Hepatic Macrophages Express Melanoma Differentiation-Associated Gene 5 in Nonalcoholic Steatohepatitis

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Abstract—The activation of innate immune system is essential for the pathogenesis of nonalcoholic steatohepatitis (NASH). Among pattern recognition receptors, it is well-characterized that toll-like receptors (TLRs) are deeply involved in the development of NASH to reflect exposure of the liver to gut-driven endotoxins. In contrast, it has not been elucidated whether retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) are similarly implicated in the disease progression. In the present study, we examined the expression of melanoma differentiation-associated antigen 5 (MDA5), known to be a member of RLRs, in a diet-induced murine model of NASH. The liver tissues were collected from C57BL/6 J mice at 1, 3, and 6 weeks after choline-deficient L-amino acid-defined high-fat diet (CDAHFD), and the expression of MDA5 was analyzed by western blotting, immunofluorescence (IF), and real-time quantitative PCR (qPCR). The results of western blotting showed that hepatic expression of MDA5 was increased at 3 and 6 weeks. In IF, MDA5-positive cells co-expressed F4/80 and CD11b, indicating they were activated macrophages, and these cells began to appear at 1 week after CDAHFD. The mRNA expression of MDA5 was significantly upregulated at 1 week. Additionally, we performed IF using liver biopsy specimens collected from 11 patients with nonalcoholic fatty liver diseases (NAFLD), and found that MDA5-positive macrophages were detected in eight out of eleven patients. In an in vitro study, MDA5 was induced upon stimulation with lipopolysaccharide in murine bone marrow-derived macrophages and THP-1 cells. Our findings suggest that MDA5 may be involved in the inflammation of NASH.

KEY WORDS: MDA5; NASH; Macrophage; F4/80; CD11b; CDAHFD.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases characterized by hepatic damage due to the excessive fat accumulation. NAFLD consists of nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH), and the latter is a more
Progressive interstitial lung disease and poor prognosis in patients with dermatomyositis (DM) [18]. Interestingly, it has been reported that all of DM patients who have liver dysfunction defined as an elevated level of alanine aminotransferase (ALT) are positive for anti-MDA5 antibody [19]. These results indicate that MDA5 is implicated in a wide range of inflammation, including sterile inflammation. Although MDA5 may be associated with metabolic inflammation, including NASH, there have been no reports regarding the expression of MDA5 in NAFLD. Herein, we examined MDA5 expression in a diet-induced murine model of NASH and in 11 patients with NAFLD, and found that MDA5 was expressed in activated macrophages in the liver of NASH.

**MATERIALS AND METHODS**

**Materials**

A rabbit polyclonal anti-MDA5 antibody (#29,020) and a rabbit polyclonal anti-GAPDH antibody (#2118) were purchased from Immuno-Biological Laboratories (Fujioka, Gunma, Japan) and Cell Signaling Technologies (Danvers, MA, USA), respectively. A rat polyclonal anti-F4/80 antibody (#MCA497GA) and a rat polyclonal anti-CD11b antibody (#GTX32495) were from Bio-Rad Laboratories (Hercules, CA, USA) and Genetex (Irvine, CA, USA), respectively. A mouse monoclonal anti-CD68 antibody (#14–0681), a rat monoclonal anti-CD3 antibody (#14–0688), a rat monoclonal anti-CD68 antibody (#14–0032), Alexa Fluor 488 anti-rabbit IgG antibody (#A11029), Alexa Fluor 488 anti-mouse IgG antibody (#A11008), Alexa Fluor 488 anti-rabbit IgG antibody (#A11009), Alexa Fluor 594 anti-rat IgG antibody (#A21471), Alexa Fluor 594 anti-rabbit IgG antibody (#A21207), and 4′,6′-diamidino-2-phenylindole (DAPI) were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Recombinant human TNF-α and Lipopolysaccharide (LPS) from *Escherichia coli* were obtained from Roche Diagnostics (San Diego, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. NucleoSpin RNA kit was purchased from Macherey–Nagel GmbH & Co. KG (Düren, Germany). M-MLV reverse transcriptase and oligo(dT)_{12-18} were from Invitrogen (Foster City, CA, USA). THUNDERBIRD Next SYBR qPCR mix was purchased from Toyobo (Osaka, Japan). Polyvinylidene fluoride (PVDF) membranes and Luminata Crescendo Western HRP substrate were from Merck Millipore (Burlington, MA, USA). Recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF) was from Immuno-Biological Laboratories (Fujioka, Gunma, Japan). THUNDERBIRD Next SYBR qPCR mix was purchased from Toyobo (Osaka, Japan). Polyvinylidene fluoride (PVDF) membranes and Luminata Crescendo Western HRP substrate were from Merck Millipore (Burlington, MA, USA). Recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF) was from Immuno-Biological Laboratories (Fujioka, Gunma, Japan).
macrophage colony-stimulating factor (GM-CSF) and sodium palmitate were from Fujifilm Wako Pure Chemical Co. (Osaka, Japan) and Nacalai tesque (Kyoto, Japan), respectively.

