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Apolipoprotein A-II Suppressed Concanavalin A-Induced Hepatitis via the Inhibition of CD4 T Cell Function

Junji Yamashita,*† Chiaki Iwamura,* Tetsuya Sasaki,*† Kunitoshi Mitsumori,‡ Kazutoshi Ohshima,† Kaori Hada,† Naoko Hara,† Munehisa Takahashi,† Yoshiaki Kaneshiro,† Hitoshi Tanaka,† Kenji Kaneko,† and Toshinori Nakayama*  

Con A-induced hepatitis has been used as a model of human autoimmune or viral hepatitis. During the process of identifying immunologically bioactive proteins in human plasma, we found that apolipoprotein A-II (ApoA-II), the second major apolipoprotein of high-density lipoprotein, inhibited the production of IFN-γ by Con A-stimulated mouse and human CD4 T cells. Con A-induced hepatitis was attenuated by the administration of ApoA-II. The beneficial effect of ApoA-II was associated with reduced leukocyte infiltration and decreased production of T cell-related cytokines and chemokines in the liver. ApoA-II inhibited the Con A-induced activation of ERK-MAPK and nuclear translocation of NFAT in CD4 T cells. Interestingly, exacerbated hepatitis was observed in ApoA-II-deficient mice, indicating that ApoA-II plays a suppressive role in Con A-induced hepatitis under physiological conditions. Moreover, the administration of ApoA-II after the onset of Con A-induced hepatitis was sufficient to suppress disease. Thus, the therapeutic effect of ApoA-II could be useful for patients with CD4 T cell-related autoimmune and viral hepatitis. *The Journal of Immunology, 2011, 186: 3410–3420.

Although nonspecific immunosuppressive drugs are widely used to inhibit autoreactive immune responses, these drugs show numerous side effects during prolonged usage and are therefore not an ideal treatment for autoimmune patients. Autoimmune hepatitis is a progressive chronic disease with occasional exacerbations (1, 2). A standard therapy with prednisolone sodium succinate (prednisolone) alone or in combination with azathioprine as immune suppressive drugs is used for nearly all autoimmune hepatitis patients. However, some patients are resistant to this standard therapy (3, 4), and thus the development of new therapeutic agents is warranted.

T cell-mediated immune responses play an important role in the development and progression of various liver diseases, including autoimmune hepatitis, viral infection, and alcoholic hepatitis (4–10). Con A-induced hepatitis is a murine experimental model of autoimmune or viral hepatitis that shares several pathological properties with the disease in humans (11). Con A-induced hepatitis has also been used as a model of T cell-mediated liver injury (12), and the infiltration of CD4 T cells into the liver is critical for the development of human autoimmune and viral hepatitis (6, 9, 10). The importance of T cells during the induction and effector phases of Con A-induced hepatitis has been well documented (12–15). Pretreatment with T cell-specific Abs or immunosuppressive agents, such as anti-thymocyte differentiation Ag 1, anti-CD4 mAb, FK506 (Tacrolimus), or cyclosporine A, inhibit Con A-induced hepatitis, indicating that CD4 T cells and their activation of TCR-mediated signaling are required for the induction of Con A-induced hepatitis (12). In addition, IFN-γ appears to be important for the development of Con A-induced hepatitis (13–15). Moreover, we reported previously that NKT cells and their production of IFN-γ play an important role in the development of Con A-induced hepatitis (16). Therefore, IFN-γ-producing CD4 T cells and NKT cells appear to be good therapeutic target cells in Con A-induced hepatitis.

High-density lipoprotein (HDL) has been implicated in several cardioprotective pathways and is thought to play a significant role in the removal of excess cholesterol from peripheral tissues and transport to the liver resulting in excretion into the bile (17, 18). HDL is classified according to its content of major apolipoproteins, apolipoprotein A-II (ApoA-II) and apolipoprotein A-I (ApoA-I). ApoA-II is synthesized in the liver and accounts for ~20% of HDL. The mean concentration of ApoA-II in human serum is ~30–35 mg/dl (19), although >20% of patients with coronary artery diseases showed a higher concentration between 40 and 60 mg/dl (19, 20). Studies of the physiological and pathophysiological effects of ApoA-II have focused on reverse cholesterol transport and antioxidant functions, mechanisms through which HDL are believed to protect against atherosclerosis (18, 21). ApoA-I is also the major apolipoprotein associated with HDL. There is abundant evidence indicating that the risk of coronary atherosclerotic cardiovascular disease is directly associated with the levels of plasma lipids and ApoA-I, as demonstrated by the analysis of ApoA-I transgenic mice (21, 22). However, the role...
of apolipoproteins in the immune response and in the pathogenesis of inflammation remains unidentified.

In this study, we demonstrate that ApoA-II has a suppressive effect on IFN-γ production by Con A-stimulated mouse and human CD4 T cells and attenuates Con A-induced hepatitis. ApoA-II could be used as an effective new therapeutic agent for CD4 T-cell-dependent autoimmune or viral hepatitis in humans.

Materials and Methods

**Mice**

Female BALB/c mice (6-wk-old) were purchased from Charles River Laboratories. Heterozygous ApoA-II knockout mice, which were purchased from The Jackson Laboratory, were bred to produce wild-type (WT) controls and homozygous ApoA-II knockout (ApoA-II−/−) mice (23, 24). All of the mice used in this study were maintained under specific pathogen-free conditions. All of the animal care and experimental protocols were conducted in accordance with the guidelines of Chiba University.

