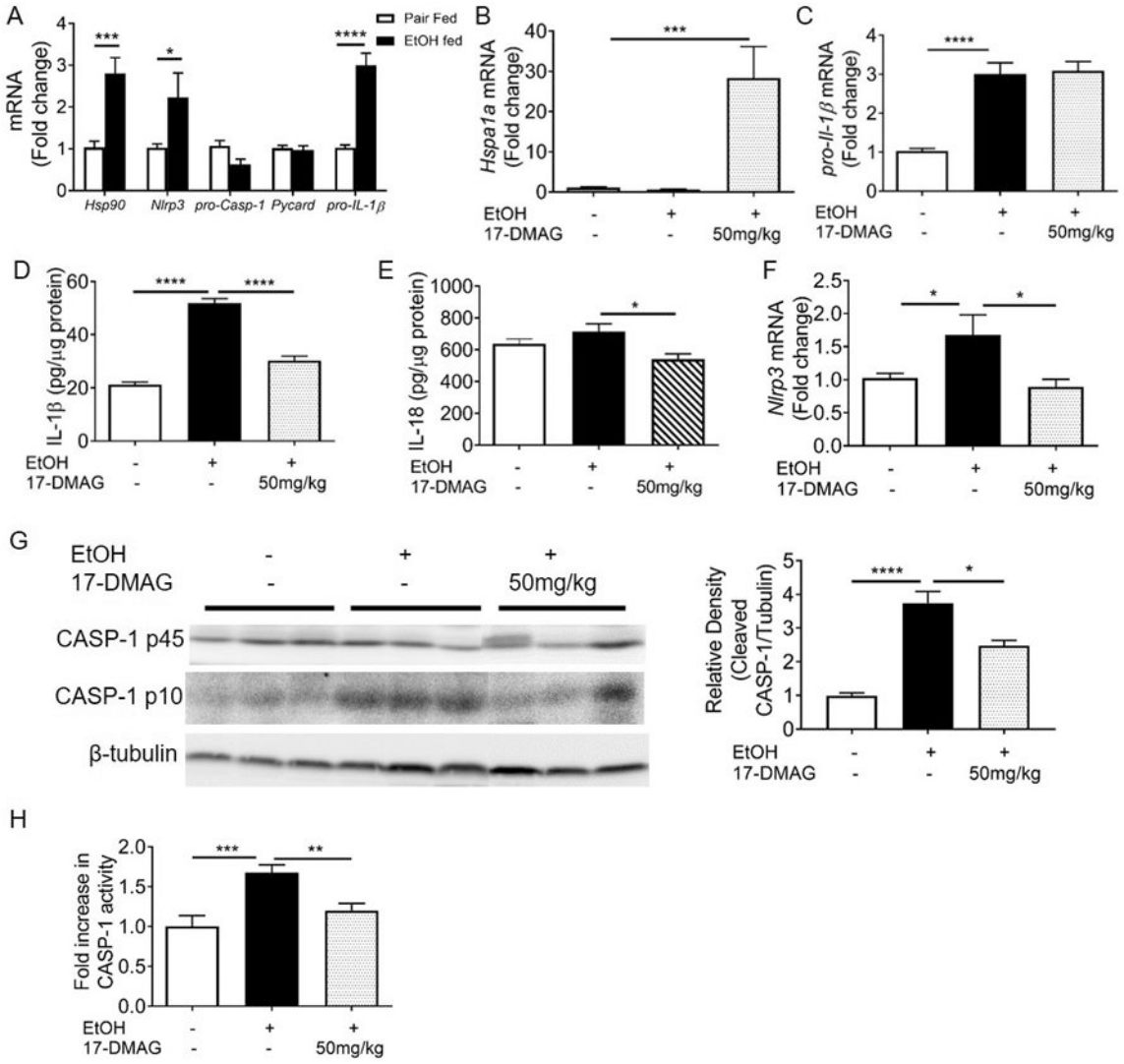

Figure 2. Therapeutic targeting of HSP90 alleviates NLRP3 activation and reduces IL-1β protein production in alcoholic livers.

