RAMP1 attenuates immune-mediated hepatitis via recruiting regulatory T cells

Tomoyoshi Inoue,[1,2,3] Mitsuko Inoue,[4] Yoshiya Ito,[1,3] Fumisato Otaka,[1,2,3] Tomohiro Betto,[1,2,3]
Sakiko Yamane,[1,2,3] Wasaburo Koizumi,[2] Masataka Majima[1,3]

[1] Department of Molecular Pharmacology, Graduate School of Medical Sciences, Kitasato University

[2] Department of Gastroenterology, Kitasato University School of Medicine

[3] Department of Pharmacology, Kitasato University School of Medicine

[4] Department of Endocrinology, Kitasato University School of Medicine

Objectives: Autoimmune hepatitis (AIH) has characteristic features of autoimmunity, and impaired immune response plays a critical role in AIH. Calcitonin gene-related peptide (CGRP), which is released from the sensory nervous system, regulates innate immune activation via receptor activity-modifying protein 1 (RAMP1), a subunit of the CGRP receptor. We investigated the role of RAMP1 in a concanavalin A (Con A)-induced model of AIH.

Methods: Experimental immune-mediated hepatitis was created by an intravenous injection of Con A (20 mg/kg) in wild-type (WT) mice and RAMP1-deficient (RAMP1$^{-/-}$) mice. Alanine transferase (ALT), accumulation of T cells, and cytokine expression were assessed.

Results: Compared with WT mice, RAMP1$^{-/-}$ mice exhibited enhanced levels of ALT, increased CD4$^+$ cells, and decreased regulatory T cells (Tregs) after Con A treatment. In RAMP1$^{-/-}$ mice, mRNA levels of pro-inflammatory cytokines including tumor necrosis factor (TNF)-α and interferon (IFN)-γ were upregulated, while anti-inflammatory cytokines including interleukin (IL)-10 and transforming growth factor (TGF)-β were downregulated. Deletion of Tregs with a neutralizing antibody against folate receptor 4 exacerbated Con A-induced liver injury, with downregulation of IL-10 and TGF-β.

Conclusion: These results suggest that RAMP1 signaling regulates immune-mediated hepatitis by enhancing TGF-β and IL-10 from Tregs.

Key words: autoimmune hepatitis, CGRP, RAMP1, Tregs

Abbreviations: AIH, autoimmune hepatitis; CGRP, calcitonin gene-related peptide; RAMP1, receptor activity-modifying protein 1; Con A, concanavalin A; WT, wild-type; RAMP1$^{-/-}$, RAMP1-deficient; ALT, alanine transferase; Tregs, regulatory T cells; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; TGF, transforming growth factor; Foxp3, forkhead box p3; FR4, folate receptor 4; IgG, immunoglobulin G; PBS, phosphate buffer solution; RT-PCR, reverse transcript-polymerase chain reaction; CXCR3, C-X-C chemokine receptor 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Introduction

AIH is a classical autoimmune liver disease that has characteristic features of autoimmunity.[1] Tregs play an important role in regulating immune homeostasis and preventing autoimmunity.[2] Tregs are characterized by expression of the transcription factor Foxp3, and the surface marker CD25.[3] Although the etiology of AIH is still unknown, the progressive imbalance between effector T cells (CD4$^+$ T cells) and Tregs is a hallmark of autoimmune diseases. Con A-induced hepatitis is used as a mouse model of immune-mediated liver injury and resembles viral and autoimmune hepatitis in humans.[4] Although Con A-induced liver injury is principally

mediated by CD4$^+$ T cells, recent studies have demonstrated the involvement of Tregs in Con A-mediated hepatitis.[5] Therefore, the induction of Tregs is a critical determinant in disease progression and activity.