**Purification of ApoA-II from human plasma**

Human plasma was fractionated by the cold ethanol method according to Cohn et al. (25). Precipitation of Cohn et al. fraction IV-1 (PIV-1) was used as the starting material, and ApoA-II was further purified from PIV-1 by an additional ethanol precipitation (26). Briefly, PIV-1 was resuspended in buffer, 100 mM Tris-HCl (pH 8) containing 6 M urea. A first extraction was performed with a 1:1 (v/v) mixture of chloroform and ethanol with stirring, and the phases were separated by centrifugation. The lower organic phase contained most of the lipoprotein Apo-A-I. For the supernatant, which contains ApoA-II and ApoA-I, ethanol was added with stirring, and the mixture was incubated. After centrifugation, the pellet was discarded, and the supernatant, now depleted of proteins with molecular masses >28 kDa, was fractionated to separate ApoA-II from ApoA-I. This was achieved by further addition of ethanol and an additional incubation. After centrifugation, the supernatant and pellet contained ApoA-II (17 kDa) and ApoA-I (28 kDa), respectively. These were dialyzed against PBS.

**SDS-PAGE and two-dimensional electrophoresis**

SDS-PAGE (5–20% gels) was used for separation of proteins. After electrophoresis, the gels were stained with bromphenol blue. Two-dimensional electrophoresis was performed as described (27) with slight modifications. Briefly, protein samples were mixed with rehydration buffer [7 M urea, 2 M thiourea, 4% CHAPS, 0.2% ampholine (pH 3–10), 20 mM DTT, and 0.001% bromphenol blue] and loaded onto Immobiline Drystrip (pH 3–10; linear; Amersham Biosciences, Uppsala, Sweden). Isoelectric focusing was performed for a total of 30,000 Vh on an IPGphor apparatus (Amersham Biosciences). After a standard SDS equilibrating step, proteins were further separated by SDS-PAGE.

**In vivo experimental design**

For the Con A-induced hepatitis experiments, 12.5 mg/kg Con A was injected into BALB/c mice i.v. as a single dose. ApoA-II (50 or 250 mg/kg, i.v.) or PBS (30 ml/kg, i.v.) was slowly administered into the mice via the tail vein 10 min before the injection of Con A. Four, 12, and 24 h after the ApoA-II or PBS treatment, hepaticized blood was collected from the heart. The plasma fraction was then separated by centrifugation. These samples were used to measure activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using an automatic analyzer (Pujifuj Medical). In vivo experiments were excluded after the ApoA-II (250 mg/kg, i.v.) or PBS treatment for flow cytometry analysis. In separate experiments, the livers were excised 12 h after the ApoA-II (250 mg/kg, i.v.) or PBS treatment for histological analysis, immunohistochemistry, and detection of mRNA expression of cytokines and chemokines.

For assessment of the physiological role of ApoA-II, ApoA-II−/− and WT mice were injected with 20 mg/kg, i.v., Con A as a single dose. Twelve hours after the injection of Con A, hepaticized blood was used to measure levels of ALT and AST, and the livers were excised for histological analysis. Where indicated, the livers from ApoA-II−/− and WT mice were excised 4 h after Con A injection and used for flow cytometry analysis. In addition, anti–CD4b–mAb (R4-6A2; 250 mg/kg) was i.p. injected into ApoA-II−/− and WT mice 30 min before Con A injection. Anti–IL-17b antibody (50104; 100 μg) was i.p. injected into ApoA-II−/− and WT mice 30 min before Con A injection.

**Histological and immunohistochemical analysis**

For histological analysis, the livers from individual mice were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E. Specimens were examined under a light microscope. For immunohistochemical analyses, livers were fixed in 4% paraformaldehyde, and tissue was embedded in Tissue-Tek OCT compound (Sakura Finetechnical). Ten-micrometer cryostat sections were treated with 5% hydrogen peroxide in PBS to quench endogenous peroxidase activity. Sections were preblocked with anti-CD16/CD32 mAb (2.4G2; BD Pharmingen) in PBS containing 2% FCS and stained with biotin-conjugated Ab for deoxyuridine triphosphate by using MEBSTATIN Apoptosis Kit II (Medical & Biological Laboratories) according to the manufacturer’s protocol or with biotin-conjugated mAbs (BD Pharmingen) for anti-CD4 (RM4-5), anti-CD11b (M170), and anti-lymphocyte Ag 6G (Ly6G) (1A8). After being washed with TNT buffer [0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.05% Tween 20], sections were treated with HRP-conjugated streptavidin (Enbridge) in TNT buffer. After another washing step, amplification of the fluorescent signal with FITC-tyramide was performed by TSA direct kit (PerkinElmer). The specimens were analyzed using a fluorescence microscope (Biorevo; Keyence).

**Quantitative PCR analysis**

Total RNA was isolated from the liver (three mice in each group) with TRIzol reagent (Sigma-Aldrich). Reverse transcription was carried out with Superscript II Reverse Transcriptase (Invitrogen). Samples were then subjected to real-time PCR analysis on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) as described (28). The primers and TaqMan probes for the detection of mouse IL-4 and hypoxanthine phosphoribosyltransferase (HPRT) were purchased from Applied Biosystems. The probes for the detection of the other genes in this study were purchased from Roche Diagnostics (Basel, Switzerland). Primers for the Roche Diagnostics probes were as follows: TNF-α (forward primer, 5′-ATGAGCCACAGAAAGACTGATC-3′; reverse primer, 5′-TAC-GGCTTTGTACCTGGAATT-3′); IFN-γ (forward primer, 5′-ATCTGGGAGAAGCAGGGAAG-3′; reverse primer, 5′-TTCAAGACCTTACCAAGGTC-3′); RANTES (forward primer, 5′-CAAAGCTTCTCAGCCGTA-3′; reverse primer, 5′-ATGAGCAACTTCCAAGCCAA-3′); IP-10 (forward primer, 5′-TTCAGAAGTTCAGGCTTCAAGT-3′; reverse primer, 5′-ATCTGGAGAAGCAGGGAAG-3′; reverse primer, 5′-GAGGAGGTGACACTCCTCTTTTAA-3′; reverse primer, 5′-CTGGAGAACTCAGGCTTCAAGT-3′; RANTES (forward primer, 5′-TGGACAGAACCCATT-3′; reverse primer, 5′-GAGGAGGTGACACTCCTCTTTTAA-3′). Gene expression was normalized using the HPRT signal.

