

Serum amyloid A1 induces apoptosis and cell survival in hepatocytes following acute liver injury

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Short Report

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Abstract

Background Acute phase protein serum amyloid A (SAA) is one of abundantly expressed protein in the liver following injury or other traumas. However, the relative contribution during inflammatory response in the liver remains largely elusive.

Method SAA1 genetic knockdown was performed by injecting siRNA/invivolectamine3.0 complex in CCl₄-treated C57BL/6 mice. Parts of the excised liver were then processed for histological analyses. Primary human hepatocytes were isolated from surgical specimens, and evaluated for cell death, luciferase activity and chemokine production after recombinant human SAA1 treatment.

Results In a mild liver injury status, after hepatocyte specific knockdown of SAA1 in mice liver, we observed reduced number of apoptotic hepatocyte at non-necrotic area. *In vitro* cell death assay revealed that in primary human hepatocytes, SAA1 induced apoptosis and cell survival simultaneously. SAA1 induced activation of phosphatidylinositol 3-kinase (PI3K), Akt and nuclear factor κ B (NF- κ B) - dependent genes, which ultimately led to cell survival. In contrast, blockade of NF- κ B revealed hepatotoxic effects of SAA1 with appearance of apoptotic signatures such as caspase-3 and PARP cleavage products. SAA1-induced expression of pro-inflammatory genes such as MCP-1 and RANTES were inhibited when two survival signals NF- κ B and PI3K were pharmacologically blocked by treatment with ActD and LY294002, respectively.

Conclusion These results indicate that cytokine-like property of SAA1 resembles TNF- α signaling pathway in hepatocytes and triggers apoptosis and cell survival responding to injury.

Introduction

Hepatocyte death is the major component of the pathology of liver diseases, including alcoholic steatohepatitis (ASH), nonalcoholic steatohepatitis (NASH), ischemia and perfusion [1, 2]. A number of factors including, chemokines, cytokines, growth factors, intracellular pathways and receptors contribute to liver disease initiation and progression through hepatocytes death [3]. Hepatocyte death can cause liver inflammation, fibrogenesis and may ultimately induce cirrhosis [4]. Hepatocyte itself releases biological active mediators, particularly damage associated molecular patterns (DAMPs), are well characterized players of almost in all kind of liver diseases [5]. DAMPs induced inflammation typically occurs in response to various different noxae that cause tissue stress and injury [6] and, it may often lead to detrimental aggravation of initial insults in the liver [7]. Pathologically, excessive deposition of DAMPs may cause massive inflammation and, in turn, the continuous production of DAMPs by newly dying hepatocytes cells in response to inflammation may lead to a vicious cycle which ultimately culminates in the development of autoimmune disease(s) [8]. In addition, acute liver failure occurs when the extent of hepatocyte death exceeds the regenerative capacity of the liver [8]. Therefore, identifying indigenous molecules induce cell death and inflammation is one of a relevant strategy for therapeutic manipulation of liver disease pathogenesis.

Serum amyloid A (SAA), predominantly released from injured hepatocytes, represents an emerging example of DAMPs [9]. It is apoprotein that has been suggested to act as a cytokine and also serve as a prominent effector of inflammation in autoimmune diseases [10]. The relative contribution of SAA1 in liver inflammation and disease pathogenesis, however, remains ill-defined. Herein, we therefore studied the role of SAA1 in toxic CCl₄-induced liver injury mice model. We found that SAA1 induces apoptosis in hepatocytes observed in non-necrotic area of mice tissue sections. In addition, SAA1 induces apoptosis and cell survival pathways simultaneously in primary human hepatocytes in similar fashion with TNF- α .

Materials And Methods

Mice and liver injury

Six to eight weeks C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China). Mice were maintained on a 12hour-light/12hour-dark cycle with free access to food and water. All animal experiments were performed under ethical condition based on procedure approved by Guangzhou Providential Department of Sciences and Technology (ethical process No: N2014050). CCl₄ mediated liver injury was induced by single IP injection of 20 % CCl₄ diluted in olive oil or olive oil alone as vehicle.

