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ORIGINAL ARTICLE

Melanoma Differentiation-Associated Gene 5 Positively Modulates TNF-α-Induced CXCL10 Expression in Cultured HuH-7 and HLE Cells

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Abstract— The molecular mechanisms of innate immunity are closely associated with the development of non-alcoholic fatty liver disease (NAFLD). TNF-α is a key cytokine involved in the pathogenesis of metabolic inflammation like NAFLD. Melanoma differentiation-associated gene 5 (MDA5) is a member of the intracellular RNA helicase family proteins that play a pivotal role in an antiviral immune response. Previous studies have demonstrated that TNF-α induces the expression of MDA5 in some types of cells. However, the correlation between TNF-α and the expression of MDA5 in hepatocytes remains unknown. In the present study, we used two human hepatocellular carcinoma cell lines, HuH-7 and HLE, and examined the expression of MDA5 in these cells upon stimulation with TNF-α. The expression of MDA5 induced by TNF-α was analyzed by quantitative real-time RT-PCR and western blotting. Next, RNA interference against MDA5 was performed and the expressions of CXCL10 and STAT1 were examined. We found that the expression of MDA5 had increased upon stimulation with TNF-α in a concentration-dependent manner. Gene silencing against MDA5 suppressed the expression of TNF-α-induced CXCL10 in both cells. In HLE cells, gene silencing of MDA5 impaired STAT1 phosphorylation 24 h after stimulation with TNF-α. On the other hand, TNF-α-induced STAT1 phosphorylation was not detected in HuH-7 cells. These results indicated that MDA5 positively modulated the TNF-α-induced expression of CXCL10 in both STAT1-dependent and -independent manner and may be associated with metabolic inflammation in the liver.

KEY WORDS: MDA5; TNF-α; CXCL10; STAT1; hepatocytes.

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INTRODUCTION

Tumor necrosis factor (TNF)-α is a multifunctional cytokine which is involved in chronic inflammation and tumorigenesis. In the liver, TNF-α plays crucial roles in the induction of antiviral responses, liver fibrosis, and hepatocyte apoptosis. It has been well established that TNF-α is essential for the progression of conditions with metabolic inflammation like non-alcoholic steatohepatitis (NASH) as

well as the innate immune responses for the hepatitis virus infection [1]. In the pathogenesis of the non-alcoholic fatty liver disease (NAFLD), TNF-α is associated with the development of the obesity-linked insulin resistance [2] and is mainly produced by the adipocytes and Kupffer cells. Obesity causes an increased secretion of adipokines such as TNF-α and interleukin-6. Impaired integrity of the intestinal barrier causes an influx of lipopolysaccharide (LPS) derived from the gut microbiome to the liver *via* the portal vein. Kupffer cells activated due to the stimuli with LPS secrete many inflammatory cytokines [3, 4]. During these processes, the hepatocytes are exposed to excessive levels of inflammatory cytokines including TNF-α. Furthermore, various chemokines are known to modulate inflammation. They play important roles in recruiting the immune cells to inflamed sites and activating Kupffer cells, hepatic stellate cells, endothelial cells, and hepatocytes [5, 6]. Among them, C-X-C motif chemokine 10 (CXCL10), predominantly secreted by the hepatocytes and liver sinusoidal endothelial cells, plays a pivotal role in the pathogenesis of NASH and is regarded as a potential therapeutic target [6, 7]. In a previous report, it has been revealed that TNF-α is a highly potent inducer of CXCL10 in hepatocytes [8].

Melanoma differentiation-associated gene 5 (MDA5) and retinoic acid-inducible gene-I (RIG-I) are members of the DExH RNA helicase family proteins [9]. MDA5 and RIG-I function as sensors for the viral RNA and consequently lead to the production of type I interferons (IFNs) [10]. Many studies have reported that MDA5 is critically involved in the antiviral responses against hepatitis B, C, and D viruses [11–14]. On the other hand, it has also been implicated in the chronic inflammation caused by non-viral stimuli. Previously, we had reported that an increase in the expression of MDA5 was observed in the gastric mucosa infected with *Helicobacter pylori* [15]. Moreover, there are increasing evidences describing the association of MDA5 with autoinflammation and autoimmune responses [16, 17]. Although it has been reported that TNF-α induces the expression of MDA5 in HO-1 melanoma cells [9], it remains unknown whether MDA5 is induced similarly in hepatocytes stimulated with TNF-α. Additionally, no reports on the association of MDA5 with CXCL10 expression in hepatocytes are available.