**Preparation of liver leukocyte cells**

Liver leukocyte cells were isolated as previously described (29, 30). Briefly, the liver was minced through a stainless steel mesh (#200) and suspended in PBS. After being washed once, the cells were resuspended in 33% Percoll solution and centrifuged. The pellet was subjected to flow cytometric analysis. For surface staining, one million cells were incubated with anti-CD16/CD32 mAb (2.4G2) and stained with the appropriate staining reagents, according to a standard method. The reagents used in this study were anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD11b (M170), and anti-Ly6G (1A8) mAbs.

**Preparation of mouse and human CD4 T cells**

For the preparation of mouse CD4 T cells, splenic CD4 T cells were purified from splenocytes using FITC-conjugated anti-CD4 mAb (RM4-5; BD Pharmingen), anti-FITC magnetic beads (Miltenyi Biotec), and autoMACS cell sorting (Miltenyi). For the preparation of human CD4 T cells, PBMCs from healthy volunteers were collected after obtaining informed consent. Peripheral blood was diluted with PBS and applied on Ficol-Paque (Amersham Biosciences). After centrifugation, mononuclear cells in the interface were harvested. Human CD4 T cells were purified from mononuclear cells using FITC-conjugated anti-CD4 mAb (RPA-T4; BD Pharmingen), anti-FITC magnetic beads, and autoMACS cell sorting.
Measurement of cytokines

Mouse splenic CD4 T cells (1 × 10^6 cells per well) or human CD4 T cells (5 × 10^6 cells per well) from PBMCs were stimulated with 5 μg/ml Con A (Sigma-Aldrich) for 24 h in the presence or absence of ApoA-II or ApoA-I. The amounts of IL-2 and IFN-γ in the culture supernatants were measured by ELISA as previously described (31). In Figs. 4C and 5C, the amounts of 23 cytokines from mouse and human CD4 T cells in the culture supernatants were detected by Bio-Plex Pro Mouse Cytokine Standard Group I 23-plex (Bio-Rad Laboratories) or Bio-Plex Pro Human Cytokine Standard Group I 23-plex according to the manufacturer’s protocol, respectively. Data from the reaction was then acquired and analyzed using the Bio-Plex suspension array system (Luminex 100 system) from Bio-Rad Laboratories.

Proliferation and cell division assay

To assay proliferation of CD4 T cells, mouse splenic CD4 T cells or human CD4 T cells were stimulated with Con A for 24 h in the presence or absence of ApoA-II or ApoA-I. [3H]Thymidine (37 kBq per well) was added to the stimulation culture for the last 16 h, and the incorporated radioactivity was measured on a β plate (32). For cell division, mouse splenic CD4 T cells or human CD4 T cells were labeled with CFSE (Molecular Probes) as described (31) and stimulated with Con A for 24, 48, and 72 h in the presence or absence of ApoA-II or ApoA-I. Flow cytometry analysis was performed on a FACSCalibur (BD Biosciences), and the results were analyzed using the FlowJo software program (Tree Star).

Phosphoprotein assay

Mouse splenic CD4 T cells were stimulated with Con A for 0, 5 min, 0.5, 1, and 3 h in the presence or absence of ApoA-II or ApoA-I. Protein lysates were prepared with a cell lysis kit (Bio-Rad Laboratories) on samples collected at the indicated time points. The presence of phosphorylation of ERK1/2, c-Jun, IκBα, and p38 MAPK was detected by Bio-Plex 4-plex Phosphoprotein assay kit (Bio-Rad Laboratories) and the Phosphoprotein Testing Reagent kit (Bio-Rad Laboratories) according to the manufacturer’s protocol. Data from the reaction was then acquired and analyzed using the Bio-Plex suspension array system.

Immunoblot assay

Mouse splenic CD4 T cells were stimulated with Con A for 0, 5 min, 0.5, 1, and 3 h in the presence or absence of ApoA-II or ApoA-I. Nuclear extracts for the detection of NFAT2 (NFATc1) or lamin were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce) on samples collected at the indicated time-points. Immunoblotting was performed with anti-NFATc1 mAb (BD Pharmingen) or anti-lamin polyclonal Ab (Santa Cruz Biotechnology). Protein levels were visualized by ECL (Amersham Biosciences) using HRP-conjugated anti-mouse IgG Ab or anti-rabbit IgG Ab (Amersham Biosciences).

Statistical analysis

All of the data are expressed as the mean ± SD. Statistical analysis was performed using GraphPad Prism, and differences were determined by unpaired two-tailed Student t test or one-way ANOVA with Dunnett’s multiple comparison tests. A p value <0.05 was considered statistically significant.

