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Interleukin-34 drives macrophage polarization to the M2 phenotype in autoimmune hepatitis

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ABSTRACT

Background: Autoimmune hepatitis is a chronic inflammatory disease, the abnormal immunological function is the main pathogenesis. Interleukin-34 is a newly identified cytokine that shares the same receptor as colony stimulating factor-1.
Methods: We used interleukin-34 knockout and wild-type mice in a Con A-induced hepatitis model and co-cultured RAW264.7 macrophage cells with interleukin-34. We then detected associated inflammatory cytokine and chemokine levels to elucidate the role of interleukin-34.
Results: In this study, we found that the loss of interleukin-34 resulted in higher sensitivity to Con A-induced hepatitis. RAW264.7 macrophage cells were able to differentiate to the M2 phenotype upon interleukin-34 stimulation.
Conclusions: We conclude that interleukin-34 may protect the liver from Con A-mediated hepatitis by driving M2 macrophage polarization and suppressing inflammation.

1. Introduction

Hepatitis is a global health problem caused by viral infections, autoimmune diseases, fatty liver diseases, and metabolic disorders. Acute hepatitis is characterized by strong inflammation, which induces hepatocyte death and can lead to liver failure [12,28]. Autoimmune hepatitis (AIH) is a chronic inflammatory disease in which unknown triggers lead to a primarily T cell-mediated immune response that targets the liver, and the main auto-antigen of this process has not yet been identified [15,24]. Therefore, AIH treatment is still based on non-selective immunosuppression and non-specific immunosuppressive agents. In addition, the diagnostic criteria of AIH has limitations for acute disease presentations as well as atypical cases, and further refinement is required [13]. Despite the lack of a standardized animal model for AIH, various murine models are based on T cell activation in the liver and adaptive and innate immune cell interactions [11]. Concanavalin A (Con A)-induced hepatitis is an appropriate animal model for drug research and immune-mediated liver injury for human hepatitis [30]. Con A activates primarily $CD4^+$ T helper cells in this model [29]. However, macrophage deletion prevents Con A-induced hepatitis [2], which indicates that Con A-mediated hepatitis involves macrophage-T_H cell interactions. Moreover, the excessive activation of macrophages induces liver damage and is the most common cause of mortality during hepatitis B virus (HBV) and hepatitis C virus (HCV) infection [20].

Macrophages are an essential component of innate immunity and play an important role in inflammation and host defense. Cells of the monocyte-macrophage lineage are characterized by considerable diversity and plasticity. In fact, diversity and plasticity are hallmarks of macrophages. Macrophages exert dual functions in liver pathology

Abbreviations: AIH, autoimmune hepatitis; Con A, concanavalin A; IL-34, interleukin-34 CSF-1, colony stimulating factor-1; CSF-1R, colony stimulating factor-1 receptor; M-CSF, Macrophage colony-stimulating factor; PBMCs, peripheral blood mono-nuclear cells; HBV, hepatitis B virus; HCV, hepatitis C virus; WT, wild type; ARG-1, Arginase-1; MRC-2, mannose receptor C type 2; IL-10, interleukin-10; CD163, CD163 antigen; IL-1, interleukin-1; INOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-alpha; ALT, alanine aminotransferase; AST, aspartate aminotransferase

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[6,16]. Macrophages can differentiate into the classical M1 and alternative M2 phenotypes in response to various signals. The M1 phenotype is characterized by high levels of pro-inflammatory cytokine expression, high reactive nitrogen and oxygen intermediate production, Th1 response promotion, and strong microbicidal and tumoricidal activity that inhibits the proliferation of surrounding cells and damages contiguous tissue. In contrast, M2 macrophages are involved in parasite containment, promote tissue remodeling and tumor progression and have immunoregulatory functions that promote the proliferation of contiguous cells and tissue repair [27,31]. In addition, the phenotype of polarized M1 and M2 macrophages can be reversed in vitro and in vivo. Therefore, identifying the molecules associated with dynamic changes in macrophage polarization and understanding their interactions are crucial for elucidating the molecular basis of disease progression and designing novel macrophage-mediated therapeutic strategies.