(A) C57BL/6 mice were fed control (pair-fed) or Lieber-DeCarli alcohol (ethanol-fed) diet and received a single dose of alcohol binge. Hepatic expression of *Hsp90*, *Nlrp3*, *pro-Casp-1*, *Pycard*, and *pro-Il-1β* was evaluated by RT-PCR. (B-F) Mice were subjected to pair-fed or Lieber-DeCarli diet with alcohol and administered with 50mg/kg 17-DMAG at the end of the feeding. mRNA expression of *Hspa1a* (B) and *pro-Il-1β* (C), protein levels of IL-1β (D) and IL-18 (E), and mRNA expression of *Nlrp3* (F) was evaluated in the liver tissues (n=6–12). (G, H) Liver homogenates assessed for (G) Caspase-1 cleavage by western blot, and expression was quantitated and normalized to livers from pair-fed mice and (H) Caspase-1 activity was determined and normalized to pair-fed mice (n=8). Data are represented as mean ± SEM, * p <0.05, ** p <0.01, ***p<0.001, **** p<0.0001. EtOH, ethanol; DMAG, 17-Dimethylaminoethylamino-17-demethoxygeldanamycin.

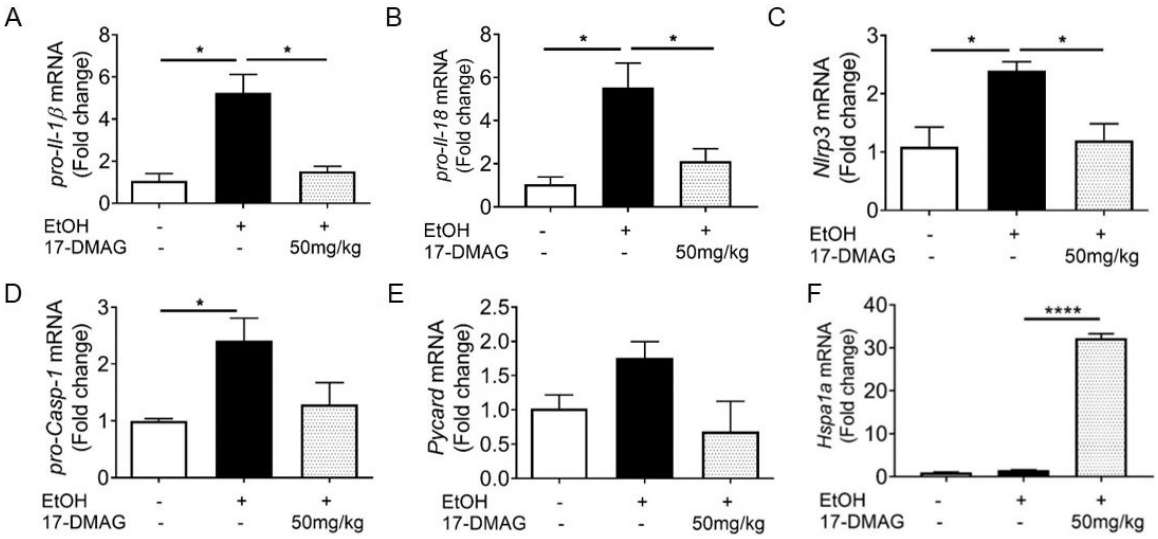

Figure 3: Inhibition of HSP90 reduces the expression of NLRP3 genes in alcoholic liver macrophages.

C57BL/6 mice were fed control (pair-fed) or Lieber-DeCarli alcohol (ethanol-fed) diet and received a single dose of alcohol binge. A group of mice were administered with 50mg/kg 17-DMAG at the end of the feeding. Hepatic macrophages isolated from these mice were assessed for the mRNA expression of *pro-Il-1β* (A), *pro-Il-18* (B), *Nlrp3* (C), *pro-Casp-1* (D), *Pycard* (E), and *Hspa1a* (F) (n=4). Error bars represent mean ± SEM. *p<0.05. EtOH, ethanol; DMAG, 17-Dimethylaminoethylamino-17-demethoxygeldanamycin.

