RAGE deficiency ameliorates autoimmune hepatitis involving inhibition of IL-6 production via suppressing protein Arid5a in mice

Xiaoxiao Li
Huazhong University of Science and Technology

Shuyao Hua
Huazhong University of Science and Technology

Dai Fang
University of South Carolina

Xiaoyuan Fei
Huazhong University of Science and Technology

Zheng Tan
Huazhong University of Science and Technology

Fang Zheng
Huazhong University of Science and Technology

Weimin Wang
Huazhong University of Science and Technology

Min Fang (minfang89@hust.edu.cn)
Huazhong University of Science and Technology

Research Article

Keywords: RAGE, ConA, IL-6, Arid5a, hepatitis

Posted Date: September 29th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2095068/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

Activation of T cells and pro-inflammatory cytokines are essential for human autoimmune hepatitis. The receptor for advanced glycation end-product (RAGE) is one of receptors for inflammatory alarm molecule high mobility group box 1 (HMGB1), and is involved in autoimmune hepatitis. However, the molecular mechanism of RAGE in setting of autoimmune hepatitis remains elusive. This study aimed to identify the function and mechanism of RAGE in autoimmune hepatitis. The RAGE deficient mouse was used to investigate the role and underlying mechanisms by which RAGE signaling-driven immune inflammatory response in the ConA-induced experimental hepatitis. We found that the RAGE deficiency protects the mouse from liver inflammatory injury caused by ConA challenge. mRNA expression of VCAM-1, IL-6, TNF-α within the livers is markedly decreased in RAGE-deficient mice compared to wild-type mice. In parallel, RAGE deficiency leads to reduced levels of serum pro-inflammatory cytokines IL-6, TNF-α as compared to wild type control mice. RAGE-deficient mice exhibits increased of hepatic NK cells and decreased CD4+ T cells than those of wild type control mice. Notably, in vivo blockade of IL-6 in wild type mice significantly protected mice from ConA induced hepatic injury. Furthermore, RAGE deficiency impaired IL-6 production is associated with decreased expression of Arid5a in liver tissues, a half-life IL-6 mRNA regulator. RAGE signalling is important in regulating the development of autoimmune hepatitis. Immune modulation of RAGE may represent a novel therapeutic strategy to prevent immune-mediated liver injury.

Introduction

RAGE (the receptor for advanced glycation end-product) is a multiligand receptor that binds structurally diverse molecules, such as AGEs (advanced glycation end products), HMGB1 (high mobility group box 1), S100 family members, amyloid-beta and DNA[12]. RAGE regulates a number of cell processes like inflammation, apoptosis, proliferation and autophagy [3]. Targeting RAGE signaling through use of inhibitors and anti-RAGE antibodies can be promising treatment strategy [4]. Indeed, we and others have showed that HMGB1/ RAGE signaling axis plays an important role in the pathogenesis of autoimmune hepatitis and liver injury [5-8]. In clinical setting, autoimmune hepatitis patients (AIH) displayed distinct profiles of serum EN-RAGE (extracellular newly identified receptor for advanced glycation end products binding protein), sRAGE (Soluble RAGE) or EN-RAGE/sRAGE compared to healthy controls. Furthermore, these three parameters exhibited potentials as novel biomarkers for AIH diagnosis and prognosis evaluation [9]. Inhibition of HMGB1/RAGE/NF-κB and HMGB1/TLR4/NLRP3 signaling pathways alleviated hepatic inflammation caused by type II diabetes in mice [10]. Additionally, earlier studies showed that RAGE is involved in hepatic ischemia/reperfusion (I/R) injury and liver cancer [11, 12]. However, the molecular mechanism by which RAGE participates in the pathogenesis of autoimmune hepatitis remains unclear. In the present study, we found that RAGE deficiency protects mice from ConA-induced hepatitis, and this protection is associated with regulation of IL-6 production. Particularly, RAGE deficiency dramatically reduces the IL-6 production evoked by ConA challenge via suppressing protein Arid5a, which stabilizing IL-6 mRAN (message RNA).
Materials And Methods

Mice

RAGE knockout mice with C57BL/6 background were a gift from Professor Fang Zheng in our department. Wild type mice that six to eight weeks old C57BL/6 male mice weighing 18–22 g were used. They were bred in specific pathogen-free conditions, and all of the experiments were performed in accordance with the guidelines of the Tongji Medical College Animal Care and Use Committee. The study protocols were specifically reviewed and approved by this ethics committee.