Interleukin-34 (IL-34) is a newly identified cytokine with only partially understood functions, although it was recently found to be correlated with the inflammatory process in certain diseases, such as rheumatoid arthritis [33], inflammatory bowel disease [10] and Sjogren's syndrome [5]. Another study revealed that IL-34 levels increased with fibrosis progression and were an independent marker for liver fibrosis [23,26]. Interestingly, emerging findings indicate that serum IL-34 levels and IL-34 mRNA levels in peripheral blood mono-nuclear cells (PBMCs) were significantly lower in patients with chronic HBV than in healthy controls, and these findings suggest that IL-34 inhibits HBV replication in vitro and in vivo [4]. Moreover, IL-34 was reported to recruit M2 tumor-associated macrophages in tumor tissues [25]. All of these studies indicate that IL-34 plays an important role in inflammation and autoimmune progression. Comprehensive proteomic analyses have shown that IL-34 shares the same receptor as colony stimulating factor-1 (CSF-1) [32]. Although it lacks appreciable similarity to CSF-1 or any other protein [19], IL-34 promotes the differentiation, proliferation and survival of monocytes, macrophages and osteoclasts by binding to CSF-1R as CSF-1 does [1]. More importantly, IL-34 drives monocyte polarization toward the immunosuppressive M2 phenotype in a manner similar to that of M-CSF [9]. Therefore, the role of IL-34 in AIH has not yet been explored. Our team found that IL-34 knockout mice developed more severe hepatitis than wild-type (WT) mice in a Con A-induced model. In addition, we attempted to reveal the mechanism of AIH and find new therapy methods for treating AIH.

2. Materials and methods

2.1. Mice

C57BL/6 mice were obtained from the Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). IL-34$^{-/-}$ mice were obtained from Beijing HFK Bioscience Co. Ltd. All animals were maintained in a temperature-controlled, specific pathogen-free room with a 12-h light and dark cycle and ad libitum access to food in the Experimental Animal Center of Medical School of Nantong University according to the National Laboratory Animal Guidelines. All mice used in the experiments were 6–9 weeks of age. All experiments were approved by the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital and Nantong Third People's Hospital Affiliated to Nantong University.

2.2. Acute liver injury

Con A-induced acute liver injury was induced in the mice as previously reported. Con A was dissolved in pyrogen-free phosphate-buffered saline (PBS) at a dose of 25 mg/kg for the experiments as indicated in the figure legends.

2.3. Cell culture and differentiation

RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Rockford, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C. A total of 2×10^5 RAW264.7 cells were seeded in a 6-well plate with 50 ng/ml IL-34 (R&D, Minneapolis, MN, USA) for 24 h and harvested for qPCR.

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using RNAiso Plus (TaKaRa, Dalian, China), and reverse transcription was performed using PrimeScript™ RT Master Mix (TaKaRa). cDNA amplification was performed using SYBR Green Premix Ex Taq™ II (TaKaRa) according to the manufacturer's instructions. All RT-qPCR procedures were performed using a CFX96 Real-Time PCR Detection System. The indicated gene expression level was normalized to β-actin. Relative expression was calculated using the $2^{-\Delta\Delta cq}$ method [17].

2.5. Detection of inflammatory cytokine and chemokine levels

At 24 h following Con A administration, all mice were sacrificed and serum and liver samples were collected. In the experiments, the serum samples were analyzed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels using a multichannel autoanalyzer. Liver tissue sections were fixed in 10% formalin and embedded in paraffin for subsequent usage. A portion of the liver samples were snap frozen for future RNA studies. The expression levels of IL-34, Arginase-1 (ARG-1), mannose receptor C type 2 (MRC-2), IL-10, CD163, F4/80, IL-1, inducible nitric oxide synthase (INOS), monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-alpha (TNF-α) in the liver were detected by qPCR.

2.6. Histology

Liver tissues were removed and immediately fixed in 10% neutral buffered formalin (NBF). The tissues were stored at −80 °C until use. Formalin-fixed tissues were embedded in paraffin and sectioned at 3–5 μm thickness. Formalin-fixed, de-waxed sections were stained with hematoxylin and eosin (HE) for histopathological observations.