In the present study, we used two human hepatocellular carcinoma (HCC) cell lines as experimental models of hepatocytes and investigated the expression of MDA5 in these cells when stimulated with TNF-α. Additionally, we assessed the effect of gene silencing against MDA5 on TNF-α-induced CXCL10 expression.

MATERIALS AND METHODS

Materials

A recombinant human TNF-α was purchased from Roche Diagnostics (Manheim, Germany). Dulbecco's modified eagle's medium (DMEM) containing Glutamax™, fetal bovine serum (FBS), Lipofectamine RNAiMAX reagent, oligo(dT)$_{12–18}$, and M-MLV reverse transcriptase were obtained from Invitrogen (Frederick, MD, USA). Small interfering RNA (siRNA) against MDA5 and IFN regulatory factor 1 (IRF1) and non-silencing negative control siRNA were purchased from Qiagen (Hilden, Germany). A siRNA against NF-κB p65 and rabbit polyclonal anti-MDA5 antibody were purchased from Cell Signaling Technologies (Danvers, MA, USA) and Immuno-Biological Laboratories (Fujioka, Gunma, Japan), respectively. Rabbit polyclonal anti-signal transducers and activators of transcription 1 (STAT1) antibody (sc-346) and mouse monoclonal anti-phosphorylated STAT1 (p-STAT1) antibody (sc-136229) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). A rabbit anti-β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-rabbit IgG HRP-conjugated antibody (#1858415), anti-mouse IgG HRP-conjugated antibody (#1858413), and BCA Protein Assay Reagent were purchased from Pierce/Thermo Scientific Inc. (Waltham, MA, USA). A SsoAdvanced Universal SYBR Green Supermix solution was obtained from Bio-Rad Laboratories Inc. (Hercules, CA, USA). A NucleoSpin RNA kit was purchased from Macherey-Nagel GmbH & Co. KG (Düren, Germany). Polyvinylidene fluoride (PVDF) membranes and Luminata Crescendo Western HRP substrate were from Millipore Corporation (Billerica, MA, USA). An enzyme-linked immunosorbent assay (ELISA) kit for CXCL10 was obtained from R&D systems (Minneapolis, MN, USA).

Cell Culture

A well-differentiated and non-differentiated human HCC cell lines, HuH-7 and HLE, respectively, were cultured in DMEM containing Glutamax™ with 10% FBS and maintained in an incubator with 5% CO_2 at 37 °C. For the RNA interference (RNAi) experiments, the cells were transfected with siRNA against MDA5, p65, IRF1, and a negative control siRNA using the Lipofectamine RNAiMAX reagent, according to the manufacturer's instructions. After 24 h of transfection, the culture medium was changed and the cells were treated with 10 ng/ml TNF-α for 24 h. Then, the culture medium was collected and the

Table 1. Oligonucleotide Primers for Real-Time Quantitative PCR

cDNA	Primers
MDA5	F: 5′-GTTGAAAAGGCTGGCTGAAAAC-3′
	R: 5′-TCGATAACTCCTGAACCACTG-3′
pan IFN-α	F: 5′-AGAATCTCTCCTTTCTCCTG-3′
	R: 5′-TCTGACAACCTCCCAGGCAC-3′
IFN-β	F: 5′-CCTGTGGCAATTGAATGGGAGGC-3′
	R: 5′-CCAGGCACAGTGACTGTACTCCTT-3′
STAT1	F: 5′-CAGTTCTCCCAAGGGAGTTAG-3′
	R: 5′-GTATGCAGTGCCACGGAAAGC-3′
IRF1	F: 5′-CGGGGCTCATCTGGATTAATAAAGAGG-3′
	R: 5′-GGATGTGCCAGTCGGGGAGAGTG-3′
CXCL10	F: 5′-TTCAAGGAGTACCTCTCTCTAG-3′
	R: 5′-CTGGATTCAGACATCTCTTCTC-3′
GAPDH	F: 5′-GCACCGTCAAGGCTGAGAAC-3′
	R: 5′-ATGGTGGTGAAGACGCCAGT-3′

cells were lysed. Analysis using quantitative real-time reverse transcription-polymerase chain reaction (qPCR) was conducted on the RNA extracted from the cells. Western blotting and ELISA were performed using cell lysates and culture medium, respectively.