ConA-induced hepatitis

Con A was dissolved in pyrogen-free phosphate-buffered saline (PBS), and intravenously (I.V.) administered to the mice at a dose of 15 mg/kg or 20 mg/kg body weight.

Biochemical and histological

Blood was collected 10 hours after ConA injection and serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurement were examined. Liver tissue was fixed in 4% paraformaldehyde and cut into 4-μm-thick sections for H&E staining.

Immunohistochemical analysis

Paraffin sections (4μm) of liver were incubated with rabbit polyclonal anti-LY6G antibody (1:1000, Servicebio, Wuhan, China) or rabbit monoclonal anti-RAGE antibody (1:4000, Abcam) overnight at 4 °C and then were stained with secondary antibody (1:200, Servicebio, Wuhan, China). The sections of liver were stained with DAB kit (Servicebio, Wuhan, China) according to the manufacturer’s instructions. After that, the cell nuclei were re-dyed and the liver sections were dehydrated and sealed.

Myeloperoxidase (MPO) assay

Liver tissues of experimental mice were measured using myeloperoxidase (MPO) test kit (Nanjing Jiancheng Bioengineering Institution, China) according to the manufacturer’s protocol.

ELISA

Serum levels of IL-6, IFN-γ and TNF-α were measured using an enzyme-linked immunosorbent assay kit (BioLegend, San Diego, CA) according to the manufacturer’s protocol.

Quantitative real-time PCR

Total RNA was extracted from the liver tissue of the experimental mice using RNAiso Plus (TAKARA, Dalian, China), followed by cDNA synthesis using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA USA). Subsequently, cDNA was used to measure the mRNA levels of IL-6, TNF-α and IFN-γ, using Fast Start universal SYBR Green Master Mix (Roche Pharma, AG, Germany). ß-
actin was used as the normalization control. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative mRNA levels. The primer sequences used were as follows

**IL-6**

forward: GAGACTTCCATCCAGTGGCC  
reverse: AAGTGCATCATCGTTGTCATACA

**TNF-α**

forward: CATCTTCTCAAATTCGAGTGACAA  
reverse: TGGGAGTAGACAAGGTACAACCC

**IFN-γ**

forward: TCAAGTGGCATACTGTGAAGAA  
reverse: TGGCTCTGCAGGATTTTCATG

**β-actin**

forward: CATCCGTAAAGACCTCTATGCCAAC  
reverse: ATGGAGCCACCGATCCACA

### Western blot analysis

Liver tissues of the experimental mice were homogenized, and the supernatant containing proteins was collected. The proteins were separated through 10% SDS–polyacrylamide gel electrophoresis and transferred onto PVDF membranes. After blocking for 1 h at room temperature in 5% milk or 5% BSA, the blots were incubated with the antibody overnight at 4°C. Rabbit monoclonal anti-VCAM-1 (1:4000, Abcam), anti-ICAM-1 (1:1000, ABclonal), anti-Arid5a (1 μg/ml, Abcam), anti-STAT3 (1:2000, Cell Signaling Technology), anti-p-STAT3 (1:2000, Cell Signaling Technology), anti-JAK2 (1:1000, Cell Signaling Technology) and anti-p-JAK2 (1:1000, Cell Signaling Technology) were used in the experiments. Secondary antibodies (1:2000, Cell Signaling Technology) were incubated at room temperature for 1.5 h. The stained blots were visualized by an enhanced chemiluminescent (ECL) (Boster) system. The blot density was quantified by Image J.