2.7. Statistical analysis

Each experiment was performed in triplicate and repeated at least three times. Data are expressed as the mean ± standard deviation and were analyzed using Student's *t*-test or one-way analysis of variance followed by Tukey's multiple comparison test. Statistical analyses were carried out using GraphPad Prism (GraphPad, La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Induction of immune-mediated hepatitis and the role of IL-34

To determine the regulatory effect of IL-34 on immune-mediated hepatitis, we used Con A to induce hepatitis in WT mice. Next, we detected IL-34 expression in the mice by qPCR and found that IL-34 expression was significantly lower in the Con A model mice than in the negative control mice (Fig. 1A). In addition, we used IL-34 knockout mice to elucidate the role of IL-34 in the Con A-induced hepatitis model. We first evaluated the areas of necrosis in the IL-34$^{-/-}$ mice and WT mice. The necrosis area was larger in the KO mice than in the WT mice (Fig. 1B and 1C).

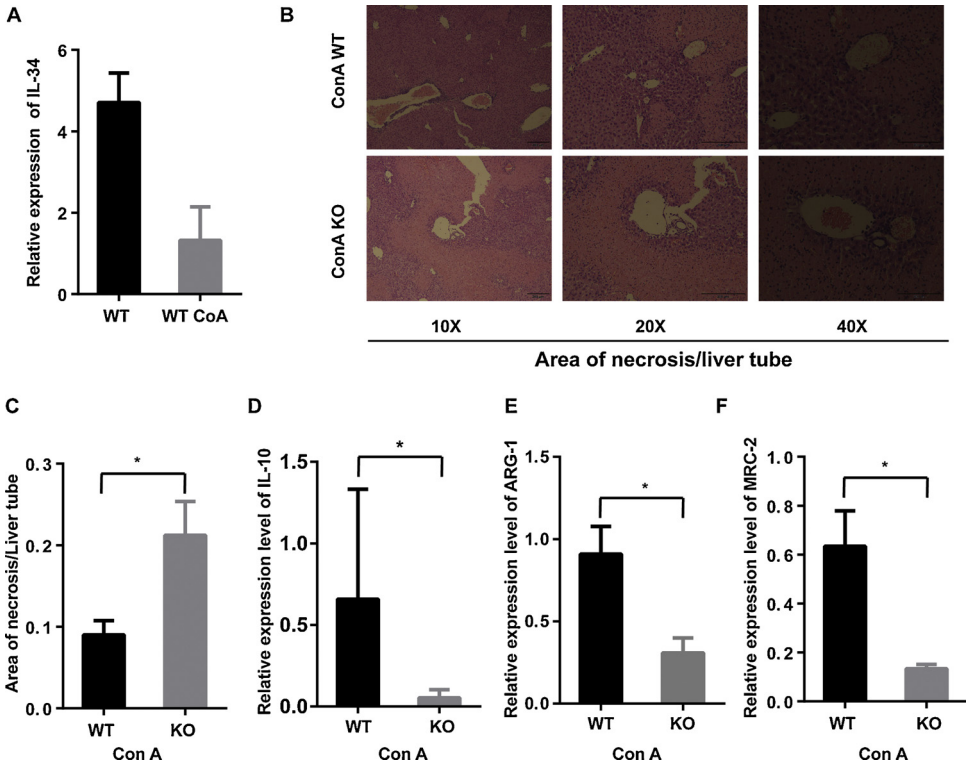

Fig. 1. Con A-mediated liver injury in WT and IL-34 knockout mice. (A) IL-34 expression in WT and Con A-treated WT mice. (B) Representative HE staining of livers in the Con A experiments (x100, x200, x400). (C) Quantification of necrotic lesions / liver tubers in the liver parenchyma, mean ± SD. (D–F) IL-10, ARG-1 and MRC-2 expression levels, mean ± SD. *p < 0.05.

3.2. ALT and AST levels in the AIH model

Firstly, we detected the serum levels of ALT and AST of WT and IL-34$^{-/-}$ mice treated with ConA. We found no differences between the two groups (Figure S. 1A and 1B).