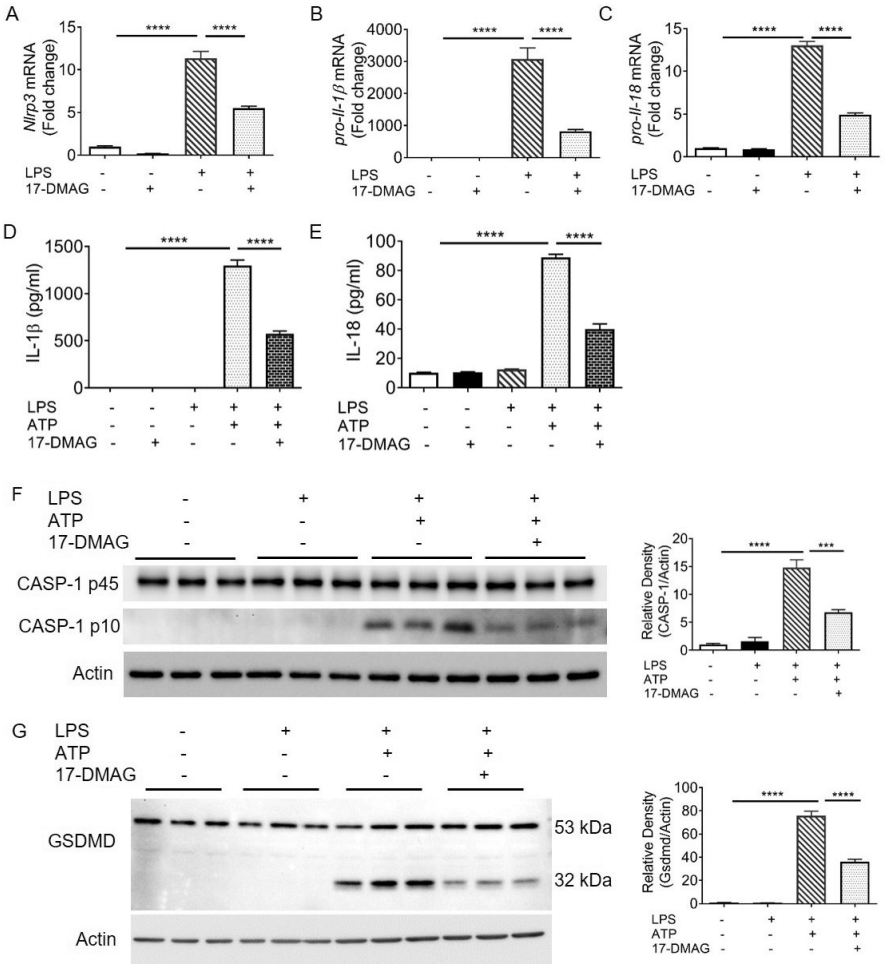

Figure 4. Inhibition of HSP90 decreases NLRP3 inflammasome activity in LPS-induced BMDMs.

(A, B) BMDMs were treated with 1μM 17-DMAG for 6h. LPS (1μg/mL) was added to indicated group for the final 3h. RNA was assessed for the expression of (A) *Nlrp3* and (B) *pro-Il-1β*. (C) For IL-18 induction, BMDMs were stimulated with 1μg/mL LPS for 8h. 1μM 17-DMAG was added for the final 6h and expression of *pro-Il-18* was assessed. (D, E) BMDMs were stimulated with LPS (1μg/mL) for 4h (D) and 8h (E). 5mM ATP was added to the indicated group of cells. 1μM 17-DMAG was added with ATP for the final 1h and release of IL-1β (D) and IL-18 (E) was assessed in the culture supernatants by ELISA (n=6). (F, G) BMDMs were stimulated with LPS (1μg/mL) for 4h and 5mM ATP with or without 1μM 17-DMAG was added to the indicated group of cells for 1h. Proteolysis of pro-CASP-1 to CASP-1 p10 fragment (F) and GSDMD cleavage (G) was evaluated by western blotting (n=6). Data are represented as mean ± SEM, ***p<0.001, **** p<0.0001. LPS, lipopolysaccharide; 17-Dimethylaminoethylamino-17-demethoxygeldanamycin