In vivo knockdown of SAA1

For *in vivo* knockdown of SAA1, invivolectamine3.0-siRNA complex was prepared and injected in to C57BL/6 mice according to the manufacturer instruction (Life science technology). The detailed procedure is described in supplementary materials and methods section.

Histological analysis

Mice were sacrificed after 24 hr of CCl₄ injection and the livers were fixed in 4 % paraformaldehyde and embedded in paraffin. The detailed procedure is described in supplementary materials and methods section.

Cell isolation and culture

Tissues were obtained by qualified medical staff, with written donor consent and the approval of the Ethics Committee according to the Declaration of Helsinki. The detailed procedure is described in supplementary materials and methods section.

Determination of cell death

Cell death in hepatocytes was measured by LDH, Caspase 3- and PARP cleavage and measuring Caspase-3 activity. The detailed procedure is described in supplementary materials and methods section.

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Annexin V/propidium iodide (PI) staining

Cells were seeded into 6-well plates and cultured for 24 hr. The detailed procedure is described in supplementary materials and methods section.

Luciferase reporter gene assay

Primary human hepatocytes were transfected with 2 µg/well of reporter plasmid for nuclear factor pGL4.32[luc2P/NF-κB-RE/Hygro] Vector (Promega cat# E849A). The detailed procedure is described in supplementary materials and methods section.

Quantitative RT-PCR

Total RNA was extracted by using TRIzol reagent (Invitrogen) as described previously [11]. The detailed procedure is described in supplementary materials and methods section.

Western blot analysis

Protein extracted from primary human hepatocyte was run on 10 % SDS acrylamide gel. The detailed procedure is described in supplementary materials and methods section.

Statistical analysis

The results are presented as mean \pm S.E of at least three independent experiments. One-way ANOVA followed by Tukey's multiple comparisons test and two tailed student's *t* test was used to calculate the significant differences between groups. A *p* value less than 0.05 is considered as statistically significant.

Results And Discussion

SAA1 promotes liver injury and apoptosis of hepatocytes in CCl₄ treated mice liver

To study the role of SAA1 *in vivo*, we used a knockdown strategy to suppress the expression of SAA1 in CCl₄-treated mice liver. For this purpose we transfected siRNA sequence targeting SAA1 by using in vivo fectamine 3 reagents one day before CCl₄ injection. After 2 days of transfection, the mRNA and protein expression of SAA1 was significantly downregulated in case of SAA1 siRNA (Fig. 1a). We next examined the area of injury in liver tissue sections with H&E staining 24 hr following CCl₄ injection. The injury area was larger in tissue sections of control and NC siRNA-treated mice liver compared with SAA1 siRNA-treated mice (Fig. 1b). Increased serum level of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are widely accepted as the marker for the intensity of hepatocyte death in the liver [12]. Given that we measured plasma level of ALT/AST 0, 6 and 12 hr following injury. We found that ALT level was significantly reduced in SAA1 siRNA sample compared with buffer- and non-targeting -

control (NC siSAA)-treated samples, however, the level of AST did not show significant difference in both control and treatment groups (Fig. 1c). To determine whether SAA1 promotes hepatocyte apoptosis *in vivo*, we examined apoptotic hepatocytes in the non-necrotic areas of liver sections with TUNEL assay in control, NC siRNA and SAA1-siRNA-treated mice. As shown in Fig. 1d, larger TUNEL-positive cells could be observed in the necrotic area of control, NC siRNA-treated mice, but they were considerably reduced in SAA1 siRNA-treated mice. This indicated that SAA1 amplifies injury and induces apoptosis in hepatocytes.