RNA Isolation and qPCR

Total RNA was extracted from the cells using the NucleoSpin RNA kit according to the manufacturer's instructions. Single-strand cDNA was synthesized from 1 μg of total RNA using the oligo $d(T)_{12–18}$ primers and M-MLV reverse transcriptase. A qPCR was performed using a Bio-Rad CFX real-time PCR thermocycler with SsoAdvanced Universal SYBR Green Supermix solution. The primer sequences used were as described in Table 1. Each sample was run in triplicate.

Western Blot Analysis

The cells were washed twice with PBS and lysed using Laemmli's reducing sample buffer. The protein concentration was measured using the BCA Protein Assay Reagent. Each protein sample of 15 μg was loaded onto

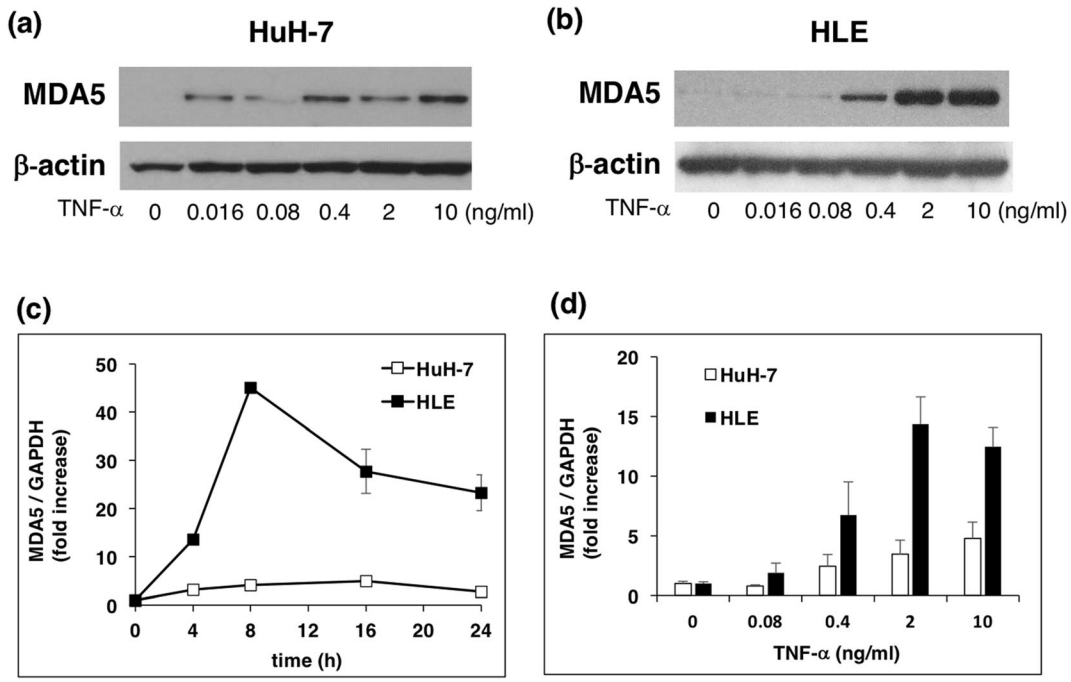

Fig. 1. TNF-α induced the expression of MDA5 in HuH-7 and HLE cells. **a** The HuH-7 cells were treated with various concentrations of TNF-α for 24 h and their cell lysates were subjected to western blotting to detect MDA5 and β-actin. **b** The HLE cells were treated with TNF-α as presented in (**a**) and western blotting of MDA5 and β-actin were performed. **c** The cells were treated with various concentrations of TNF-α for up to 24 h. Total RNA was extracted from the cells and the mRNA expression of MDA5 was analyzed by qPCR. **d** The cells were treated with 10 ng/ml TNF-α for 24 h. Total RNA was extracted from cells and the mRNA expression of MDA5 was analyzed by qPCR.

a 7.5% or 10–20% SDS-polyacrylamide gel and subjected to electrophoresis, after which, the separated proteins were transferred onto a PVDF membrane. The membrane was blocked for 120 min in Tris-buffered saline with Tween 20 (TBS-T; 50 mM Tris-HCl, 250 mM NaCl and 0.1% Tween 20) containing 5% nonfat dry milk and incubated overnight at 4 °C with an antibody against MDA5 (1:1000), p-STAT1 (1:1000), STAT1 (1:1000), or β-actin (1:2000), followed by incubation with a horseradish peroxidase-labeled secondary antibody for 1 h at room temperature. Immunodetection was carried out using Luminata Crescendo substrate.

ELISA

ELISA was performed to examine the secretion of CXCL10 by HuH-7 and HLE cells. The concentration of CXCL10 in the supernatant of culture media was determined using an ELISA kit, according to the manufacturer's instructions.