3.3. Cytokine expression levels in Con A-induced hepatitis

We next detected the levels of AIH-associated cytokines in the Con A-induced hepatitis mice. We used qPCR to detect the IL-10 expression in the Con A-treated IL-34$^{-/-}$ and WT mice. As shown in Fig. 1D, IL-10 expression was lower in the IL-34$^{-/-}$ mice than in the WT mice. Then, we detected the expression of ARG-1 and MRC-2, which are M2 macrophage markers, in the livers of the Con A-treated IL-34$^{-/-}$ and Con A-treated WT mice by qPCR. Both markers were significantly lower in the Con A-treated IL-34$^{-/-}$ mice than the Con A-treated WT mice (Fig. 1E and F). Obvious differences were not observed in the expression levels of CD163, F4/80, IL-1, INOS, MCP-1, or TNF-α between the Con A-treated IL-34$^{-/-}$ mice and the Con A-treated WT mice (Figure S. 1C-I).

3.4. IL-34 induces the polarization of RAW264.7 macrophages to the M2 phenotype

To further identify the role of IL-34 in Con A-mediated AIH, we evaluated whether RAW264.7 macrophage cells differentiated into the M1 or M2 phenotype upon IL-34 stimulation. We performed qPCR to detect expression of MRC-2 and ARG-1, which are expressed by M2 macrophages, as well as INOs and MCP-1, which are M1 macrophage markers. The results showed that ARG-1 and MRC-2 expression was remarkably higher in the IL-34 cocultured RAW264.7 macrophages than in the control cells (Fig. 2A and B). However, INOS and MCP-1 expression was remarkably lower in the IL-34 cocultured RAW264.7 macrophages than in the control cells (Fig. 2C and D). There were no obvious differences in IL-10 and IL-6 expression between the two groups (Figure S. 1J and 1K).

4. Discussion

In our study, we assessed the role of IL-34 in Con A-induced hepatitis model. We first found that the expression of IL-34 in the liver was lower in Con A-treated WT mice than in the negative control mice which indicated IL-34 may act as a protective factor in Con A-induced liver injury. Then, we found the area of necrosis was larger in the IL-34$^{-/-}$ mice treated with Con A compare to WT. We concluded that the loss of IL-34 resulted in higher sensitivity to Con A-induced hepatitis. However, the level of serum ALT and AST did not differ between the Con A-treated IL-34$^{-/-}$ mice and WT mice. ALT is primarily localized in the liver, with lower enzymatic activities found in skeletal muscle and heart tissue. AST is localized in the heart, brain, skeletal muscle and liver tissue. The level of serum ALT and AST do not indicate the severity of liver injury because increases in serum ALT and AST enzymatic levels can also arise from extra-hepatic injury, particularly to skeletal muscle [22]. This finding suggests that the IL-34 signaling pathway is critically involved in the pathogenesis of Con A-induced liver damage.

In the Con A-induced hepatitis model, we found that expression of IL-10 which was mainly produced by M2 macrophages, was markedly lower in the Con A-treated IL-34$^{-/-}$ mice than the Con A-treated WT mice. Recently, a study revealed that IL-10 suppresses liver immunopathology by preventing CD8 T_E apoptosis [7]. This report is consistent with the necrosis area data for the Con A-treated IL-34$^{-/-}$ and WT mice. IL-10 mitigates liver injury, and IL-34 loss weakened the expression of IL-10 in this model.

In the Con A model, the expression level of ARG-1, which is an M2 macrophage marker, was remarkably lower in IL-34$^{-/-}$ mice than in WT mice. We concluded that IL-34 loss inhibited the expression of ARG-1, an important anti-inflammatory cytokine in acute liver injury [14]. In addition, we found that the expression of MRC-2, which is expressed in hepatic stellate cells (HSCs) and efficiently takes up denatured collagen, was remarkably decreased [18]. The expression level of CD163 should be reduced in the Con A-treated IL-34$^{-/-}$ mice. However, the expression level of CD163, which is the marker of M2 macrophages, did not differ between the Con A-treated IL-34$^{-/-}$ mice and WT mice. However, the CD163 expression level was suppressed by