SAA1 induces apoptosis in hepatocytes after NF- κ B inhibition

To further validate the *in vivo* results, we performed cell death assay to assess whether SAA1 induces apoptosis in human primary hepatocytes. Previous reports have highlighted that many of the signaling pathways, including apoptosis, induced by SAA1 resemble the ones activated by TNF- α [13]. We, therefore, investigated if SAA1 can induce apoptosis in human primary hepatocyte in a similar fashion of TNF- α signaling. Since human primary hepatocytes are resistant to TNF- α -induced cell death, we investigated SAA1-induced apoptosis in primary human hepatocytes that were pharmacologically (Actinomycin D, ActD) pre-treated to transcriptional inhibitor that prevents NF- κ B activation, a characteristic feature necessary for making them susceptible to TNF- α -induced apoptosis. As expected, there was no induction of cell death in human primary hepatocytes that were treated with rhSAA1 alone. However, pharmacological inhibition of NF- κ B led to almost 55% cell death after rhSAA1 treatment (Fig. 2a). To further characterize whether cell death was apoptotic, we measured the activation of the caspase3, an important executioner caspase [14], and we observed significant increased activity of caspase 3 (Fig. 2b). Through flow cytometry analysis, the apoptosis rate of human primary hepatocyte cells was significantly increased after treatment with TNF- α and rhSAA1 (Fig. 2c). In addition, we performed Western blot analysis to check the presences of cleaved caspase 3 as well as PARP cleavage fragment. As shown in Fig. 2d, we detected caspase-3 products at 17-19 kDa as well as the cleaved form of PARP at 89 kDa. Previous reports mainly emphasize the cytokine-like properties of SAA1 in various cell types [15-17]. Nevertheless, its potential to induce apoptosis is relatively an under explored area. One previous study highlighted that SAA1 may cause apoptotic cell death in human amniotic WISH cells, although the underlying mechanism was not investigated in detail [18]. Therefore, in this study we demonstrated for the first time that SAA1 induces hepatocyte apoptosis in similar fashion of TNF- α , which requires the switch of NF- κ B activity that ultimately causes cell lethality [19].

SAA1 induces transcription of chemokine through NF- κ B and PI3K/Akt pathways

Following acute liver injury, necrotic hepatocytes are capable of secreting various kinds of chemokines [20]. To determine if SAA1 can induce chemokine production in hepatocytes, we screened mRNA expression of pro-inflammatory chemokines by RT-qPCR. The results showed up-regulation of prominent pro-inflammatory chemokines after rhSAA1 treatment of primary human hepatocytes (Fig. 3a). Especially, there was a significant increase in the mRNA levels of MCP-1 and RANTES, which was also confirmed at protein level by Western blot analysis (Fig. 3b). We next determined whether SAA1-induced production of

chemokines in human primary hepatocytes was NF- κ B-dependent manner. For this purpose, we first assessed activation of NF- κ B luciferase activity that was found to be 5-15 fold increased after dose-dependent stimulation of human hepatocytes with rhSAA1 (Fig. 3c). Interestingly, Western blot analysis of lysates from rhSAA1-treated hepatocytes demonstrated that SAA1 induces phosphorylation of the p65 subunit of NF- κ B (Fig. 3d). Moreover, blockage of NF- κ B activity by using pharmacological inhibitor, ActD, resulted in a complete inhibition of chemokine production in hepatocytes (Fig. 3e). This confirmed the hypothesis that SAA1 induces apoptosis and cell survival simultaneously at which NF- κ B acts as a switch between SAA1-induced apoptosis and gene transcription in primary human hepatocytes. Similar to the TNF- α -mediated apoptotic pathway in hepatocytes, SAA1-induced activation of NF- κ B-dependent genes regulates the survival; whereas, inhibition of NF- κ B activation causes lethality [21]. This unique property of SAA1 suggests that it may act as double-edged sword following initial insult in the liver: exacerbates inflammation, and induces cell death when the NF- κ B-dependent genes cannot be transcribed.