Results

Inhibition of IFN-γ production in Con A-stimulated mouse and human CD4 T cells by ApoA-II

To identify new therapeutic agents to suppress T cell-dependent inflammatory responses with better safety profiles than the currently used drugs, we purified the protein fraction from healthy human plasma using an ethanol precipitation method as reported by Cohn et al. (25), designated PIV-1. The albumin and IgG fraction were also purified from plasma by ethanol precipitation. As shown in Fig. 1A, the PIV-1 fraction significantly suppressed IFN-γ production by Con A-stimulated mouse CD4 T cells. No significant change was observed after the addition of the albumin or IgG fraction. Next, we compared the protein content of whole plasma and PIV-1 using SDS-PAGE. A spot corresponding to a 17-kDa protein (indicated by circles) showed increased intensity in PIV-1 compared with plasma (Fig. 1B, compare lanes 1 and 2 and lanes 5 and 6). Furthermore, the two-dimensional electrophoresis revealed that the 17-kDa protein appears to contain several proteins. The spots circled in Fig. 1C were identified as apolipoproteins, ApoA-II and ApoA-I, using the databases of SWISS-2DPAGE Viewer. To examine the inhibitory effect of apolipoproteins on IFN-γ production, ApoA-II was further purified from PIV-1 by an additional ethanol precipitation (Fig. 1D). As shown in Fig. 1E, ApoA-II (17 kDa) purified from the PIV-1 fraction showed a potent suppressive effect on IFN-γ production from Con A-stimulated mouse and human CD4 T cells.

Attenuation of Con A-induced hepatitis by ApoA-II administration

Next, we examined whether the administration of ApoA-II inhibits its liver injury induced by i.v. injection of Con A. Hepatitis was evaluated by measuring the levels of AST and ALT in plasma 4, 12, and 24 h after Con A injection (12.5 mg/kg). In the Con A-induced hepatitis model, the levels of AST and ALT increased 4 h after Con A injection and thereafter increased rapidly, reaching peak values at the 12-h time point (Fig. 2A). The administration of ApoA-II (50 or 250 mg/kg) before injection of Con A significantly and dose-dependently suppressed the increased levels of AST and ALT. We then evaluated the histological and immunohistochemical changes in the liver 12 h after Con A injection. Histological examination of the liver by H&E staining revealed focal or massive severe necrosis in the area between the central veins and the portal tracts of Con A-injected mice (Fig. 2B, second row, top and middle panels). In addition, TUNEL staining indicated Con A-induced apoptosis of hepatocytes (Fig. 2B, second row, bottom panels, green-stained cells). Preadministration of ApoA-II clearly suppressed the severe necrosis and apoptosis in the liver (Fig. 2B). We then examined the expression of cytokines and chemokines in the liver. The mRNA expression of TNF-α, IFN-γ, IL-4, MIP-1α, MIP-1β, and RANTES in the liver 12 h after Con A injection was significantly lower in the mice administered ApoA-II (Fig. 2C). Programmed death 1 and T cell Ig mucin 3 expression was not upregulated by the administration of ApoA-II (data not shown). The treatment of normal mice with ApoA-II alone had no effects on the levels of AST or ALT (data not shown).

Suppression of Con A-induced leukocyte infiltration into the liver by ApoA-II administration

ApoA-II suppressed the mRNA expression of MIP-1α, MIP-1β, and RANTES that attract several types of leukocytes including CD4 T cells into the liver (33, 34). We thus evaluated the number of leukocytes infiltrating the liver. As shown in Fig. 3A, administration of ApoA-II significantly suppressed leukocyte infiltration into the liver 12 h after Con A injection. Immunohistochemical analyses revealed a massive infiltration of CD4+ and CD11b+ cells into the liver and RANTES that attract several types of leukocytes including CD4 T cells into the liver (33, 34). We thus evaluated the number of leukocytes infiltrating the liver. As shown in Fig. 3A, administration of ApoA-II significantly suppressed leukocyte infiltration into the liver 12 h after Con A injection. Immunohistochemical analyses revealed a massive infiltration of CD4+, CD11b+, and Ly6G+ cells 12 h after Con A injection, and this was inhibited by the administration of ApoA-II (Fig. 3B). The flow cytometry analysis revealed that the number of CD4 T cells, CD8 T cells, CD11b+/Ly6G– macrophages, and CD11b+/Ly6G+ neutrophils in the liver increased after Con A injection, and these increases were significantly suppressed at the 12-h time point by the administration of ApoA-II (Fig. 3C). In normal mice, treatment with ApoA-II had no effects on the proportion or the absolute numbers of these cells in the liver (data not shown).

Suppression of the activation and function of mouse CD4 T cells by ApoA-II

To clarify the mechanisms underlying the ApoA-II–induced inhibition of Con A-induced hepatitis, we assessed the effect of human ApoA-II on the activation and function of mouse CD4
T cells in vitro. We used ApoA-I, which is the major apolipoprotein associated with HDL, purified from human plasma as a control. As shown in Fig. 4A, ApoA-II, but not ApoA-I, significantly and dose-dependently suppressed [3H]thymidine uptake by Con A-stimulated CD4 T cells. Moreover, the rate of cell division of Con A-stimulated CD4 T cells was clearly and dose-dependently suppressed by the addition of ApoA-II but not ApoA-I (Fig. 4B). In addition, ApoA-II also suppressed the proliferation of CD4 T cells stimulated with anti-TCRβ mAb plus anti-CD28 mAb (Supplemental Fig. 1). Next, we examined whether ApoA-II suppressed the production of cytokines and chemokines from CD4 T cells stimulated with Con A (Fig. 4C). Among the cytokines and chemokines tested, the production of IL-2, IL-13, IFN-γ, MIP-1α, MIP-1β, and RANTES was significantly suppressed by the addition of ApoA-II. However, ApoA-I did not suppress the production of any cytokines or chemokines by Con A-stimulated CD4 T cells. Moreover, we examined whether the treatment of CD4 T cells with ApoA-II inhibited the signal transduction pathways downstream of the TCR. As shown in Fig. 4D, the phosphorylation of ERK1/2 induced by Con A was selectively inhibited by the external addition of ApoA-II.