**Preparation of Palmitate Solution**

Palmitate was prepared as previously described [20]. Briefly, 20% stock solution of fatty acid-free bovine serum albumin (BSA) was prepared by dissolving 750 mg of BSA in 3.75 mL of phosphate buffered salts (PBS). Then, 5.6 mg of sodium palmitate was dissolved in 1 mL ethanol, and palmitate-BSA complex was prepared by mixing 1 mL of palmitate solution with 3.3 mL of BSA 20% stock solution. The complex was added to a 15.7 mL cell culture medium at 37 °C. The sterile filtered solution was used as a 1 mM palmitate solution.

**Animal Study**

Male C57BL/6 J mice (6–8 weeks old) were obtained from CLEA Japan (Tokyo, Japan). All mice were maintained on a 12-h light/dark cycle at 22 °C in a specific pathogen-free environment. After 1 week of acclimation, the mice were divided into a normal chow (NC) group and a choline-deficient, l-amino acid-defined high-fat diet (CDAHFD) (#A06071302, Research Diets Inc., New Brunswick, NJ, USA) group [21]. After 1, 3, or 6 weeks, mice were sacrificed and the livers were harvested for histological and biochemical analyses. All animal experiments were conducted in accordance with the Guidelines for Animal Experimentation of the Hirosaki University ( Permit number: M20016).

**Cell Culture**

THP-1, a human monocytic leukemia cell line, was cultured in Roswell Park Memorial Institute (RPMI)1640 medium supplemented with Glutamax™ (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS), 100 mg/mL penicillin, and 100 mg/mL streptomycin under 5% CO₂ at 37 °C. To differentiate into macrophage-like cells, THP-1 cells were treated with 10 ng/mL of phorbol 12-myristate 13-acetate (PMA) for 48 h. After another 24 h of incubation without PMA, the cells were stimulated with LPS, TNF-α, or palmitate.

**Isolation and Culture of Murine Bone Marrow-Derived Macrophages**

C57BL/6 J mice (6–10 weeks old) were sacrificed, and the femurs were removed. The bone marrow (BM) cavity was flushed with ice-cold PBS using a 23G needle, and the cells were collected. After passing through a 100 μm cell strainer, the cells were cultured in RPMI 1640 medium containing 10% FBS, 50 μM 2-mercaptoethanol, 100 mg/mL penicillin, 100 mg/mL streptomycin, and 10 ng/mL GM-CSF to differentiate into BM-derived macrophages (BMDM). After 7 days of incubation, the cells were plated at a density of 1 × 10⁶ cells/mL in 12-well or 96-well plates and stimulated with LPS, TNF-α, or palmitate.

**RNA Isolation and Real-Time Quantitative PCR**

Total RNA was extracted from cells and murine liver tissues using a NucleoSpin RNA kit according to the manufacturer’s instructions, and cDNA was synthesized using the oligo(dT)₁₈ primer and M-MLV reverse transcriptase. A quantitative PCR qPCR was performed using a Bio-Rad CFX real-time PCR thermocycler with THUNDERBIRD™ Next SYBR® qPCR mix (45 cycles, annealing 60 °C). The sequences of primers used are listed in Table 1. Each sample was run in triplicate.