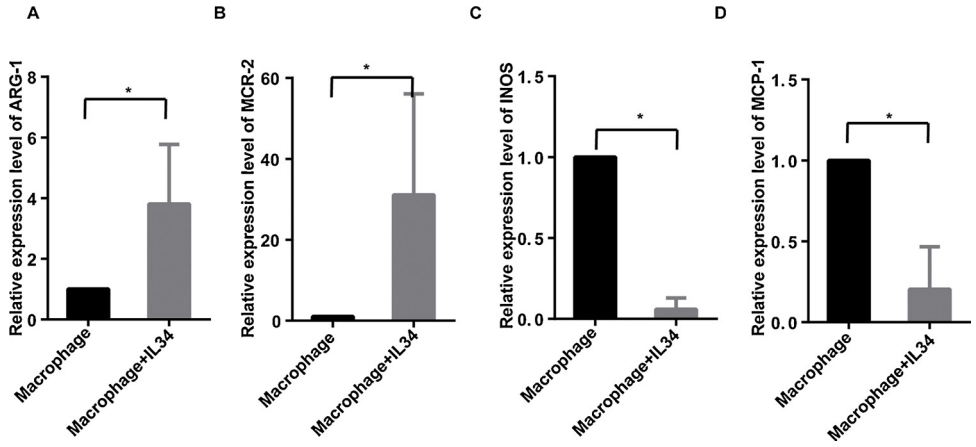

Fig. 2. RAW246.7 macrophages co-cultured with IL-34. (A) ARG-1 expression levels, mean ± SD. (B) MRC-2 expression levels, mean ± SD. (C) INOS expression levels, mean ± SD. (D) MCP-1 expression levels, mean ± SD. *p < 0.05.

proinflammatory mediators, such as lipopolysaccharide, interferon-gamma, and TNF-α, whereas IL-6 and the anti-inflammatory cytokine IL-10 strongly upregulate CD163 mRNA in monocytes and macrophages [3]. CD163 expression is regulated by proinflammatory and anti-inflammatory factors, which may explain why the expression level of CD163 did not differ between the Con A-treated IL-34$^{-/-}$ mice and WT mice. All of these results revealed that the loss of IL-34 inhibited M2 macrophage activation in Con A-mediated hepatitis and aggravated liver tissue necrosis.

In our study, we cocultured RAW264.7 macrophages with IL-34 and then wondered whether the cells could differentiate into M2 macrophages. We detected M1 and M2 macrophage markers and found that the expression level of MRC-2 and ARG-1, which are markers of M2 macrophages, was upregulated in the RAW264.7 macrophages treated with IL-34, whereas the expression level of INOS and MCP-1, which are markers of M1 macrophages, was reduced in the RAW264.7 macrophages cocultured with IL-34. M2 macrophages, which are anti-inflammatory and immunoregulatory, are polarized by Th2 cytokines, such as IL-4 and IL-13. We found that IL-34 also promoted the differentiation of RAW264.7 macrophages into the M2 phenotype. IL-34-polarized M2 macrophages were able to switch memory T cells into Th17 cells and suppress inflammation [8]. Moreover, the M2 polarization of peritoneal macrophages induced regulatory cytokine production and suppressed T cell proliferation in vitro [21]. Although, levels of ALT and AST and the CD163 expression don't quite fit with their model, all of these data indicate that IL-34 may drive the polarization of macrophages toward the M2 phenotype and M2 macrophages may produce anti-inflammation cytokines and suppress T cell proliferation. In order to draw more definite conclusion, we will increase the number of mice and cytokine IL-34 will be injected into the WT mice or IL-34$^{-/-}$ mice before they were treated with Con A in the future. And then, we will compare the levels of ALT, AST and the CD163 expression of IL-34 injected WT mice or IL-34$^{-/-}$ mice treated with Con A with WT mice or IL-34$^{-/-}$ mice only treated with Con A. We concluded that IL-34 may suppress inflammation via driving macrophage polarization toward the M2 phenotype.

5. Conclusions

IL-34 protects the liver from Con A-mediated hepatitis by driving M2 macrophage polarization and suppressing inflammation.

Declaration of competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.152493.

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