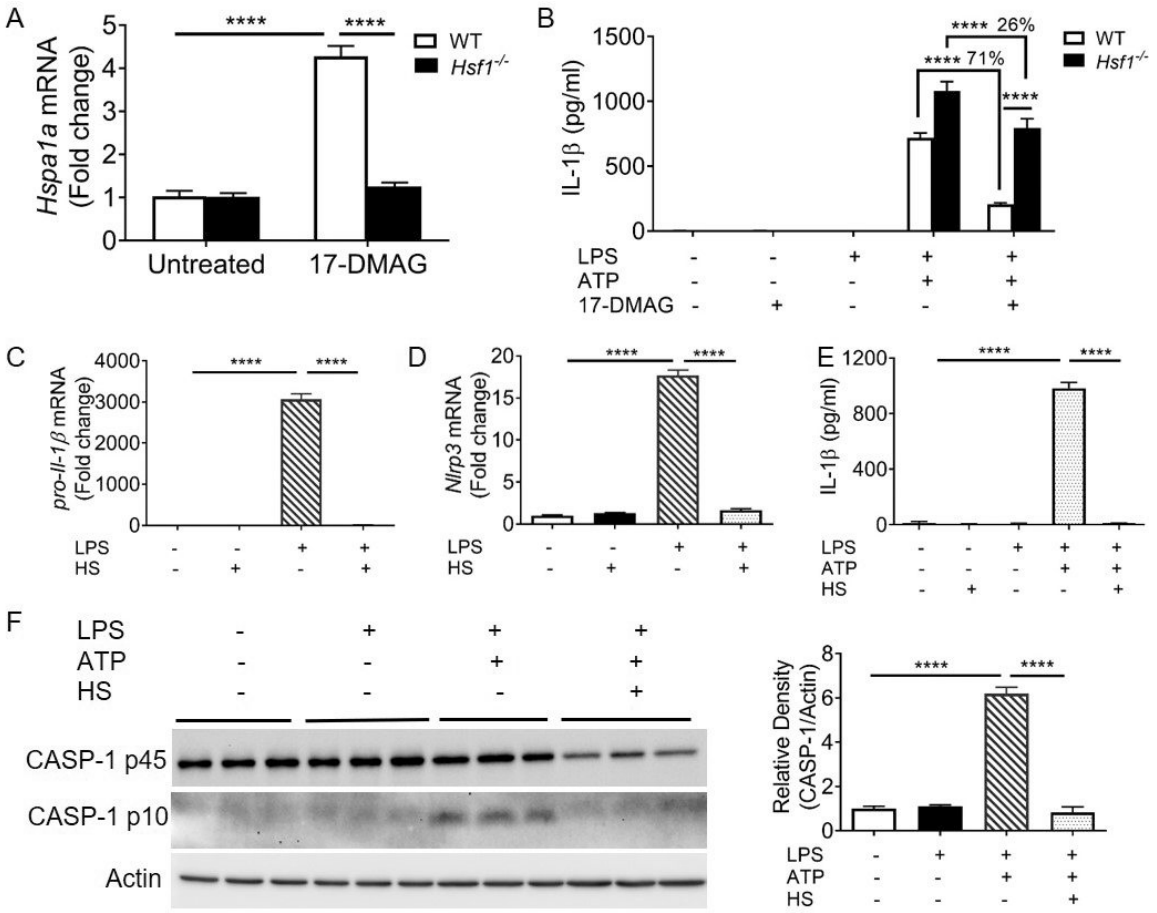

Figure 5. HSF1/HSPA1A axis contributes in decrease in LPS-mediated NLRP3 inflammasome signaling.

(A) WT and *Hsf1*$^{-/-}$ BMDMs were heat shocked at 42°C for 1h and expression of *Hspa1a* was assessed (n=6). (B) WT and *Hsf1*$^{-/-}$ BMDMs were stimulated with LPS (1μg/mL) for 4h and 5mM ATP was added to the indicated group of cells for 1h. 1μM 17-DMAG was added alongside ATP and release of IL-1β was evaluated by ELISA (n=6). (C, D) BMDMs were heat shocked at 42°C for 1h and stimulated with LPS (1μg/mL) for 2h. Real time PCR was carried out to evaluate the expression of C) pro-*Il-1β* and D) *Nlrp3*. (E, F) BMDMs were exposed to LPS (1μg/mL) for 4h before stimulation with 5mM ATP for 1h. Prior to ATP stimulation cells were heat shocked at 42°C for 1h and allowed to recover for 1h. (E) Culture supernatants were evaluated for secreted IL-1β. (F) Cell lysates were analyzed for Caspase-1 cleavage by western blotting. Data are represented as mean ± SEM, ***p<0.001, **** p<0.0001. WT, wild-type; LPS, lipopolysaccharide; HS, heat shock.