**Western Blotting**

THP-1 cells, BMDM, and murine liver tissues were lysed with RIPA buffer containing 0.2% proteinase inhibitors, and the lysates were centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatants were collected, and after determination of the protein concentration using BCA Protein Assay Reagent, equal amounts of the protein (5–50 μg) were loaded on a 10–20% sodium dodecyl sulfate (SDS) polyacrylamide gel for electrophoresis. The separated proteins were transferred onto a PVDF membrane. After the membranes were blocked with Tris-buffered saline with Tween 20 (TBS-T, pH7.4) containing 5% nonfat dry milk, they were incubated overnight at 4 °C with a primary antibody against MDA5 (1:1000) or GAPDH (1:2000). The membranes were then incubated with an HRP-conjugated secondary antibody for 1 h at room temperature. Immunodetection was carried out using
Luminata Crescendo substrate. The density of each band was measured using the ImageJ software [22].

Histological Analysis of Human Samples

Liver samples were obtained from 11 patients (4 men and 7 women, median age 47.0 ± 15.9) who had undergone ultrasound-guided liver biopsy for the diagnosis of NASH at the Hirosaki University Hospital. The biopsy specimens were fixed in 10% formalin and embedded in paraffin. The sections were subjected to conventional hematoxylin and eosin (H&E) staining and immunofluorescence. In addition, 8 patients (5 men and 3 women, median age 64.0 ± 7.0) who underwent liver biopsy for metastatic liver tumors without any existing liver diseases were included in this study, and the uninvolved area in the specimen was regarded as a normal control. The summary of clinical characteristics of NAFLD patients and control cases is provided in Table 2. This study was approved by the Ethics Committee of the Hirosaki University Graduate School of Medicine (Permit number: 2018–152).

Immunofluorescence

After heat-induced antigen retrieval followed by blocking of endogenous peroxidase with 3% hydrogen peroxide, the tissue sections were blocked with 5% normal goat serum and 5% BSA in TBS-T for 1 h. Sections were then incubated with a rabbit anti-MDA5 (1:250) and either a rat anti-F4/80 (1:250), a rat anti-CD11b (1:250), a rat anti-CD68 (1:250), a mouse anti-CD68 (1:250), or a rat anti-CD3 (1:250) antibodies overnight at 4 °C. After washing, the cells were stained with Alexa Fluor 488-conjugated anti-rabbit IgG antibody (1:250) and Alexa Fluor 594-conjugated anti-rat IgG antibody (1:250) for 1 h at room temperature. After staining the nuclei with DAPI, the sections were visualized using confocal laser scanning microscopy (C1si; Nikon, Tokyo, Japan).

Statistical Analysis

All data are presented as the mean ± SEM. Statistical differences were analyzed using Welch’s t test, or one-way analysis of variance (ANOVA), while Wilcoxon rank sum test was used for non-parametric value. Categorical comparisons were analyzed using chi-squared test. Statistical significance was set at p < 0.05.
RESULTS

Increased Expression of MDA5 in the Liver of CDAHFD-Fed Mice

First, we analyzed MDA5 expression in the livers of CDAHFD-fed mice at 6 weeks. As shown in Fig. 1a, MDA5 expression was significantly higher in CDAHFD mice than in NC mice. In immunofluorescence (IF), the immunoreactivity of MDA5 was not detected in the liver of NC mice, whereas patchy aggregation of MDA5-positive cells was detected in the liver of CDAHFD mice (Fig. 1b). Notably, we confirmed that MDA5-positive cells accumulated around the large lipid droplets (Fig. 1c). In the liver of NASH, the distinguishing histological feature is the presence of activated macrophages aggregated around hepatocytes with fat degeneration known as “hepatic crown-like structure” [23]. To investigate whether these MDA5-positive cells were macrophages or not, we performed double staining with antibodies against MDA5 and macrophage markers, F4/80, CD11b, and CD68. As shown in Fig. 2a, b, and c, each of these markers was co-localized with MDA5, indicating that MDA5 was expressed in the activated macrophages. Some of these cells were expressing CD3 (Fig. 2d). As it has been reported that 15% of MDMϕ are positive for CD3 [24], these cells might include T lymphocytes or a subpopulation of CD3+ macrophages.