### Flow cytometry

Hepatic cell suspension was obtained by grinding on 200 mesh steel net, and liver mononuclear cells were isolated by 40% and 70% Percoll (Solarbio, Beijing, China) gradient centrifugation. Splenic lymphocytes were obtained by grinding and lysing red blood cells. The cells were collected and used for
flow cytometry. Liver mononuclear cells and spleen lymphocytes were divided into several parts for cell surface staining and intracellular staining. As for the surface staining, first the cells were stained with Fixable Viability Stain 510(BD), then blocked with CD16/32 antibody and then stained with surface antibody (CD45, CD3, CD4, CD8, NK1.1, CD69) for 30 minutes at 4°C, and finally washed with PBS. For detection of RAGE by flow cytometry, anti-RAGE (R&D) were first stained and then secondary antibodies were stained. As for intracellular staining, the cells were stimulated with RPMI 1640 medium containing 10% FBS and Cell Activation Cocktail (with Brefeldin A) (1:500, BioLegend) for 5h. After stimulation, the cells were stained with surface markers (CD45, CD3, CD4, CD11C and F4/80) for 30 min at 4 °C. Subsequently, the cells were fixed and permeabilized with Cytofix/Cytoperm (BD) for 20 min at 4 °C and then incubated with intracellular antibodies (IL-17A, IFN-γ, IL-4 and/or IL-6) for 50 min at 4 °C. All antibodies were purchased from BioLegend (San Diego, CA, USA) except for IL-6(BD). Flow cytometry data were collected by BD Verse cytometer and analysed by FlowJo X.

T cell proliferation in vitro

The spleen lymphocytes were labeled with CFSE (5μM/ml, BioLegend) and stimulated with ConA (2μg/ml) in vitro. The cells stained with CD3 were collected at 48h and 72h respectively for flow cytometry.

Statistical analysis

Results are shown as means ± standard deviation (SD). Statistical significance of differences was analyzed by a one-way ANOVA or Student’s t test. Data were analyzed using GraphPad Prism 8 software. Values of p < 0.05 were considered significant.

Results

Expression of RAGE increases in liver tissues and T cells by Con A challenge

We first examined the RAGE expression levels by liver tissues and T cells after injection of Con A. Immunochemical staining for RAGE in liver tissues showed that the RAGE expression was significantly increased in ConA challenged mice than that of PBS injected control animals (Fig.1A, B). Also, RAGE expressed by CD4+ T cells isolated from hepatic mononuclear lymphocytes as well as spleens was markedly increased in ConA challenged group as compared with control mice with PBS injection (Fig.1C). Interestingly, flow cytometric analysis revealed that RAGE expression by CD8+ T cells generated from both livers and spleens did not changed irrespective of ConA challenge or PBS control injection (Fig.1D).

RAGE deficiency protects mice from ConA-induced hepatitis

Next, the survival rate of mice with ConA challenge was determined on both RAGE deficiency mice and wild-type (WT) control mice. Mice with RAGE deficiency survived significantly longer than that of wild type (WT) control mice after ConA challenge (Fig.2A). In parallel, serum aminotransferases activities (ALT and

Page 5/20
AST) evoked by ConA injection in mice with RAGE deficiency were significantly lower than that of WT control mice (Fig.2B). As shown liver and spleen samples, these organs derived from RAGE deficient mice exhibited lesser damages after ConA treatment compared with those of from wild type control mice (Fig.2C). Consistently, histological analysis revealed that the liver tissues generated from RAGE-deficient mice displayed lesser inflammation and necrosis as compared with that of WT control mice (Fig.2 D).

**Defect in RAGE signalling inhibits infiltration of neutrophil and the production of pro-inflammatory cytokines**

Previously, we demonstrated the implication of HMGB1 in ConA-induced acute hepatitis [5]. We now sought to address the role of RAGE, one of receptors for HMGB1 in inflammatory response during ConA-induced hepatitis. As shown in Fig.3, RAGE deficiency resulted in significantly decrease of inflammation-related adhesion molecule VCAM-1 (vascular cell adhesion molecule-1, VCAM-1) expression in liver tissues upon ConA challenge, while another adhesion molecule ICAM-1 (intercellular adhesion molecule-1, ICAM-1) did not changed between RAGE deficient mice and WT controls after ConA treatment (Fig.3A). Moreover, in contrast to controls, the infiltration of neutrophils in RAGE-deficient liver was dramatically attenuated upon administration of ConA (Fig.3B). Quantitative RT-PCR analysis of IL-6 in the liver after Con A administration revealed a remarkable decrease of mRNA levels of IL-6 after RAGE deficiency, while mRNA levels of IFN-γ and TNF-α did not reach the significantly change between RAGE deficient mice and WT controls (Fig.3C). Interestingly, serum pro-inflammatory cytokines IL-6 and TNF-α protein levels remarkably decreased in RAGE deficient mice as compared with WT control animals (Fig.3D).