**FIGURE 1.** ApoA-II purified from human plasma inhibits IFN-γ production by Con A-stimulated mouse and human CD4 T cells. A, Purified mouse splenic CD4 T cells were stimulated with Con A (5 μg/ml) for 24 h in the presence of plasma, PIV-1, albumin, or IgG (1 mg/ml), and the amount of IFN-γ in the culture supernatant was assessed by ELISA. The results are expressed as mean ± SD (n = 5). *p<0.05, compared with PBS-added CD4 T cells. B, SDS-PAGE gel stained with bromphenol blue. Lanes 1 and 5, plasma; lanes 2 and 6, PIV-1; lanes 3 and 7, albumin; lanes 4 and 8, IgG. Circles indicate the increased spots of biologically active proteins in PIV-1. C, A representative two-dimensional electrophoresis pattern of plasma and PIV-1. The circle indicates the increased spots of biologically active proteins in PIV-1. D, SDS-PAGE gel stained with bromphenol blue showing ApoA-II purified from human plasma. E, Purified mouse splenic CD4 T cells or human CD4 T cells were stimulated with Con A in the presence of ApoA-II, and the amount of IFN-γ in the culture supernatant was assessed by ELISA. The results are expressed as mean ± SD (n = 5). *p<0.05, compared with PBS-added CD4 T cells. Similar data were obtained from three independent experiments.
addition of ApoA-II. Increased phosphorylation of c-Jun was detected 3 h after Con A stimulation and was inhibited by ApoA-II but not ApoA-I. The phosphorylation of IkBα, an indicator of the activation of the NF-κB signaling pathway, was not affected by the addition of ApoA-II. We did not detect any increase in the phosphorylation of p38 MAPK after Con A stimulation. The nuclear translocation of NFATc1 was detected 5 min after Con A stimulation and was significantly inhibited by the addition of

**FIGURE 3.** Suppression of Con A-induced leukocyte infiltration into the liver by ApoA-II administration. A, Total leukocyte cell numbers in the liver. Con A (12.5 mg/kg, i.v.) and vehicle (PBS, i.v.) or Con A and ApoA-II (250 mg/kg, i.v.) were injected into BALB/c mice. Livers were collected 4, 12, and 24 h after Con A injection. The results are expressed as mean ± SD (n = 8). *p < 0.05, compared with PBS-administered mice. B, Livers were collected 12 h after Con A injection, and leukocyte migration into the liver was evaluated by staining with Abs specific for CD4, CD11b, and Ly6G. Scale bars, 50 µm. C, Flow cytometric analysis of leukocytes migrated in the liver was performed. The results are expressed as mean ± SD (n = 8). *p < 0.05, compared with PBS-administered mice.
ApoA-II (Fig. 4E). In contrast, ApoA-I had very little effect on the activation of signaling molecules. These results indicate that ApoA-II suppresses the activation and function of mouse CD4 T cells by inhibiting the ERK–MAPK pathway and NFAT signaling pathway activated by Con A stimulation.

**Suppression of the activation of human CD4 T cells by ApoA-II**

Next, we examined whether ApoA-II suppressed the activation of human CD4 T cells by inhibiting the ERK–MAPK pathway and NFAT signaling pathway activated by Con A. The proliferative response of mouse CD4 T cells was determined by [3H]thymidine uptake. Purified splenic CD4 T cells were stimulated with Con A (5 μg/ml) for 40 h in the presence of ApoA-II or ApoA-I (0.2 or 1 mg/ml). The results are expressed as mean ± SD (n = 5). *p < 0.05, compared with PBS-added CD4 T cells.

**Exacerbation of Con A-induced hepatitis in ApoA-II−/− mice**

We examined the physiological roles of ApoA-II in Con A-induced hepatitis using ApoA-II−/− mice. No spontaneous pathological AST and ALT levels or leukocyte infiltration were observed in ApoA-II−/− mice maintained under physiological conditions (Fig. 6A, 6C). However, once Con A was injected, ApoA-II−/− mice showed dramatically increased levels of AST and ALT as compared with those of WT mice (Fig. 6A). We also performed histological analysis of the liver. Without Con A injection, the livers of ApoA-II−/− mice showed slightly increased areas of glycogen accumulation (Fig. 6B, bottom row, left). After Con A injection, liver damage accompanied by increased numbers of apoptotic hepatocytes was apparently more severe in ApoA-II−/− mice as compared with that observed in WT mice (Fig. 6B, right four panels). Moreover, leukocyte infiltration into the liver 4 h after Con A injection was significantly higher in ApoA-II−/− mice (Fig. 6B, right four panels).
6C), and the increase in number of CD4 T cells in the liver of ApoA-II−/− mice was greater than that of WT mice (Fig. 6D). There was no significant difference in infiltration of macrophages or neutrophils into the liver 12 h after Con A injection between ApoA-II−/− and WT mice (Supplemental Fig. 2). The levels of IFN-γ production by Con A-stimulated ApoA-II−/− CD4 T cells were significantly higher than those in WT CD4 T cells, and the production of IFN-γ was equivalently inhibited by the addition of ApoA-II in WT and ApoA-II−/− groups (Fig. 6E). No significant difference in the production of IL-2 or the proliferation of CD4 T cells was detected between ApoA-II−/− and WT mice, whereas similar inhibition by ApoA-II was observed in CD4 T cells prepared from WT and ApoA-II−/− mice (Supplemental Fig. 3). Next, to confirm the critical role of IFN-γ– or IL-17–producing CD4 T cells in vivo in contributing to the liver injury in ApoA-II−/− mice during Con A-induced hepatitis, we depleted CD4 T cells, neutralized IFN-γ or IL-17 in ApoA-II−/− mice by the administration of anti-CD4, anti–IFN-γ, or anti–IL-17 mAb, and assessed the liver damage in response to Con A injection. Con A-induced hepatitis was protected almost completely by the injection of either anti-CD4 or anti–IFN-γ mAb in both WT and ApoA-II−/− mice, although no obvious protection after Con A injection was mediated by the injection of anti–IL-17 mAb (Fig. 6F). These results indicate that IFN-γ–producing CD4 T cells play an important role in the development and progression of Con A-induced hepatitis in both WT and ApoA-II−/− mice.