Next, we examined whether MDA5 was induced in the early phase of NASH. In western blotting, there was no difference in the expression of MDA5 between NC mice and CDAHFD mice (Fig. 3a), whereas in IF, MDA5-positive cells began to appear even at 1 week after CDAHFD (Fig. 3b). After 3 weeks of breeding, the MDA5 protein level in CDAHFD mice was significant than that in NC mice (Fig. 3c), and infiltrating MDA5-positive cells were markedly increased in the liver of CDAHFD mice (Fig. 3d). These cells were expressing both F4/80 and CD11b (Fig. 4a, b),
suggesting that MDA5-positive MDMϕ were infiltrated into the liver in the early phase of NASH. Additionally, the results of qPCR revealed that hepatic expression of MDA5, as well as C–C motif chemokine 2 (CCL2) mRNA, was significantly upregulated in CDAHFD mice even at 1 week (Fig. 5a), whereas the expression of proinflammatory cytokines, TNF-α, IL-6, and IL-12p40 remained almost unchanged. The mRNA expression of TNF-α, IL-12p40, CCL2, and C-X-C motif chemokine ligand 10 (CXCL10) mRNA started to increase after 3 weeks (Fig. 5b), and at 6 weeks, the mRNA expression of any cytokines and chemokines was significantly upregulated in CDAHFD-fed mice (Fig. 5c). These results indicated that the expression of MDA5 mRNA was upregulated prior to the enhanced expression of proinflammatory cytokines.

Hepatic Macrophages Express MDA5 in Human NAFLD

We performed IF with human liver tissues from 11 patients with NAFLD and 8 control cases. Immunoreactivity of MDA5 was detectable in 25% (2/8) of control cases.
whereas MDA5-positive cells were detected in 73% (8/11) of the patients with NAFLD (Fig. 6a). MDA5-positive area in NAFLD patients (8.4%) was significantly larger than that in control cases (1.7%) (Fig. 6b). As shown in Fig. 6c and 6d, MDA5-positive cells co-expressed CD68 or CD11b as noted in the examinations in mice, suggesting that the infiltrating macrophages expressed MDA5 in human NAFLD also.

**Stimulation with LPS Induces the Expression of MDA5 in Cultured Macrophages**

We have previously reported that MDA5 expression is upregulated upon stimulation with LPS or TNF-α in some cell types [25–27]. To further elucidate the mechanisms mediating MDA5 expression in macrophages, we used cultured THP-1-derived...
macrophage-like cells and primary murine BMDM. As shown in Fig. 7a, the expression of MDA5 mRNA was increased upon stimulation with either LPS or TNF-α in a concentration-dependent manner in THP-1, whereas the stimulation with saturated fatty acid palmitate failed to induce MDA5 expression. In BMDM, MDA5 expression was upregulated in response to LPS, while the stimulation with neither TNF-α nor palmitate led to the increased expression of MDA5 (Fig. 7b). Consistently, the protein level of MDA5 was markedly increased upon stimulation with LPS in both cells, whereas palmitate did not affect the expression in either cell (Fig. 7c, d). Stimulation with TNF-α resulted in the increased expression of MDA5 protein in THP-1, but not in BMDM (Fig. 7c, d).

DISCUSSION

Hepatic macrophages play a key role in maintaining homeostasis and in the disease development in NAFLD [28]. In the present study, we demonstrated that F4/80+ hepatic macrophages express MDA5 in CDAHFD-fed mice. F4/80+ macrophages are thought to be a heterogeneous cell population. F4/80+ CD11b+ CD68+ cells have potent phagocytic activity, whereas F4/80+
CD11b⁺ CD68⁺ cells highly produce proinflammatory cytokines such as TNF-α and IL-12 in response to LPS [29]. Interestingly, resident KCs undergo apoptosis during the NASH diet, and the loss of KCs is compensated by infiltrating MDMϕ [30]. As MDMϕ are known to be CD11b⁺ [6], it is thought that this subpopulation is infiltrated MDMϕ in NASH liver. CD11b⁺ macrophages are involved in high-fat diet-induced insulin resistance [31]. Moreover, depletion of CD11b⁺ macrophages by exposure to irradiation leads to marked inhibition of liver inflammation regardless of steatosis [32], suggesting that CD11b⁺ macrophages promote the inflammation in NASH. Therefore, it is important to elucidate the precise functions of these subsets of cells to better understand the pathogenesis of NASH. It should be noted that MDA5 was expressed in CD11b⁺ macrophages and detected at 1 week after CDAHFD, that is, in the early phase of NASH. We confirmed that the area with steatosis was less than one-third of the liver on average, and that the mRNA expression of proinflammatory cytokines had not yet been upregulated at 1 week after CDAHFD. Although it has not been clarified when MDA5 is induced in the macrophages, MDA5 may have an influence on the immunological function of the cells. In addition, we found that some of the MDA5-positive cells co-expressed a mature T cell marker CD3. It can be speculated that intrahepatic T cells may express MDA5 as well as macrophages, or they may be a subpopulation of CD3⁺ macrophages as previously reported [24]. Since MDA5 was almost completely co-localized with F4/80, CD11b, and CD68, we believe that MDA5-expressing cells are predominantly comprised of MDMϕ. Further analysis using