Next, we sought to determine the signaling pathway downstream of SAA1 responsible for driving chemokine(s) production. We determined the involvement of major inflammatory pathways, such as PI3K and Akt, which are up-stream of NF- κ B and responsible for TNF- α -induced production of cytokine in hepatocytes [22]. The Western blot analysis revealed that PI3K phosphorylation was strongly induced within 10-30 min after SAA1 treatment (Fig. 3f). Similarly, there was strong activation of Akt as well, as indicated by increased level of SAA1-induced S473 phosphorylation which lasted from 10 to 30 min (Fig. 3f). To further confirm that PI3K and Akt are the survival pathways induced by SAA1, we treated SAA1-stimulated hepatocytes with LY294002, the inhibitor that blocks the phosphorylation of PI3K. Interestingly, we observed a remarkable inhibition of chemokine production from rhSAA1-stimulated hepatocytes (Fig. 3g). Together these results suggest that PI3K is upstream of its downstream effector Akt and mediates NF- κ B-dependent transcription of pro-inflammatory chemokines in primary human hepatocytes.

Based upon the data described in this study, one may generally infer that SAA1 somehow mediates the crosstalk between necrotic and healthy hepatocytes in a paracrine manner and optionally induces apoptotic and/or inflammatory pathways in the nearby hepatocytes. Finally, cell death and increased inflammation are the key drivers of hepatic disease progression, suggesting that SAA1 may provide a link between initial inflammatory response and fibrogenesis in the settings of chronic injury.

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Declarations

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Conflict of interest

Authors declared that there is no conflict of interest.

Availability of data and material

Non applicable

Code availability

Non applicable

Consent to publication

Non applicable

Author contribution

Conceptualization, methodology and investigations: A.G, X.H, C.Z, N.A, Z.Y, J.T, Y.Z and S.L. Resource: F.Y and Y.C: writing and editing: AG and MH. Reviewing: Y.L.