Statistical Analysis

All data was presented as the mean ± SD. The statistical significance was determined using a Student's *t* test. A P value of < 0.05 was considered to be statistically significant.

RESULTS

TNF-α Induces the Expression of MDA5 in HuH-7 and HLE

Firstly, we examined whether TNF-α could upregulate the expression of MDA5 in two human hepatocellular carcinoma cell lines HuH-7 and HLE. As shown in Fig. 1a and b, MDA5 protein was induced in a concentration-dependent manner upon stimulation with TNF-α. In the HLE cells, the mRNA of MDA5 began to increase at 4 h after the treatment with TNF-α reached the maximal level at 8 h and then decreased gradually (Fig. 1c). In the HuH-7

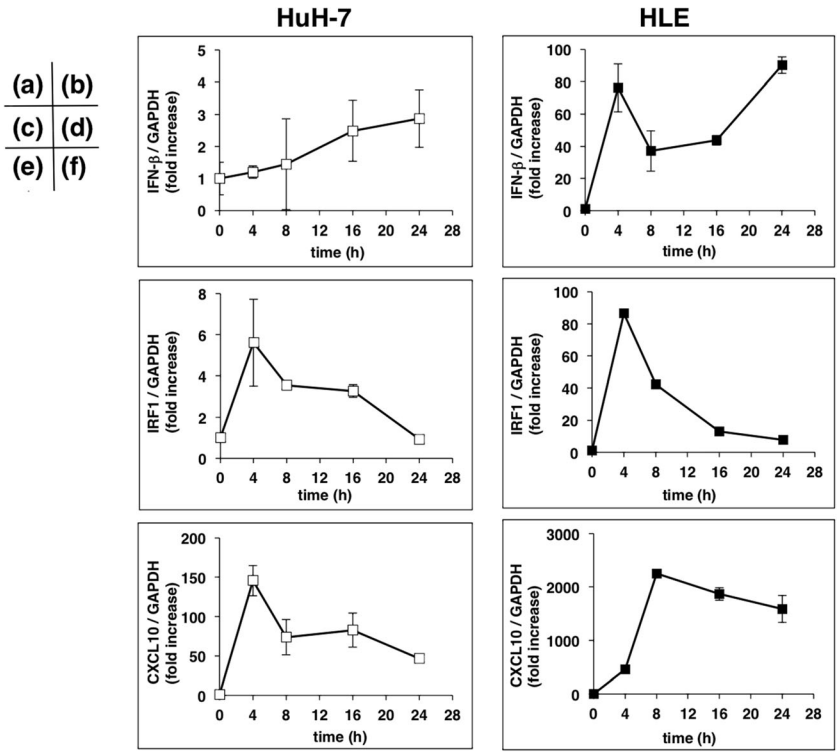

Fig. 2. The cells were treated with 10 ng/ml TNF-α for up to 24 h. Total RNA was extracted from the cells and the mRNA expression was analyzed by qPCR. The mRNA expression of IFN-β (a), IRF1 (c), and CXCL10 (e) in HuH-7 and HLE (b, d, and f, respectively) were examined.

cells, the MDA5 mRNA continued to increase until 16 h and then decreased (Fig. 1c). The expression level of the MDA5 mRNA in HuH-7 was substantially lower than that in HLE (Fig. 1d). Next, we analyzed the mRNA expression of IFN-β, IRF1, and CXCL10 in these cell lines stimulated by TNF-α. The results showed that the expression level of the IFN-β mRNA was lower in HuH-7 (Fig. 2a) than in HLE (Fig. 2b). The results of mRNA expression for IRF1 (Fig. 2c, d) and CXCL10 (Fig. 2e, f) were also found to be similar. Thus, it can be suggested that the responsiveness to TNF-α was lower in HuH-7 than in HLE and the induction of IFN-β was modest in HuH-7.

TNF-α-Induced MDA5 Expression Is Mediated by NF-κB

Thereafter, we examined the effects of NF-κB and IRF1 on the expression of MDA5 in HuH-7 and HLE stimulated with TNF-α. The results showed that gene silencing against NF-κB p65 almost suppressed the MDA5 expression induced by TNF-α in both cells (Fig. 3a, b). On the other hand, gene silencing against IRF1 significantly suppressed the expression of MDA5 in HLE (Fig. 3b, d), but not in HuH-7 (Fig. 3a, c). Therefore, it can be suggested that the expression of MDA5 induced by TNF-α was mediated by NF-κB and IRF1 was also involved in its expression in some types of cells.