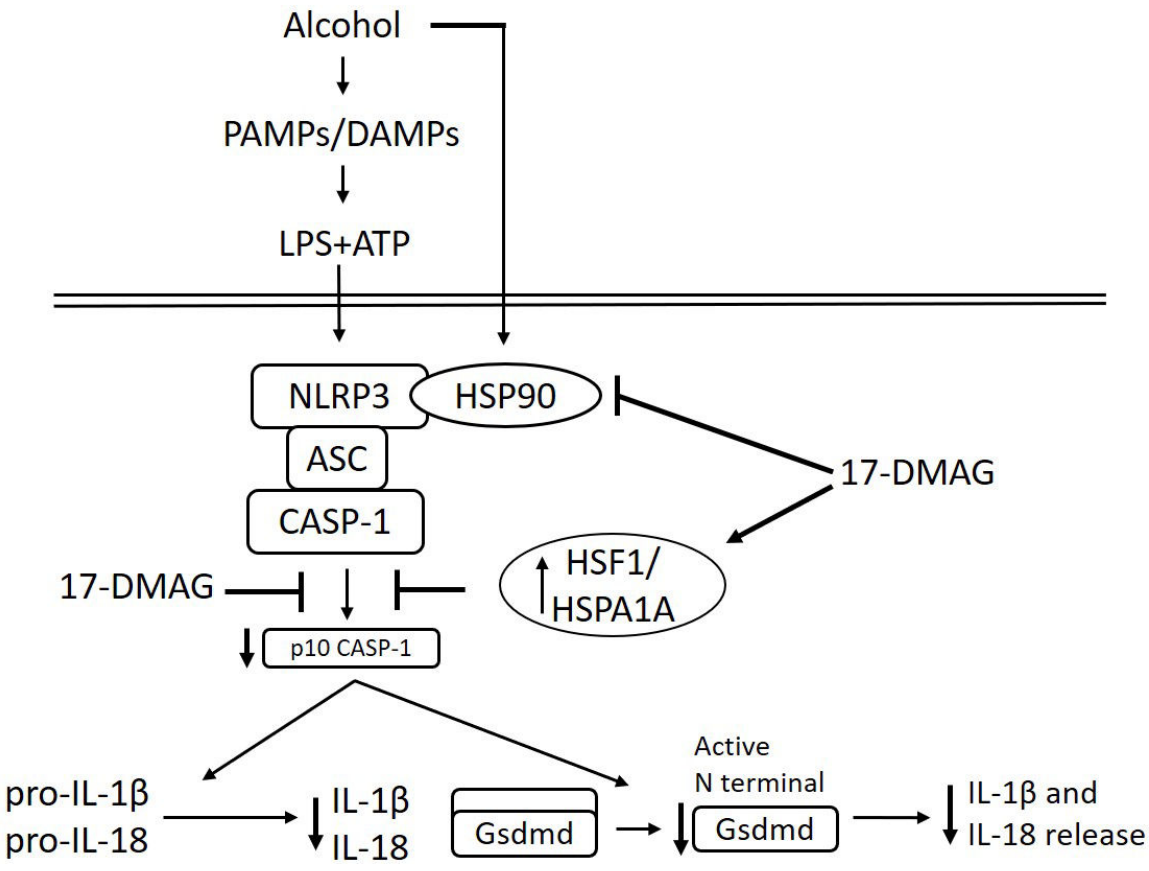

Figure 6:
Schematic representation demonstrating the effect of 17-DMAG in ameliorating NLRP3-mediated inflammasome activity.

Table 1:

Biochemical profiles of patients with alcoholic hepatitis and control patients included in the study.

	Normal livers	Early ASH	Severe AH	Severe AH (explants)
Age - median	32(28.8–50.7)	52(48.2–58.7)	51(47.2–57.7)	48.9(48–56)
Gender - male n (%)	7(70)	7(58.3)	11(61.1)	7(63.6)
AST (U/L)	21.5(18.8–26.5)	107(64–154)	114.5 (62.5–158.3)	170(131–279)
ALT (U/L)	25(14.8–34.3)	70.3(53.5–89.8)	32.5 (20–44)	N/D
Bilirubin mg/dL	0.6(0.5–0.7)	1.2(0.7–1.5)	19 (12.3–26.7)	16.3(11.1–24.3)
GGT (U/L)	17(13.5–24.8)	388(200–723)	406(165–721)	N/D
ALP (U/L)	147(106–191)	100(62–141)	386(147–491)	N/D
Albumin (g/dL)	4.6(4.3–4.6)	4.5(4.2–4.7)	2.9 (2.3–3.3)	2.4(2–3)
Creatinine mg/dL	0.8(0.74–0.9)	0.6(0.59–0.77)	0.79 (0.61–1)	0.69(0.53–0.73)

Table 2:

Biochemical profiles of patients with alcoholic cirrhosis and control patients included in the study.

	Controls (n=15)	**Alcoholic Cirrhosis (n=18)**
Age (years)	43.8 ± 19.9 (19–70)	53.8 ± 9.03 (38–65)
Gender (% male)	13/15 (86.6)	16/18 (88.8)
AST (IU/L)	-	89.1 ± 109.3 (31–492)
Bilirubin (mg/dL)	-	8.7 ± 7 (1.1–20.7)
Alkaline phosphatase (IU/L)	-	202.6 ± 102.9 (17.4–386)