Corresponding Author: Yin-xiong Li

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Figures

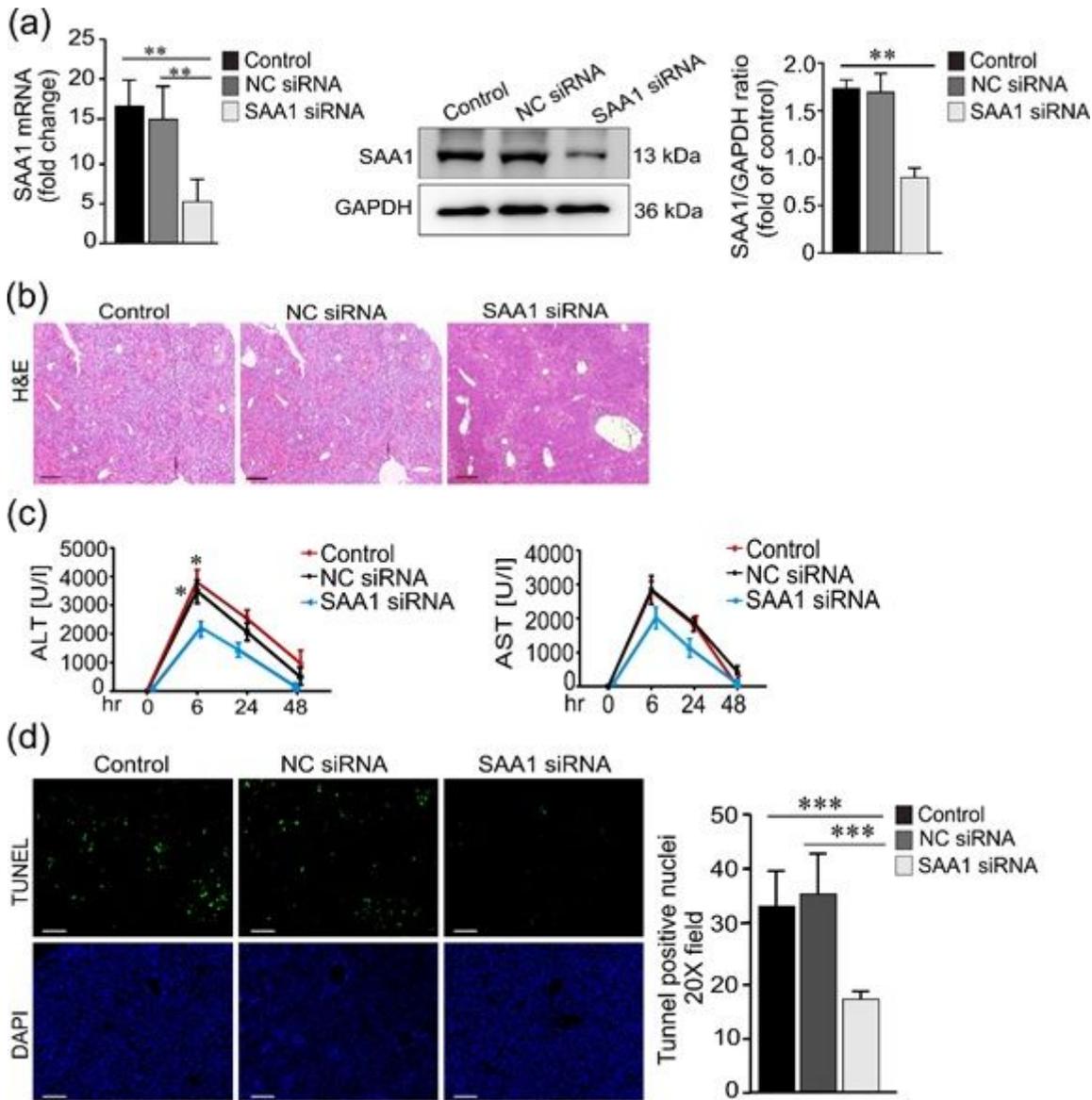


Figure 1

SAA1 amplifies injury and induces apoptosis in hepatocytes in CCl₄ treated mice liver. a relative expression of liver SAA1 mRNA and protein after injection of 3 different (control, NC siRNA and SAA1 siRNA) sequences, b representative images of H&E staining showing injury status of samples obtained from control, NC siRNA and SAA1 siRNA samples, c plasma level of ALT/AST in control, NC siRNA and SAA1 siRNA samples, d representative Images of TUNEL- positive cells in non-necrotic area. Bar graph shows quantification of TUNEL positive cells. (Scale bars = 200 μ m). Where applicable, data represents mean \pm SEM, *P < 0.05; ***P < 0.0001.