Gene Silencing against MDA5 Attenuates TNF-α-Induced CXCL10 Expression

We carried out gene silencing against MDA5 to investigate its influence on the expression of CXCL10 induced by TNF-α. Effective knockdown was confirmed by qPCR and western blotting (Fig. 3a–d). As shown in Fig. 4a, gene silencing against MDA5 elicited a significant decrease of CXCL10 mRNA expression in HuH-7 stimulated by TNF-α, and similar results were obtained in HLE as well (Fig. 4b). The knockdown of MDA5 reduced TNF-α-induced CXCL10 protein production by approximately 75% and 50% in HuH-7 and HLE, respectively (Fig. 4c, d).

Gene Silencing Against MDA5 Suppresses TNF-α-Induced STAT1 Phosphorylation in HLE

STAT-1 is a key transcriptional factor required for the expression of CXCL10 in hepatocytes. Therefore, we

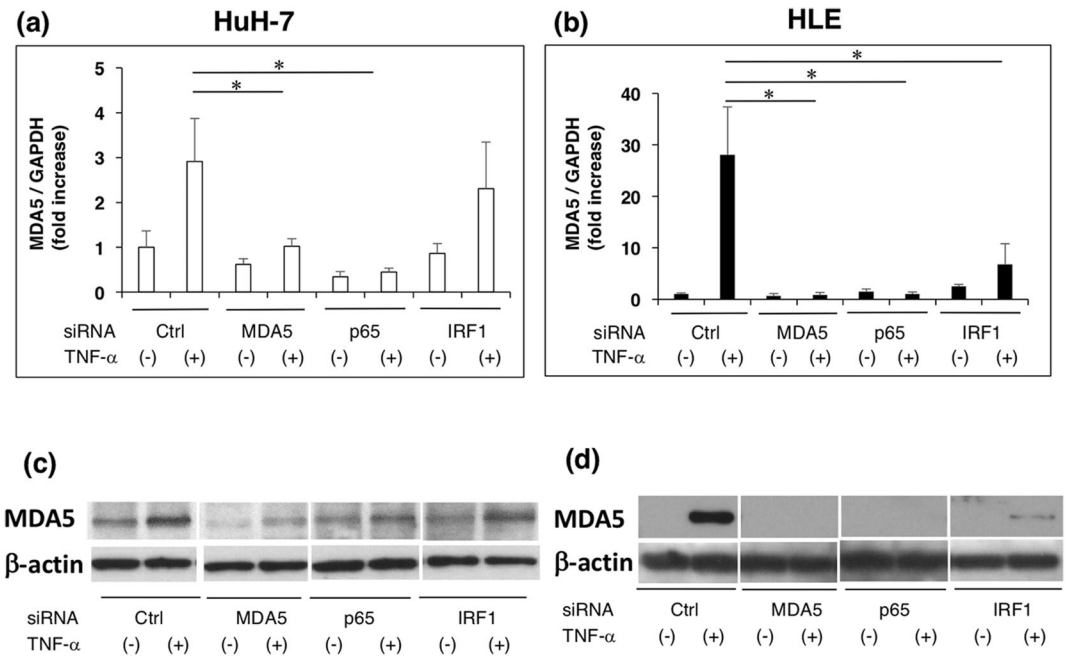

Fig. 3. TNF-α-induced MDA5 expression is mediated by NF-κB. The cells were transfected with siRNA against MDA5, NF-κB p65, IRF1, or non-silencing negative control and incubated for 24 h. After changing the culture medium, the cells were treated with 10 ng/ml TNF-α for 24 h. Total RNA was extracted from the cells and the mRNA expression of MDA5 in **a** HuH-7 and **b** HLE was analyzed by qPCR ($n = 3$, $^*p < 0.01$). The cell lysates were subjected to western blotting for detection of MDA5 and β-actin in **c** HuH-7 and **d** HLE.