**Attenuation of Con A-induced hepatitis by postadministration of ApoA-II**

Finally, we assessed whether ApoA-II inhibits Con A-induced hepatitis even after the onset of hepatitis. We administered ApoA-II 2 h after injection of Con A, because approximately one and a half times the number of CD4 T cells was observed in the liver by this time (data not shown), and compared the efficacy of ApoA-II and a clinically used dose of prednisolone (4 mg/kg, i.v.) that is a standard treatment for autoimmune hepatitis patients. Preadministration of ApoA-II or prednisolone significantly suppressed the Con A-induced increase in the levels of AST and ALT, and the efficacy of prednisolone given before Con A injection was more potent than ApoA-II. Interestingly, postadministration of ApoA-II but not prednisolone significantly suppressed the Con A-induced increase in the levels of AST and ALT (Fig. 7A). We also observed no improvements using a higher dose (20 mg/kg, i.v.) of postadministration of prednisolone (data not shown). In addition,
Con A-induced histological damage such as severe necrosis and apoptosis of hepatocytes in the liver was also suppressed by postadministration of ApoA-II but not of prednisolone (Fig. 7B).

The increased infiltration of leukocytes (Fig. 7C) and CD4 and CD8 T cells (Fig. 7D) into the liver was significantly suppressed by the postadministration of ApoA-II but not prednisolone (Fig. 7C, D). These results indicate that Con A-induced hepatitis is inhibited by ApoA-II administration even after the onset of hepatitis.

Discussion
In this report, we demonstrate clear evidence indicating that ApoA-II, which is the second major HDL in human plasma, has a suppressive effect on Con A-induced hepatitis. Exacerbated hepatitis was observed in ApoA-II−/− mice, indicating a physiological role for ApoA-II in the protection of Con A-induced hepatitis. The suppressive effect of ApoA-II was observed even after the onset of Con A-induced hepatitis. ApoA-II showed a potent suppressive effect on both mouse and human CD4 T cells. Therefore, ApoA-II could be used as a new relatively safe therapeutic agent for CD4 T cell-dependent autoimmune or viral hepatitis in humans.

Activated T cells and subsequent production of cytokines play a critical role in the pathogenesis of hepatitis. Upregulation of proinflammatory cytokines such as IFN-γ and TNF-α by Con A injection directly induce hepatocellular apoptosis and necrosis (13, 15, 35), with relatively more critical roles for IFN-γ having been suggested (36, 37). Although significantly decreased expression of both IFN-γ and TNF-α in the liver was detected (Fig. 2C), the in vitro experiments revealed that ApoA-II suppressed the production of IFN-γ but not TNF-α in Con A-stimulated mouse and human CD4 T cells (Figs. 4C, 5C). Activated CD8 T cells, which produce a high amount of IFN-γ, also contribute to the development of Con A-induced hepatitis, however less so as compared with CD4 T cells (12). ApoA-II also inhibited the production of IFN-γ in Con A-activated CD8 T cells (Supplemental Fig. 4). It is also known that macrophages and neutrophils are involved in the induction of Con A-induced hepatitis (38, 39),
because these cells can produce various cytokines and chemokines, leading to liver injury. However, the production of TNF-α from IFN-γ-stimulated peritoneal macrophages was not changed by ApoA-II (Supplemental Fig. 5A). ApoA-II also did not suppress TNF-α-induced activation of peritoneal neutrophils (Supplemental Fig. 5B), which was evaluated by the expression of activation markers for neutrophils, such as CD62L and CD11b (40). Previously, it was reported that IL-17–producing CD4 T cells also contributed to the induction of Con A-induced hepatitis (41).

ApoA-II was capable of suppressing IL-17 production in activated CD4 T cells (Supplemental Fig. 6). However, no obvious protection in both WT and ApoA-II−/− mice after Con A injection was observed by the injection of anti–IL-17 mAb (Fig. 6F). In addition, ApoA-II injection did not alter the number of Foxp3+ regulatory T cells (Tregs) among CD4 T cells infiltrating in the liver even after Con A injection, and also the number of Foxp3+ Tregs was not reduced in the ApoA-II−/− mice (J. Yamashita, K. Kaneko, and T. Nakayama, unpublished observations). Thus, Th17 cells and Tregs may not play a major role in the attenuation of Con A-induced hepatitis by ApoA-II. Taken together, ApoA-II appears to attenuate Con A-induced hepatitis largely by the suppression of IFN-γ production by CD4 T cells.