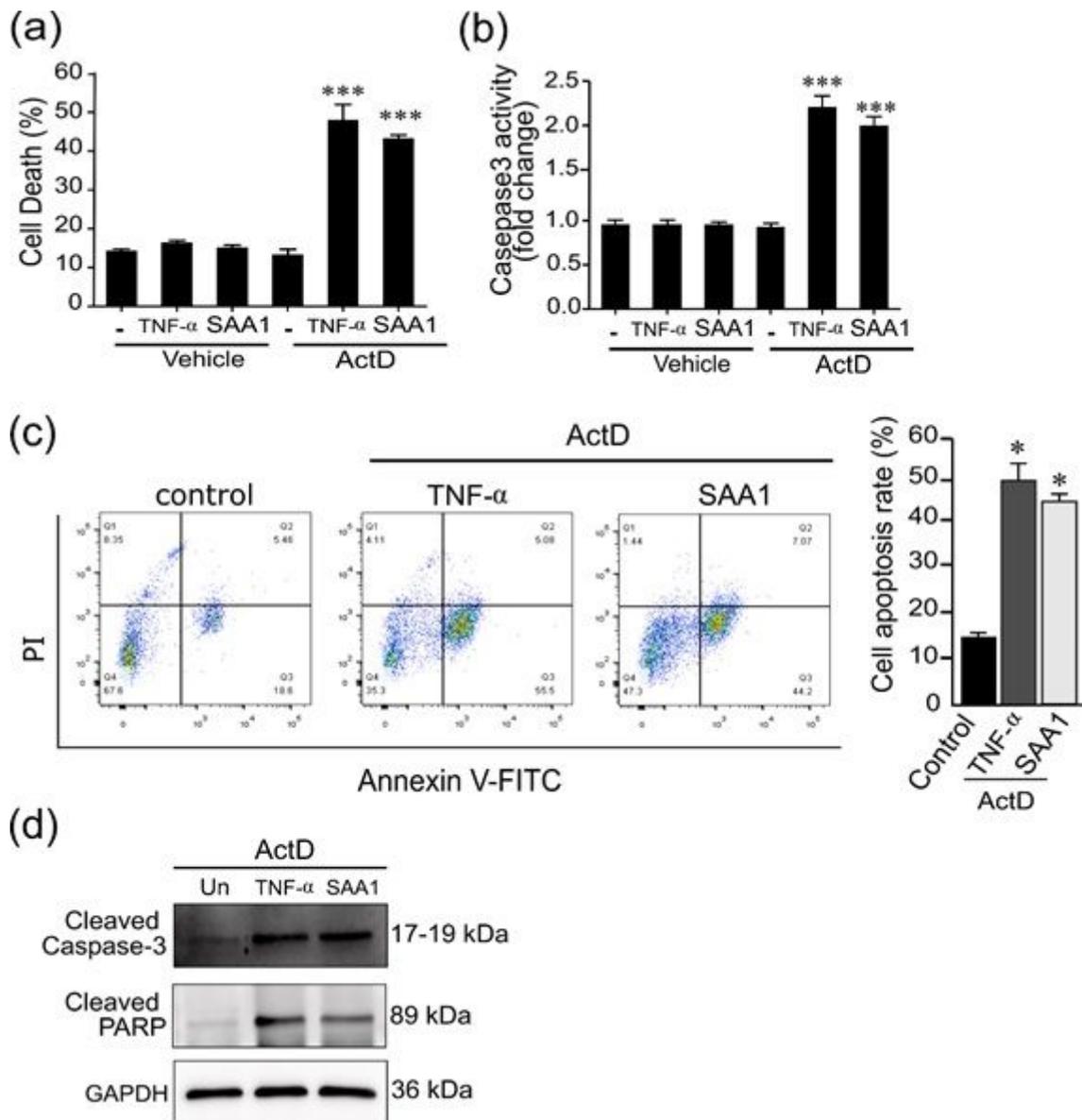


Figure 2

SAA1 induced apoptosis in hepatocytes sensitized by inhibition of NF- κ B activation. a Cell death was determined by LDH activity assay, b caspase activity was measured, c quantitative analysis of apoptotic cells by flow cytometry after Annexin-V/PI staining. Bar graph showing quantification of cell death from Annexin-V/PI staining, d caspase 3 and PARP cleavage were determined by Western blot analysis. Where applicable, data represents mean \pm SEM, *P < 0.05; ***P < 0.0001.