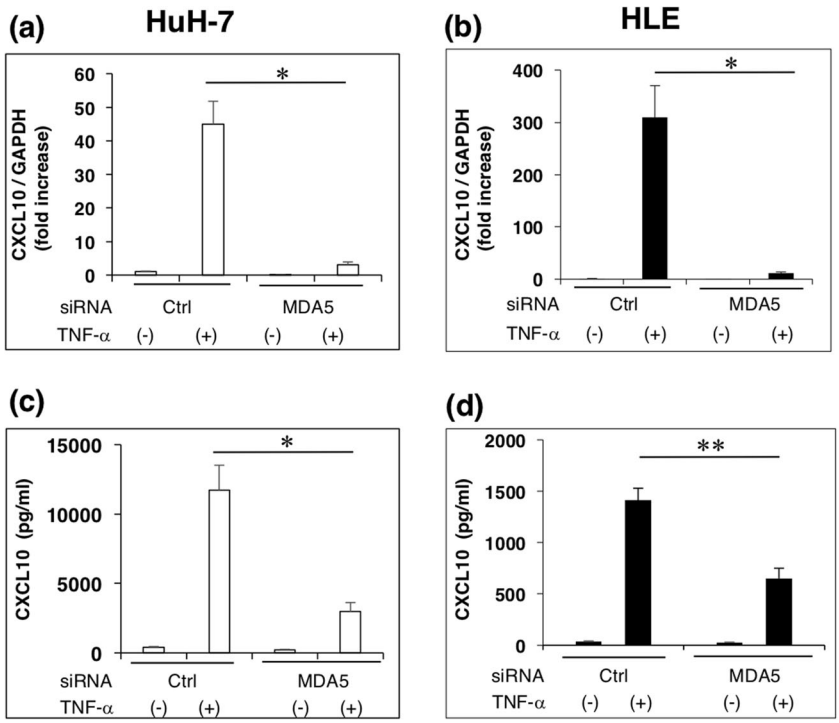

Fig. 4. Gene silencing against MDA5 attenuated TNF-α-induced CXCL10 expression. The cells were transfected with siRNA against MDA5 or non-silencing negative control and incubated for 24 h. After changing the culture medium, they were treated with 10 ng/ml TNF-α for 24 h. Total RNA was extracted from the cells and the mRNA expression of CXCL10 was analyzed by qPCR. The expression levels of CXCL10 mRNA in **a** HuH-7 and in **b** HLE ($n = 3$, $^{*}p < 0.01$). The concentration of CXCL10 protein in the culture media of **c** HuH-7 and **d** HLE was examined by ELISA ($n = 3$, $^{*}p < 0.01$, $^{**}p < 0.05$).

performed gene silencing against MDA5 and investigated the occurrence of phosphorylation of the STAT1 protein. The p-STAT1 protein was not detected in HuH-7 upon stimulation with TNF-α, whereas the phosphorylation of the STAT1 protein was detected in HLE at 24 h after treatment with TNF-α (Fig. 5a). The knockdown of MDA5 resulted in the inhibition of phosphorylation of the STAT1 protein in HLE. The results of qPCR revealed that the mRNA level of STAT1 was not increased in HuH-7 stimulated with TNF-α (Fig. 5b), whereas the marked upregulation of STAT1 mRNA was detected in HLE (Fig. 5c). Moreover, the gene silencing against MDA5 significantly suppressed the expression of STAT1 in HLE (Fig. 5c). Since it is widely known that type I or II IFN was essential for the activation of STAT1, we evaluated the expression of IFN-α and -β mRNA in both cells. The expression of IFN-α mRNA was analyzed by qPCR using pan-specific primers. The mRNA level of pan IFN-α and IFN-β was almost unchanged in HuH-7 upon stimulation with TNF-α (Fig. 6a, c). Contrary to expectations, transfection of siRNA against MDA5 resulted in the upregulation of pan IFN-α mRNA in HLE (Fig. 6b). Nevertheless, the expression levels of STAT1 mRNA and protein were not affected by the transfection as shown in Fig. 5. Thus, it can be suggested that this cellular response is a non-specific reaction and has no direct influence on the present results. In contrast, the expression of IFN-β mRNA was enhanced by the treatment with TNF-α and significantly suppressed by the knockdown of MDA5 in HLE (Fig. 6d).

DISCUSSION

RIG-I-like receptor (RLR) is a term used commonly to describe three RNA helicases, RIG-I, MDA5, and laboratory of genetics and physiology 2 (LGP2), and plays a critical role in sensing double-stranded RNA during viral infection. Many studies have revealed that RLRs are essential to antiviral responses. On the other hand, there are increasing evidences to prove that the innate immune system may be an important regulator of metabolic and