**Fig. 5** Hepatic mRNA expression at 1 week (a), 3 weeks (b), or 6 weeks (c). RNA was extracted from the liver, and mRNA expression of MDA5, TNF-α, IL-6, IL-12p40, CCL2, and CXCL10 was analyzed by qPCR. Results are presented as mean ± SEM of three to five mice in each group. ***p < 0.001, **p < 0.01, *p < 0.05 vs. NC mice.
flow cytometry or immunohistochemistry is required to confirm which types of cells express MDA5.

MDA5-positive cells were detected in human NAFLD. The immunoreactivity of MDA5 was positive in more than 70% of human NAFLD cases, and the positive staining area was significantly larger in NAFLD patients compared with controls. MDA5 co-existed with CD68 and CD11b in the same manner as murine NASH, suggesting that MDMϕ express MDA5 in human NAFLD. In this study, both NAFL patients without any fibrosis and NASH patients were included, and MDA5 expression was independent of the clinical stage of NAFLD, suggesting that MDA5 can be induced in the early phase of the disease. This finding is consistent with the results obtained from the murine NASH samples as described above.

In an in vitro study, we demonstrated that saturated fatty acid palmitate failed to induce MDA5 expression in THP-1 cells and murine BMDM, while the stimulation with LPS led to upregulation of the expression in both cells. These findings suggest that gut microbiota-derived antigen is essential for the induction of MDA5.

Fig. 6 Hepatic macrophages express MDA5 in human NAFLD. Liver biopsy specimens were obtained from 11 patients with NAFLD and 8 cases without any existing liver diseases (control), and the sections were subjected to immunofluorescence (IF). a The ratio of MDA5-positive cases was significantly higher in NAFLD group compared with control group (*p<0.05 vs. control). b MDA5-positive area in NAFLD cases was significantly larger in than that in controls (*p<0.05 vs. control). c IF was performed using anti-MDA5 (red) and anti-CD68 (green) antibodies. Cell nuclei were stained with DAPI (blue). Representative images of NAFLD and control cases are shown. Original magnification: ×200. d IF was performed using anti-MDA5 (green) and anti-CD11b (red) antibodies. Cell nuclei were stained with DAPI (blue). Representative images are shown. Original magnification: ×200.
been reported that high fat diet for only 1 week results in dysbiosis and bacterial translocation into the liver in a diet-induced murine model of NASH [33]. Thus, the upregulation of MDA5 may occur on the background of increased gut permeability and dysbiosis in NAFLD. Further analysis on the association between hepatic MDA5 and gut microbiota is required to confirm our hypothesis. Another limitation of this study is that we did not determine the effects of interaction between macrophages and other types of cells including hepatocytes and the intracellular signaling for the induction of MDA5.

The mechanism through which MDA5 modulates the inflammation remains to be elucidated. Activated macrophages secrete a variety of chemokines that infiltrate immune cells in inflamed sites. We have previously shown that LPS-induced C-X-C
motif chemokine ligand 10 (CXCL10) production is significantly suppressed by knockdown of MDA5 in human mesangial cells and U373MG human astrocytoma cells [25, 26]. Moreover, MDA5 knockdown resulted in a significant decrease in TNF-α-induced CXCL10 expression in HuH-7 and HLE human HCC cells [27]. These results suggest that MDA5 may positively modulate TLR4 signaling and TNF-α-mediated immune responses. We speculate that MDMϕ may exacerbate TLR4-mediated inflammation in the liver via MDA5. The functional role of MDA5 in the pathogenesis of NASH should be further investigated in future studies.