**RAGE deficiency changes the proportions of leukocytes in the liver challenged by ConA**

Resident leukocytes in the liver plays a critical role for the autoimmune hepatitis, therefore, the effect of RAGE deficiency on the development of leukocytes in the liver was examined. It was found that RAGE deficiency led to hepatic T cells significantly lower than those of WT control mice, while the proportion of NKT cells was comparable between RAGE deficient mice and WT control mice upon ConA injection (Fig.4A). In contrast, NK cells marked increased in RAGE deficient mice as compared to WT control mice after ConA challenge (Fig.4A). Moreover, mice challenged with ConA, in the hepatic T-cell subset, mice with RAGE deficiency exhibited significantly decreased CD4⁺ T cells than that of WT control mice. However, hepatic CD8⁺ T cells have no change between RAGE defect mice and control mice (Fig.4B).

**T cells without RAGE affects T-cell subsets activation and proliferation evoked by Con A challenge**

To examine the effect of RAGE deficiency on T-cell activation and proliferation stimulation by ConA, flow cytometry staining analysis (CD69 as activation marker) revealed that RAGE deficiency did not affect the activation of T cells in the liver upon ConA stimulation (Fig.5A). Interestingly, as compare with WT control, defect in RAGE significantly inhibited the activation of spleen CD4⁺ T cells while did not affect the activation of spleen CD8⁺ T cells by Con A stimulation (Fig.5B). CFSE staining analysis revealed that RAGE deficiency decreased proliferation of spleen T cells as compared with WT T cells (Fig.5C).
Defect of RAGE inhibits the differentiation of Th17 cells in liver

To further define the influence of RAGE deficiency on the CD4\(^+\) T-cell subsets differentiation, the cytokines profiles produced by T cells were evaluated by flow cytometry using intracellular cytokines staining. It was found that defect of RAGE did not affect the proportion of Th1 and Th2 cytokines secretion by T cells activated by ConA as compared to WT control T cells (Fig. 6A-D). However, RAGE deficiency significantly inhibited the proportion of Th17 cells with the capacity of IL-17 production (Fig.6E, F).

Blockade of IL-6 alleviates the hepatitis in RAGE competent mice challenged with ConA

To confirm that IL-6 was a critical pro-inflammatory cytokine impaired by RAGE deficiency, anti-IL-6 antibody was used to block the effect of IL-6 caused hepatitis in vivo. Blockade of IL-6 markedly reduced serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in WT control mice to the levels comparable with RAGE-deficient mice (Fig.7A). Consistently, pathological analysis revealed that blockade of IL-6 significantly ameliorated the hepatitis induced by ConA injection in wild type mice (Fig.7B). Collectively, these results confirmed our hypothesis that IL-6 was pivotal in the effect of RAGE on Con A-induced hepatitis.

Decrease of expression of ARID5A protein expression without RAGE results in reduction of IL-6 production in liver

Finally, to explore the molecular mechanism by which RAGE deficiency results in reduced IL-6 production in the liver, which alleviates the hepatitis in mice induced by ConA challenge (Fig.8A), macrophages and T cells in the liver were examined to produce IL-6 by intracellular cytokine staining. As shown in Fig.8B and C, hepatic Kupffer cells and T cells (both CD4\(^+\) and CD8\(^+\) T cells) separated from mice with RAGE deficiency exhibited significantly decreased production of IL-6 as compared to those cells generated from wild type control mice upon ConA challenge. Due to the AT-rich interactive domain-containing protein 5a (Arid5a) plays a critical role in regulating the half-life of Interleukin-6 (IL-6) mRNA [13]. Western blotting analysis revealed that hepatic tissues with RAGE deficiency displayed a significant decreased expression of Arid5a than that of control wild type mice after Con A challenge (Fig.8D). Furthermore, inflammation related signaling pathways molecules expression such as STAT3 with phosphorylation (p-STAT3) and JAK2 with phosphorylation (p-JAK2) in hepatic tissues come from mice with RAGE deficiency significantly lower as compared to that of wild type control mice (Fig.8E).