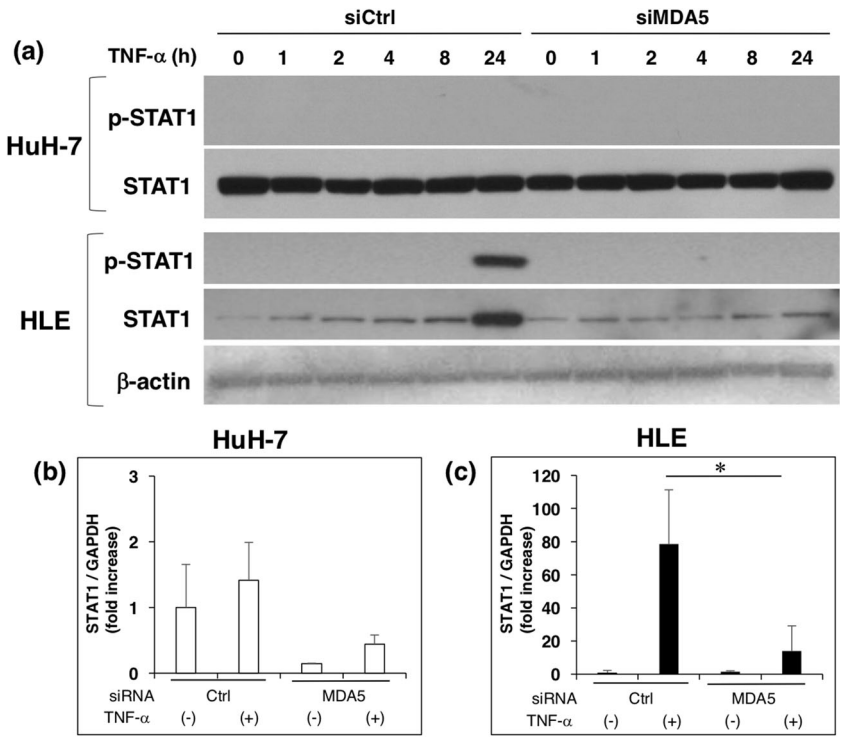

Fig. 5. Silencing of the MDA5 gene suppressed TNF-α-induced STAT1 phosphorylation in HLE. The cells were transfected with siRNA against MDA5 and incubated for 24 h. After changing the culture medium, the cells were treated with 10 ng/ml TNF-α for up to 24 h. **a** The cell lysates were subjected to western blotting to analyze the expression of p-STAT1, STAT1, and β-actin. Total RNA was extracted from cells and the expression of STAT1 mRNA in **b** HuH-7 and **c** HLE was examined by qPCR ($n = 3$, $^*p < 0.01$).

cardiovascular diseases [18]. Pan *et al*. have reported that RIG-I was upregulated in rodent models of type 2 diabetes mellitus [19]. We have reported that RIG-I was strongly expressed in the macrophages of the atherosclerotic lesions [20]. Toyoda *et al*. have reported that RIG-I was correlated with the occurrence of hepatic steatosis in patients with chronic hepatitis C [21]. Although there is a possibility that MDA5 may be associated with the development of metabolic inflammation, it has not yet been elucidated.

In the present study, we focused on the function of TNF-α as a key inducer of MDA5 because it plays a pivotal role in the development of metabolic inflammation, and we have previously reported that TNF-α had induced the expression of RLRs in some types of cells like macrophages and synoviocytes [20, 22]. In this study, we found that TNF-α could induce the expression of MDA5 in human HCC cell lines HuH-7 and HLE without any viral stimuli and was mediated by NF-κB. Interestingly, IRF1 was involved in its expression only in HLE. Previous reports have shown that IRF1, originally identified as a regulator of IFN-β gene [23], was induced upon stimulation with TNF-α in hepatocytes [24] and *IFIH1* gene, which encodes MDA5 that is known to be one of the IFN-stimulated genes. Therefore, it is suggested that the NF-κB/IRF1/IFN-β pathway is important for the expression of MDA5 in HLE. Moreover, the expression of IFN-β and the phosphorylation of STAT1 were inhibited by the knockdown of MDA5 suggesting that it enhanced the IFN-β/STAT1 signaling and the possibility of the presence of a positive feedback loop between IFN-β and MDA5 in HLE. On the other hand, it is confirmed that IRF1 was not required for the expression of MDA5 in HuH-7, suggesting that the IFN-β/STAT1 signaling was not deeply involved in its expression in HuH-7. It is assumed that the expression of MDA5 was affected by the activation level of the IFN-β/STAT1 signaling in response to TNF-α because the expression level of MDA5 was substantially lower in HuH-7 than in HLE. We could not clarify the molecular mechanisms associated with the difference in responsiveness to TNF-α between HuH-7 and HLE. It can be speculated that differences in genetic backgrounds, such as a mutation of p53, may influence the