CONCLUSION

MDA5 was upregulated in the NASH liver. Hepatic macrophages expressed MDA5 in human NAFLD patients and CDAHFD-fed mice. MDA5 may contribute not only to viral infection but also to metabolic inflammation in the liver.

AUTHOR CONTRIBUTION

S. Kawaguchi: conceptualization, resources, investigation, writing—original draft. H. Sakuraba: conceptualization, resources. M. Horiuchi, J. Ding, T. Matsumiya, and K. Seya: investigation, writing—review and editing. C. Iino and T. Endo: investigation, resources. H. Kikuchi: conceptualization, resources. S. Yoshida and H. Hiraga: methodology. S. Fukuda: resources, supervision. T. Imaizumi: writing—review and editing, resources, project administration.

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DECLARATIONS

Ethics Approval and Consent to Participate All animal experiments were conducted in accordance with the Guidelines for Animal Experimentation of the Hirosaki University ( Permit number: M20016). In retrospective studies using human liver biopsy specimens, informed consent was obtained from all patients in an opt-out format. This study protocol was approved by the Ethics Committee of the Hirosaki University Graduate School of Medicine ( Permit number: 2018–152), and the study was performed in accordance with the 1964 declaration of Helsinki and its later amendments.

Consent for Publication Yes.

Competing Interests The authors declare no competing interests.

REFERENCES

1. White, D.L., F. Kanwal, and H.B. El-Serag. 2012. Association between nonalcoholic fatty liver disease and risk for hepatocellular cancer, based on systematic review. Clinical Gastroenterology and Hepatology 10 (12): 1342–1359.
2. TILG, H., and A.R. Moschen. 2010. Evolution of inflammation in nonalcoholic fatty liver disease: The multiple parallel hits hypothesis. Hepatology 52 (5): 1836–1846.
3. Marra, F., and G. Svegliati-Baroni. 2018. Lipotoxicity and the gut-liver axis in NASH pathogenesis. Journal of Hepatology 68 (2): 280–295.
4. Hui, J.M., A. Hodge, G.C. Farrell, J.G. Kench, A. Kriketos, and J. George. 2004. Beyond insulin resistance in NASH: TNF-alpha or adiponectin? Hepatology 40 (1): 46–54.
5. Chassaing, B., L. Etienne-Mesmin, and A.T. Gewirtz. 2014. Microbiota-liver axis in hepatic disease. Hepatology 59 (1): 328–339.
6. Wen, Y., J. Lambrecht, C. Ju, and F. Tacke. 2021. Hepatic macrophages in liver homeostasis and disease-diversity, plasticity and therapeutic opportunities. Cellular & Molecular Immunology 18 (1): 45–56.
7. Seki, E., and D.A. Brenner. 2008. Toll-like receptors and adaptor molecules in liver disease: Update. Hepatology 48 (1): 322–335.
8. Blériot, C., and F. Ginhoux. 2019. Understanding the heterogeneity of resident liver macrophages. Frontiers in Immunology 10: 2694.
9. Karlmark, K.R., R. Weiskirchen, H.W. Zimmermann, N. Gassler, F. Ginhoux, C. Weber, M. Merad, T. Luedde, C. Trautwein, and F. Tacke. 2009. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. Hepatology 50 (1): 261–274.
10. Rivera, C.A., P. Adegboyega, N. van Rooijen, A. Tagalicud, M. Allman, and M. Wallace. 2007. Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. Journal of Hepatology 47 (4): 571–579.
11. Miura, K., L. Yang, N. van Rooijen, H. Ohnishi, and E. Seki. 2012. Hepatic recruitment of macrophages promotes nonalcoholic steatohepatitis through CCR2. American Journal of Physiology. Gastrointestinal and Liver Physiology 302 (11): G1310-1321.
12. Yoneyama, M., M. Kikuchi, K. Matsumoto, T. Imaizumi, M. Miyagishi, K. Taira, E. Foy, Y.M. Loo, M. Gale Jr., S. Akira, S. Yonehara, A. Kato, and T. Fujita. 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in...
antiviral innate immunity. *The Journal of Immunology* 175 (5): 2851–2858.