Discussion

In the present study, we investigated the effects and mechanisms of RAGE deficiency on the pathogenesis of hepatitis in a mouse autoimmune hepatitis model. We demonstrated that deficiency of RAGE in the mice resulted in an ameliorated hepatitis. This protection of ConA-induced hepatitis in RAGE-deficient mice was caused by decreased IL-6 secretion by RAGE-deficient hepatic kupffers cells and T cells.
The biological function of HMGB1 is mediated by multiple receptors, including the receptor for advanced glycation end products (RAGE) and Toll-like receptors (TLRs), which are expressed in different hepatic cells [14]. Previous studies have shown that innate immune cells such as macrophages, dendritic cells, and neutrophils express RAGE expression [15-17]. In this study, we observed that mice injected with ConA induced the expression of RAGE in inflammatory liver tissues. Interestingly, ConA stimulation particularly promoted the expression of RAGE on CD4\(^+\) T cells rather than CD8\(^+\) T cells isolated from both liver and spleen.

We previously have demonstrated that pro-inflammatory cytokines TNF-\(\alpha\) and IFN-\(\gamma\) plays an important roles in detrimental inflammation in hepatitis caused in ConA challenge [5, 18]. Now we showed that RAGE deficiency impaired the expression of IL-6 by hepatic Kupffer cells and CD4\(^+\) T cells to attenuate hepatitis. To our knowledge, this is first time to report that RAGE deficiency to reduce the secretion of pro-inflammatory IL-6 by hepatic immune cells. Indeed, previous studies have suggestion that IL-6 participates in the pathogenesis of inflammation and tumor in the liver, and hepatitis viral B has the capability of stimulating IL-6 secretion by hepatocytes [19, 20]. In addition, RAGE deficiency down-regulates the expression of adhesion molecule VCAM-1, and decreases the infiltration of neutrophils to alleviate autoimmune hepatitis caused by ConA CAM-1.

In addition to aberrant Th1/Th2 T-cell responses in the liver involving in hepatic inflammation, Th17 cells was a recently discovered subtype of CD4\(^+\) T-helper cells. TGF-\(\beta\) and IL-6, two cytokines abundantly present in injured liver promote Th17 cells differentiation to participate in hepatic inflammation [21]. In this study, we observed that RAGE deficiency not only inhibits the production of IL-6 by hepatic Kupffer cells and CD4\(^+\) T cells, but also the decreased IL-6 impaired the generation of hepatic Th17 cells, which further ameliorated autoimmune hepatitis by ConA challenge. Importantly, in vivo blockade of IL-6 using anti-IL-6 blocking antibody dramatically alleviated the hepatitis induced by ConA injection. This further confirmed that IL-6 plays a critical role in the context of RAGE deficiency protects mouse against ConA-induced hepatitis.

What is the mechanism by which RAGE deficiency inhibits the secretion of IL-6 by hepatic immune cells? To answer this question we checked the expression of Arid5a as a unique RNA binding protein in liver tissues, which stabilizes IL-6 mRNA through binding to the 3’ untranslated region of IL-6 mRNA [22]. We found that RAGE deficiency impaired the hepatic expression of Arid5a, and as a result, inhibited the elevation of IL-6 serum level in ConA-treated mice. In line with our finding, it has been shown that Arid5a not only regulates the expression of IL-6 and generation of Th17, but also involves in inflammatory diseases [23, 24]. In parallel, our investigation was supported by Weinlage et al’s study [25]. Moreover, in the present study, we provided a novel molecular explanation that pro-inflammatory cytokine IL-6 plays a critical role in RAGE signaling axis in the pathogenesis of autoimmune hepatitis.

In conclusion, we have presented strong evidence to support that RAGE signaling plays a crucial role in regulating the secretion of IL-6 by hepatic immune cells in the context of ConA-induced autoimmune
hepatitis. This is a new finding that demonstrates a regulatory role of RAGE in autoimmune hepatitis, providing a reference value for the basic research and clinical treatment of autoimmune hepatitis.

Abbreviations

RAGE the receptor for advanced glycation end-product
AGEs advanced glycation end products
HMGB1 high mobility group box 1
AIH autoimmune hepatitis patients
EN-RAGE extracellular newly identified receptor for advanced glycation end products binding protein
sRAGE soluble RAGE
ConA Concanavalin A
I/R hepatic ischemia/reperfusion
ALT alanine aminotransferase
AST aspartate aminotransferase
MPO Myeloperoxidase
ICAM-1 intercellular adhesion molecule-1
VCAM-1 vascular cell adhesion molecule-1
WT wild type mice
Arid5a AT-rich interactive domain-containing protein 5a

Declarations

Ethics approval and consent to participate The study was approved by the Ethics Committee of the Tongji Medical College (Huazhong university of Science and Technology). This study and all the experiments were carried out in accordance with the declaration of Helsinki.