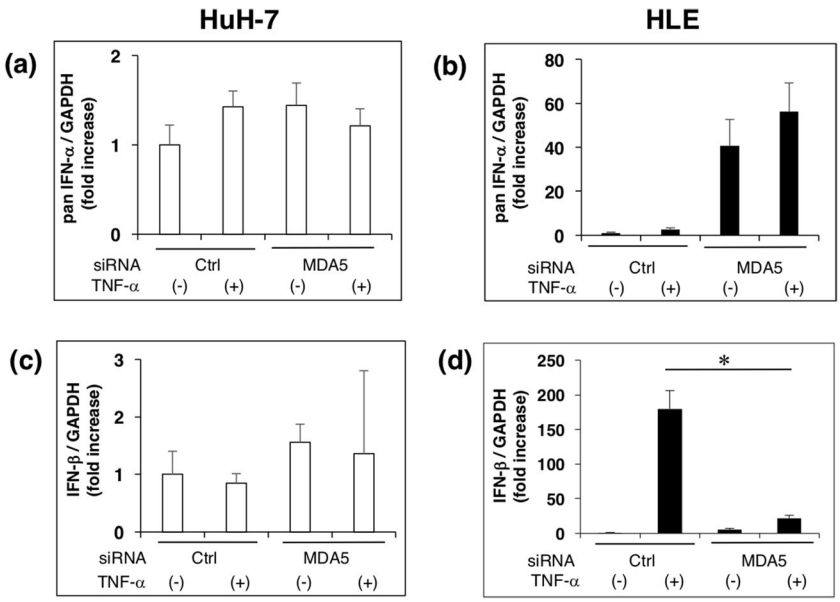

Fig. 6. The cells were transfected with siRNA against MDA5 and incubated for 24 h. After changing the culture medium, they were treated with 10 ng/ml TNF-α for 24 h and total RNA was extracted from the cells. The expression levels of pan **a** IFN-α mRNA and **c** IFN-β in **b** HuH-7 and **d** HLE were analyzed by qPCR ($n = 3$, $^*p < 0.01$).

cellular responses. Although NF-κB was essential for the expression of MDA5 in both cells, we could not elucidate whether the induction of MDA5 was directly mediated by NF-κB in this study. Further investigations are needed.

In our previous studies, we have demonstrated that MDA5 modulated the expression of CXCL10 in human mesangial cells and astrocytoma cells [25, 26]. In the present study, we have analyzed the role of MDA5 induced by TNF-α in the expression of CXCL10 in hepatoma cell lines. It was observed that a knockdown of the MDA5 gene resulted in a marked decline of CXCL10 production in HuH-7 and HLE cells stimulated by TNF-α, suggesting that MDA5 positively modulated the expression of CXCL10. Because it has been reported that STAT1 is critically involved in the induction of CXCL10 in hepatocytes [27], it is possible that MDA5 can modulate the CXCL10 expression by enhancing the IFN-β/STAT1 pathway. In this study, we demonstrated that gene silencing against MDA5 impaired STAT1 phosphorylation in HLE upon stimulation with TNF-α. On the other hand, TNF-α-induced STAT1 phosphorylation was not detected in HuH-7, suggesting that IFN-β/STAT1 signaling was not activated by TNF-α in the cells. These results indicated that MDA5 positively modulated the TNF-α-induced expression of CXCL10 in both STAT1-dependent and -independent manner. Although the precise mechanisms of STAT1-independent pathway remain unclear, we speculate that MDA5 may exert some influence on the activity of a transcriptional factor other than STAT1 or the post-transcriptional regulation such as the stabilization of the mRNA.

CXCL10 is closely associated with the development of various kinds of chronic liver diseases. In murine NASH, CXCL10-deficient mice exhibit significantly attenuated liver inflammation compared to the wild type mice, which occurs due to a reduction in the number of infiltrated macrophages [28]. Zhang *et al*. reported that the hepatic levels of CXCL10 mRNA were significantly high in patients with NASH [7]. These reports suggested that CXCL10 could be deeply involved in the pathogenesis of NAFLD and can be regarded as a strong candidate to therapeutically target NAFLD. Therefore, it can be suggested that MDA5 modulates the inflammation of NAFLD *via* CXCL10 involvement and might be a potential therapeutic target.

CONCLUSION

In the present study, we found that MDA5 was induced by TNF-α and positively modulated TNF-α-induced CXCL10 expression in HuH-7 and HLE cells. MDA5 may be critically involved in hepatic inflammation.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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