13. Crampton, S.P., J.A. Deane, L. Feigenbaum, and S. Bolland. 2012. Ifih1 gene dose effect reveals MDA5-mediated chronic type I IFN gene signature, viral resistance, and accelerated autoimmunity. *The Journal of Immunology* 188 (3): 1451–1459.

14. Asdonk, T., M. Steinhertz, A. Großmann, C. Ströcker, C. Lahrmann, I. Motz, K. Pauli-Krahe, A. Frenzel, T. Schmitz, W. Barchet, G. Hartmann, G. Nickenig, and S. Zimmer. 2016. MDA-5 activation by cytoplasmic double-stranded RNA impairs endothelial function and aggravates atherosclerosis. *Journal of Cellular and Molecular Medicine* 20 (9): 1696–1705.

15. Matsui, T., T. Imaizumi, T. Shimoyama, M. Sawaya, T. Kunikazu, T. Matsumiya, K. Satoh, and S. Fukuda. 2012. Expression of melanoma differentiation-associated gene 5 is increased in human gastric mucosa infected with Helicobacter pylori. *Journal of Clinical Pathology* 65 (9): 839–843.

16. Imaizumi, T., T. Aizawa-Yashiro, K. Tsuruga, H. Tanaka, T. Matsumiya, H. Yoshida, T. Matsui, F. Xing, R. Hayakari, and K. Satoh. 2012. Melanoma differentiation-associated gene 5 regulates the expression of a chemokine CXCL10 in human mesangial cells: Implications for chronic inflammatory renal diseases. *Tohoku Journal of Experimental Medicine* 228 (1): 17–26.

17. Sadler, A.J. 2018. The role of MDA5 in the development of autoimmune disease. *Journal of Leukocyte Biology* 103 (2): 185–192.

18. Nakashima, R., Y. Imura, S. Koyabashy, N. Yokawa, H. Yoshifumi, D. Kawabata, K. Ohmura, T. Usui, T. Fujii, K. Okawa, and T. Mimori. 2010. The RIG-I-like receptor IFIH1/MDA5 is a dermatomyositis-specific autoantigen identified by the anti-CADM-140 antibody. *Rheumatol. 49 (3): 433–440.

19. Nagashima, T., Y. Kamata, M. Iwamoto, H. Okazaki, N. Fukushima, and S. Minota. 2019. Liver dysfunction in anti-melanoma differentiation-associated gene 5 antibody-positive patients with dermatomyositis. *Rheumatology International* 39 (5): 901–909.

20. Nissar, A.U., L. Sharma, M.A. Mudasir, L.A. Nazir, S.A. Umar, P.R. Sharma, R.A. Vishwakarma, and S.A. Tadque. 2017. Chemical chaperone 4-phenyl butyric acid (4-PBA) reduces hepatocellular lipid accumulation and lipotoxicity through induction of autophagy. *Journal of Lipid Research* 58 (9): 1855–1868.

21. Matsutomo, M., N. Hada, Y. Sokamaki, A. Uno, T. Shiga, C. Tanaka, T. Ito, A. Katsume, and M. Sudoh. 2013. An improved mouse model that rapidly develops fibrosis in non-alcoholic steatohepatitis. *International Journal of Experimental Pathology* 94 (2): 93–103.

22. Schneider, C.A., W.S. Rasband, and K.W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9: 671–675.

23. Itoh, M., H. Kato, T. Suganami, K. Konuma, Y. Marumoto, S. Terai, H. Sakugawa, K. Kanai, M. Hamaguchi, T. Fukushima, S. Aoe, K. Akiyoshi, Y. Komohara, M. Takeya, I. Sakaida, and Y. Ogawa. 2013. Hepatic crown-like structure: a unique histological feature in non-alcoholic steatohepatitis in mice and humans. *PLoS ONE* 8 (12): e82163.