The administration of ApoA-II suppressed the migration of CD4 T cells, CD8 T cells, macrophages, and neutrophils into the liver after Con A injection (Fig. 3B, 3C). We measured the expression of several chemoattractant factors and found that ApoA-II significantly suppressed the mRNA expression of MIP-1α, MIP-1β, and RANTES that attract CD4 T cells, CD8 T cells, and macrophages in the liver after Con A injection (Fig. 2C). Indeed, the increased production of MIP-1α, MIP-1β, and RANTES was reported in chronic hepatitis C (42–44), alcoholic hepatitis and cirrhosis (45), and transplanted liver (46, 47) in humans. In the mouse model, these chemokines also have an important role in the induction of hepatitis (33, 34). The infiltration of CD4 or CD8 T cells into the liver after Con A injection was more rapid as compared with that of macrophages and neutrophils (Fig. 3C).

Therefore, the suppression of chemokine production from CD4 T cells by ApoA-II at the early stage of hepatitis could reduce the migration of macrophages and neutrophils, both of which are known to be involved in the pathogenesis of Con A-induced hepatitis. Previously, we and others reported that Vα14iNKT cells are rapidly activated, produce large amounts of IFN-γ after the injection of Con A, and can contribute to the development of Con A-induced hepatitis (16, 48, 49). Although we observed a decrease in the number of Vα14iNKT cells in the liver after Con A injection, the levels were equivalent between ApoA-II– and PBS-treated groups (Supplemental Fig. 7A). In addition, no obvious difference in the number of IFN-γ-producing Vα14iNKT cells in the spleen between ApoA-II– and PBS-treated mice was observed (Supplemental Fig. 7B). Thus, Vα14iNKT cells may not be involved in the process of inhibition of hepatitis by ApoA-II.

The activation of nuclear transcription factors such as AP-1, NFAT, and NF-κB is essential for the activation of T cells and the transcriptional upregulation of the various cytokine genes (50–53). ApoA-II inhibited the phosphorylation of ERK1/2 and c-Jun, a member of the AP-1 transcription factor family, and suppressed the nuclear translocation of NFATc1 in Con A-stimulated CD4 T cells (Fig. 4D, 4E). Thus, this could be the mechanism by which...
ApoA-II inhibited the activation and IFN-γ production in CD4 T cells. In fact, cyclosporine A and FK506 (Tacrolimus) inhibited Con A-induced hepatitis through the inhibition of the activation of calcineurin, the upstream signaling molecule of NFAT activation and nuclear translocation (12). We demonstrate a physiological role for ApoA-II in the protection from Con A-induced hepatitis using ApoA-II−/− mice (Fig. 6). CD4 T cells may play a more important role in this protection as compared with CD8 T cells, because selectively increased infiltration of CD4 T cells and their enhanced IFN-γ production were observed in ApoA-II−/− mice (Fig. 6D, 6E). In the liver of ApoA-II−/− mice, increased areas of glycogen accumulation were observed (Fig. 6B). Thus, the changes in lipoprotein metabolism in ApoA-II−/− mice could induce the malfunction of CD4 T cells leading to enhanced Con A-induced hepatitis. However, the upregulated CD4 T cell function, such as enhanced IFN-γ production, observed in ApoA-II−/−/− CD4 T cells was normalized by the addition of ApoA-II (Fig. 6E). Con A-induced production of IL-2 and proliferation were not significantly altered in ApoA-II−/−/− CD4 T cells and were equivalently inhibited by ApoA-II (Supplemental Fig. 3). Therefore, the basic function of CD4 T cells developed in ApoA-II−/− mice appeared to be normal. In any event, CD4 T cells appear to be the major target cells for the inhibitory effect of ApoA-II in Con A-induced hepatitis under physiological conditions. Patients with autoimmune hepatitis usually require immunosuppressive therapy for many years. The immunosuppressive drugs, primarily glucocorticoids, serve as the standard therapy for autoimmune hepatitis (3, 4, 54, 55). Therefore, we compared the ability of ApoA-II and prednisolone to suppress Con A-induced hepatitis. Interestingly, postadministration of ApoA-II attenuated the ability of ApoA-II and prednisolone reduced leukocyte infiltration including CD4 and CD8 T cells into the liver (Fig. 7C, 7D). Thus, the mechanisms underlying the inhibition of Con A-induced hepatitis appeared to be distinct between ApoA-II and prednisolone. Because ApoA-II is a component of normal human plasma, side effects induced by the administration of ApoA-II could be marginal. In fact, preliminary preclinical experiments suggest that only marginal side effects are observed (J. Yamashita, K. Kaneko, and T. Nakayama, unpublished observations). Therefore, a combination therapy of ApoA-II with a low dose glucocorticoid and/or other immunosuppressive agents may prevent the severe side effects and consequently may prove that ApoA-II is an effective therapeutic agent for autoimmune hepatitis.