Consent for publication Not applicable
Availabili ty of data and materials The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests The authors declare that they have no competing interests.

Funding This work was supported by grants from the National Natural Science Foundation of China (91542110, 81373167 to M. Fang).

Authors' contributions Xiaoxiao Li, Dai Fang and Min Fang wrote the manuscript. Xiaoxiao Li, Shuyao Hua, Dai Fang, Xiaoyuan Fei, Zheng Tan, Fang Zheng, Weimin Wang prepared the figures 1-8. All authors reviewed the manuscript.

Acknowledgements We thank Mr. Yong Xu and Zhihui Liang for technical assistance with flow cytometry.

References


Figures
Figure 1

The expression of RAGE in mice following ConA challenge

(A) RAGE mRNA expression in liver treated with PBS or ConA for 10h were measured by real-time quantitative PCR. n=3 or 5 per group. (B) The expression of RAGE in liver tissue treated with PBS or ConA for 10h were detected by immunohistochemistry. n=3 per group. (C) RAGE expression in CD4\(^+\)T cells of
the spleen and liver treated with PBS or ConA for 10h. (D) RAGE expression in CD8+ T cells of the spleen and liver treated with PBS or ConA for 10h. Data are presented as means±SD of three to six individual mice per group and represent>3 independent experimental repeats. *p<0.05 vs Control group mice; NS: no significant.

Figure 2

RAGE deficiency protects mice from ConA-induced hepatitis.

(A) Survival was monitored for 36 h after ConA (20 μg/g body weight) injection. n=10 per group. (B) Serum levels of ALT and AST in WT mice and RAGE-deficient mice after intravenous injection of ConA (15 μg/g body weight). n=3 or 6 per group. (C) Liver and spleen of WT mice and RAGE−/− mice displayed different pathological features after ConA injection. (D) Liver sections from RAGE−/− mice and WT mice were stained with H&E 10 h post ConA injection. Bar=100μm. Mice treated with PBS were the control group. Data are presented as means±SD of three to six individual mice per group. Similar results were obtained for three independent experiments. *p<0.05; **p<0.01 vs Control group mice.
Figure 3

RAGE deficiency attenuates the hepatic inflammation response. (A) Western blot of liver tissue of VCAM-1 and ICAM-1 in WT mice and RAGE−/− mice. Representative blots from three independent experiments are shown. (B) Immunohistochemistry and myeloperoxidase activity were used to detect neutrophil infiltration in liver tissue in WT mice and RAGE−/− mice after intravenous injection of ConA. n=3 or 6 per group. (C) Hepatic mRNA levels of IL-6, IFN-γ and TNF-α in WT and RAGE−/− mice following ConA injection.
were measured by real-time quantitative PCR. n=3 or 6 per group. (D) Serum levels of IL-6, IFN-γ and TNF-α in WT mice and RAGE−/− mice after intravenous injection of ConA were measured by ELISA. n=3 or 6 per group. Mice treated with PBS were the control group. Data are presented as means±SD of three to six individual mice per group. Similar results were obtained for three independent experiments. NS: no significant; *p<0.05; **p<0.01; *** p<0.001 vs Control group mice.

**Figure 4**

**RAGE deficiency inhibits the proportion of T cells recruited in the liver following ConA injection.** (A) RAGE deficiency affects T, NK and NKT cell recruitment following ConA injection. Hepatic mononuclear cells were stained with CD3 and NK1.1. The right bar chart shows the percentage of NKT, T and NK cells in liver CD45+ cells from the WT and RAGE−/− mice treated with PBS or ConA for 10h. n=2-5 per group. (B) RAGE deficiency affects CD4+T and CD8+T cells recruitment following ConA injection. The right bar chart shows
the percentage of CD4⁺T and CD8⁺T in liver CD3⁺T cells from the WT and RAGE⁻/⁻ mice treated with PBS or ConA for 10h. Data are presented as means±SD of three to six individual mice per group. Similar results were obtained for three independent experiments. NS: no significant; *p<0.05; **p<0.01 vs Control group mice.