24. Rodriguez-Cruz, A., D. Vesin, L. Ramon-Lueng, J. Zúñiga, V.F.J. Quesniaux, B. Ryffel, R. Lascurain, I. García, and L. Chávez-Galán. 2019. CD3+ macrophages deliver proinflammatory cytokines by a CD3- and transmembrane TNF-dependent pathway and are increased at the BCG-infection site. *Frontiers in Immunology* 10: 2550.

25. Imaizumi, T., T. Aizawa-Yashiro, S. Watanabe, T. Matsumiya, H. Yoshida, T. Matsui, F. Xing, P. Meng, R. Hayakari, K. Tsuruga, and H. Tanaka. 2013. TLR4 signaling induces retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5 in mesangial cells. *Journal of Nephrology* 26 (5): 886–893.

26. Imaizumi, T., K. Murakami, K. Ohta, H. Seki, T. Matsumiya, P. Meng, R. Hayakari, F. Xing, T. Aizawa-Yashiro, T. Matsui, H. Yoshida, and H. Kijima. 2013. MDA5 and ISG56 mediate CXCL10 expression induced by toll-like receptor 4 activation in U373MG human astrocytoma cells. *Neuroscience Research* 76 (4): 195–206.

27. Kawaguchi, S., H. Sakuraba, T. Haga, T. Matsumiya, K. Sera, T. Endo, N. Sawada, C. Iino, H. Kikuchi, H. Hiraga, S. Fukuda, and T. Imaizumi. 2019. Melanoma differentiation-associated gene 5 positively modulates TNF-a-induced CXCL10 expression in cultured HuH-7 and HLE cells. *Inflammation* 42 (6): 2095–2104.

28. Li, H., Y. Zhou, H. Wang, M. Zhang, P. Qiu, M. Zhang, R. Zhang, Q. Zhao, and J. Liu. 2020. Crosstalk between liver macrophages and surrounding cells in nonalcoholic steatohepatitis. *Frontiers in Immunology* 11: 1169.

29. Kinoshita, M., T. Uchida, A. Sato, M. Nakashima, S. Shono, Y. Habs, H. Miyazaki, S. Hiroi, and S. Seki. 2010. Characterization of two F4/80-positive Kupffer cell subsets by their function and phenotype in mice. *Journal of Hepatology* 53 (5): 903–910.

30. Seidman, J.S., T.D. Troutman, M. Sakai, A. Gola, N.J. Spann, H. Bennett, C.M. Bruni, Z. Ouyang, R.Z. Li, X. Sun, B.T. Vu, M.P. Pasillas, K.M. Ego, D. Gosselin, V.M. Link, L.W. Chong, R.M. Evans, B.M. Thompson, J.G. McDonald, M. Hosseini, J.L. Witzum, R.N. Gemain, and C.K. Glass. 2020. Niche-specific reprogramming of epigenetic landscapes derives myeloid cell diversity in nonalcoholic steatohepatitis. *Immunity* 52 (6): 1057–1074.e7.

31. Zheng, C., Q. Yang, C. Xu, P. Shou, J. Cao, M. Jiang, Q. Chen, G. Cao, Y. Han, F. Li, W. Cao, L. Zhang, L. Zhang, Y. Shi, and Y. Wang. 2015. CD11b regulates obesity-induced insulin resistance via limiting alternative activation and proliferation of adipose tissue macrophages. *Proc Natl Acad Sci U S A* 112 (52): E7239–E7248.

32. Nakashima, H., M. Nakashima, M. Kinoshita, M. Ikarashi, H. Miyazaki, H. Hanaka, J. Imaki, and S. Seki. 2016. Activation and increase of radio-sensitive CD11b+ recruited Kupffer cells / macrophages in diet-induced steatohepatitis in FGF5 deficient mice. *Science and Reports* 6: 34466.

33. Moursies, J., P. Brescia, A. Silvestri, I. Spadoni, M. Sorribas, R. Wiest, E. Mileti, M. Galbiati, P. Invernizzi, L. Adorini, G. Penna, and M. Rescigno. 2019. Microbiota-driven gut vascular barrier disruption is a prerequisite for non-alcoholic steatohepatitis development. *Journal of Hepatology* 71 (6): 1216–1228.