In summary, we showed that ApoA-II protected mice from Con A-induced hepatitis by suppressing the function of activated CD4 T cells and reducing the intraphepatic infiltration of inflammatory cells. Although we used ApoA-II prepared from human plasma in this study, we recently found that rApoA-II also attenuated Con A-induced hepatitis (J. Yamashita, K. Kaneko, and T. Nakayama, unpublished observations). Hence, our study offers new perspectives for the treatment of CD4 T cell-related autoimmune or viral hepatitis with ApoA-II in humans.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. ApoA-II suppresses the function of mouse CD4 T cells stimulated with anti-TCRβ plus anti-CD28. Purified mouse splenic CD4 T cells were labeled with CFSE and stimulated with immobilized anti-TCRβ mAb plus anti-CD28 mAb in the presence of ApoA-II (1 mg/ml) for 24, 48 and 72 h, and the amounts of IL-2 and IFN-γ in the culture supernatants after 24 h stimulation were assessed by ELISA. The results are expressed as mean ± SD. *P < 0.05, compared with PBS-added CD4 T cells.
Supplemental Figure 2. Normal Con A-induced infiltration of macrophages and neutrophils into the liver in ApoA-II−/− mice. Con A (20 mg/kg, i.v.) was injected into ApoA-II−/− mice. Flow cytometric analysis was performed to assess the infiltration of macrophages and neutrophils into the liver 12 h after Con A injection. The numbers of CD11b+/Ly6G+ cells and CD11b+/Ly6G+ cells and were calculated using total leukocyte cell counts and flow cytometric analysis data. The results are expressed as mean ± SD (n=6).
Supplemental Figure 3. The suppressive effect of ApoA-II is observed for both IL-2 production and proliferation in Con A-stimulated ApoA-II<sup>−/−</sup> CD4<sup>+</sup> T cells. (A), Purified splenic CD4<sup>+</sup> T cells from ApoA-II<sup>−/−</sup> and WT mice were stimulated with Con A (5 μg/ml) for 24 h in the presence of ApoA-II, and the amount of IL-2 in the culture supernatant was assessed by ELISA. The results are expressed as mean ± SD. (B), The proliferative response of splenic CD4<sup>+</sup> T cells was examined by [<sup>3</sup>H]-thymidine uptake. Purified splenic CD4<sup>+</sup> T cells from ApoA-II<sup>−/−</sup> and WT mice were stimulated with Con A for 40 h in the presence of ApoA-II. The results are expressed as mean ± SD. Similar data were obtained from three independent experiments.
Supplemental Figure 4. Suppression of the function of mouse CD8 T cells by ApoA-II. Splenic CD8 T cells were purified from BALB/c splenocytes using PE-conjugated anti-CD8 mAb, anti-PE magnetic beads (Miltenyi Biotec), and Auto-MACS cell Sorter (Miltenyi Biotec). The purified CD8 T cells were stimulated with Con A (5 μg/ml) for 24 h in the presence of ApoA-II (0.2 or 1 mg/ml), and the amounts of IL-2 and IFN-γ in the culture supernatant were assessed by ELISA. The results are expressed as mean ± SD. *P < 0.05, compared with PBS-added CD8 T cells. Similar data were obtained from three independent experiments.
Supplemental Figure 5. ApoA-II does not suppress the function of macrophages or the activation of neutrophils. (A), To collect macrophages, 1 ml of 4% fluid thioglycollate medium (Sigma-Aldrich) was injected intraperitoneally into BALB/c mice. The peritoneal lavage cells were harvested on day 4 after the thioglycollate injection. Macrophages were purified using FITC-conjugated anti-CD11b mAb, anti-FITC magnetic beads, and Auto-MACS cell Sorting. Purified CD11b-positive cells consisted of more than 95% macrophages identified by flow cytometry and Wright-Giemsa staining, respectively (data not shown). Purified peritoneal macrophages were stimulated with Con A (5 μg/ml) or IFN-γ (2.5 ng/ml) for 24 h in the presence of ApoA-II or ApoA-I (1 mg/ml), and the amount of TNF-α in the culture supernatant was assessed by ELISA. The results are expressed as mean ± SD (n=5). (B), To collect neutrophils, 1 ml of 4% fluid thioglycollate medium was injected intraperitoneally into BALB/c mice. The peritoneal lavage cells were harvested 4 h after thioglycollate injection. Neutrophils were purified using PE-conjugated anti-Ly6G mAb, anti-PE magnetic beads, and Auto-MACS cell Sorting. The purified Ly6G-positive cells consisted of more than 95% neutrophils identified by flow cytometry and Wright-Giemsa staining (data not shown). Purified peritoneal neutrophils were stimulated with TNF-α (1 ng/ml) for 0.5 h in the presence of ApoA-II or ApoA-I, and the expression of CD11b and CD62L was assessed using flow cytometry.
Supplemental Figure 6. The suppressive effect of ApoA-II administration on IL-17 production by mouse liver-infiltrating CD4 T cells after Con A injection. Con A (12.5 mg/kg, i.v.) and vehicle, or Con A and ApoA-II (250 mg/kg, i.v.) were administered into BALB/c mice. 12 h after Con A injection, liver CD4 T cells were purified from mice using FITC-conjugated anti-CD4 mAb, anti-FITC magnetic beads, and AutoMACS cell Sorter. The purified CD4 T cells were stimulated with Con A (5 μg/ml) for 24 h, and the amounts of IFN-γ and IL-17 in the culture supernatant were assessed by ELISA. The results are expressed mean ± SD (n=4). *P < 0.05, compared with PBS-administrated mice.
Supplemental Figure 7. ApoA-II administration did not change the activation of Vα14 NKT cell in the liver. (A), Con A (12.5 mg/kg, i.v.) and vehicle, or Con A and ApoA-II (250 mg/kg, i.v.) were administered into BALB/c mice. Flow cytometric analysis of Vα14 NKT cells in the liver 4, 12, and 24 h after Con A injection was performed to determine the expression profiles of α-GalCer/CD1d-tetramer⁺ and TCRβ⁺ cells. The number of α-GalCer/CD1d-tetramer⁺/TCRβ⁺ cells was calculated based on the total leukocyte cell counts and flow cytometric analysis data. The results are expressed as mean ± SD (n=8). (B), The intracellular expression of IFN-γ in α-GalCer/CD1d-tetramer⁺ NKT cells or CD4 T cells in spleen 12 h after Con A injection were analyzed using a Cytofix/Cytoperm Kit Plus (with Golgistop; BD Biosciences) according to the manufacturer’s instructions.