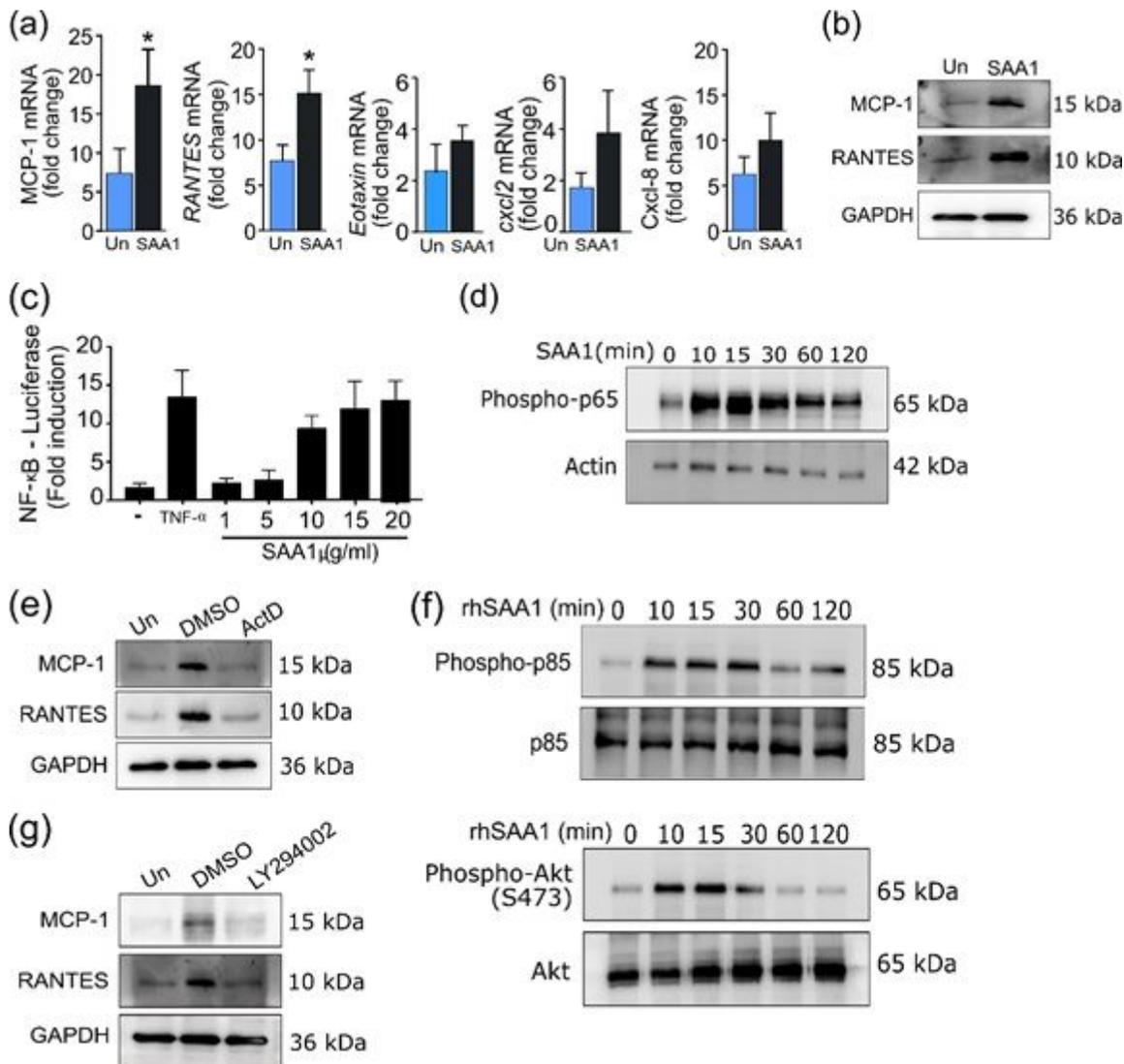


Figure 3

SAA1 induces chemokine production in primary human hepatocytes through NF-κB and PI3K /Akt pathways. a fold change of chemokines in human primary hepatocyte after rhSAA1 treatment, b protein level of MCP-1 and RANTES was determined by Western blot analysis, c NF-κB luciferase activity in fold induction, d Western blots analysis of activation of p65 subunit of NF-κB, e Western blot analysis of NF-κB after treatment with inhibitor ActD (0.2 μg/ml), f phosphorylation of PI3K at p85 subunit was determined by Western blot analysis, g Phosphorylation of Akt at S473 was determined by Western blot analysis, h Western blot analysis of chemokines after treatment of the cells with PI3K inhibitor LY294002 (10 μM). Where applicable, data represents at least three independent experiments mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001.

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