**Figure 5**

**RAGE deficiency affects T cell activation and proliferation following ConA injection.** (A) RAGE deficiency does not affect T cell activation in liver. The right bar chart shows the percentage of CD69 in liver CD4⁺T cells and CD8⁺T cells from the WT and RAGE⁻/⁻ mice treated with PBS or ConA for 10h. n=3 or 6 per group.
(B) RAGE deficiency affects CD4+ T cell activation in spleen. The right bar chart shows the percentage of CD69 in spleen CD4+ T cells and CD8+ T cells from the WT and RAGE−/− mice treated with PBS or ConA for 10h. n=3 or 6 per group. (C) CFSE-labeled WT mice and RAGE−/− mice spleen T cell proliferation in vitro was detected by flow cytometry at 48h and 72h. Lymphocytes were stained with CD3. n=10 per group. Data are presented as means±SD. Similar results were obtained for three independent experiments. NS: no significant; *p<0.05; **p<0.01; *** p<0.001; **** p<0.0001 vs Control group mice.
Figure 6

RAGE deficiency reduces the differentiation of Th17 cells in liver following ConA injection. Hepatic mononuclear cells were prepared, and subjected to flow cytometric analysis. Intracellular cytokine staining was employed to evaluate the changes of Th1 (IFN-γ), Th2 (IL-4) and Th17 (IL-17) cell subsets. (A) RAGE deficiency does not affect Th1 cells. B The right bar chart shows the percentage of IFN-γ⁺ cells in liver CD4⁺T cells from the WT and RAGE⁻/⁻ mice treated with PBS or ConA for 10h. C RAGE deficiency does not affect Th2 cells. D The right bar chart shows the percentage of IL-4⁺ cells in liver CD4⁺T cells from the WT and RAGE⁻/⁻ mice treated with PBS or ConA for 10h. (E) RAGE deficiency reduces Th17 cells. (F) The right bar chart shows the percentage of IL-17⁺ cells in liver CD4⁺T cells from the WT and RAGE⁻/⁻ mice treated with PBS or ConA for 10h. Data are presented as means±SD of three to six individual mice per group. Similar results were obtained for three independent experiments. NS: no significant; *p<0.05; *** p<0.001 vs Control group mice.

Figure 7

Reduction of IL-6 alleviated liver injury of WT mice after ConA injection. (A) Serum levels of ALT and AST in WT mice and RAGE⁻/⁻ mice were showed after intravenous injection of ConA and anti-IL-6 or IgG antibody. Anti-IL-6 (100 μg) or IgG (100 μg) antibody is injected 2 hours after ConA injection. n=3-5 per group. (B) Liver sections from WT mice and RAGE⁻/⁻ mice were stained with H&E 10 h after ConA and anti-IL-6 or IgG antibody injection. Data are presented as means±SD of three mice. Similar results were obtained for twice independent experiments. NS: no significant; *p<0.05 vs Control group mice.
Figure 8

RAGE deficiency reduces the expression of ARID5A protein in liver, which in turn reduces the expression of IL-6.

Hepatic mononuclear from the WT and RAGE−/− mice were isolated after ConA injection (A) Intracellular staining of IL-6 in liver DC was performed. The right bar chart shows the percentage of IL-6+ cells in liver
CD11C^+ cells from the WT and RAGE^−/− mice treated with PBS or ConA for 10h. (B) Intracellular staining of IL-6 in liver KCs was performed. The right bar chart shows the percentage of IL-6^+ cells in liver F4/80^+ cells from the WT and RAGE^−/− mice treated with PBS or ConA for 10h. n=3-6 per group. (C) Intracellular staining of IL-6 in liver CD3^+ T cells, CD4^+ T cells and CD8^+ T cells were performed. The bar charts below show the percentage of IL-6^+ cells in liver T cells from the WT and RAGE^−/− mice treated with PBS or ConA for 10h. n=3 or 5 per group. (D) Western blot of Arid5a in liver tissue of WT mice and RAGE^−/− mice. Representative blots from three independent experiments are shown. (E) Western blots of the total proteins and phosphorylated proteins of JAK2 and STAT3 in liver tissue of WT mice and RAGE^−/− mice. Representative blots from three independent experiments are shown. Data are presented as means±SD of three to six individual mice per group. Similar results were obtained for three independent experiments. NS: no significant; *p<0.05; **p<0.01; *** p<0.001; **** p<0.0001 vs Control group mice.