Sensory neurons communicate actively with the immune system. The immune system is also tightly regulated by the nervous system, which releases several mediators including neurotransmitters. CGRP, a 37 amino acid peptide, is produced in the neural body of dorsal root ganglion cells and released from sensory nerve endings.[6] CGRP binds to a specific receptor, a complex formed by RAMP1 and calcitonin receptor-like receptor.[7] Our recent studies show that CGRP down-regulates the function of T cells[8] and macrophages[9,10] via RAMP1 signaling during inflammation. Recently, it is reported that CGRP induces Tregs.[11] However, it remains unknown whether or not CGRP/RAMP1 regulates immune-mediated liver injury by induction of Tregs. The objective of the present study was therefore conducted to examine the role of RAMP1 signaling in immune-mediated hepatitis caused by Con A through recruitment of Tregs.

Materials and Methods

Animals
Male C57Bl/6 WT mice were obtained from Clea Japan (Tokyo). Male RAMP1$^{-/-}$ mice were developed previously.[12] All experimental procedures were approved by the Animal Experimentation and Ethics Committee of the Kitasato University School of Medicine, and were performed in accordance with the guidelines for animal experiments set down by the Kitasato University School of Medicine, which are in accordance with the "Guidelines for Proper Conduct of Animal Experiments" published by the Science Council of Japan.

Animal procedures
Animals were intravenously injected (via the tail vein) with 20 mg/kg Con A (Merck KGaA, Darmstadt, Germany) to induce hepatitis.[13] Levels of ALT were measured using a Dri-Chem 7000 Chemistry Analyzer System (Fujifilm, Tokyo).

Depletion of Tregs
Experimental animals were depleted of Tregs by an intraperitoneal injection of a neutralizing antibody against folate FR4 (50 μg per mouse, clone eBio TH6; eBioscience, San Diego, CA, USA) at 24 hours before Con A administration. Control animals were treated with IgG isotype control antibodies.

Immunofluorescence analysis
Excised liver tissues were fixed for 4 hours at 4℃ with 4% neutral buffered paraformaldehyde. Following cryoprotection with 30% sucrose/0.1 M PBS (pH 7.2), 4 μm-thick cryostat sections were cut, washed in PBS, and incubated for 1 hour at room temperature in Protein Block Serum-Free PBS (Abcam, Cambridge, MA, USA). After blocking, sections were incubated overnight at 4℃ with a rabbit anti-mouse RAMP1 polyclonal antibody (Bioss Antibodies, Woburn, MA, USA), a rabbit or rat anti-mouse CD4 monoclonal antibody (Abcam) or a rat anti-mouse Foxp3, the transcription factor of Tregs (eBioscience). After washing three times in PBS, the sections were incubated with a mixture of the following secondary antibodies for 1 hour at room temperature: Alexa Fluor 488-conjugated donkey anti-rat IgG (Molecular Probes, OR, USA), Alexa Fluor 594-conjugated donkey anti-rabbit IgG (Molecular Probes), and Alexa Fluor 488-conjugated donkey anti-goat IgG (Molecular Probes). Images were captured under a fluorescence microscope (Biozero BZ-9000 Series; Keyence, Osaka). After labeling, six low-power optical fields (200× magnification) were randomly selected, and the number of positive cells was counted.

Real-time RT-PCR
Transcripts encoding TNF-α, IFN-γ, IL-10, TGF-β Foxp3, CXCR3, and GAPDH were quantiied by real-time RT-PCR analysis. Quantitative PCR amplification was performed using SYBR Premix Ex Taq II (Tli RNaseH Plus; Takara Bio, Shiga). The gene-specific primers used for real-time RT-PCR were designed using Primer 3 software (http:primer3.sourceforge.net) based on data from GenBank.
The primers were:
5'-TCTTCTCATTCCTGCTTGTGG-3' (sense) and 5'-GATCTGAGTGTGAGGGTCTGG-3' (antisense) for TNF-α; 5'-ATCTGGAGGAACTGGCAAAAG-3' (sense) and 5'-CGCTTATGTTGTTGCTGATGG-3' (antisense) for IFN-γ; 5'-CGGAAATGATCCAGTTTTACC-3' (sense) and 5'-TGAGGGTCTTCAGCTTCTCAC-3' (antisense) for IL-10; 5'-AACAATTCCTGGCGTTACCTT-3' (sense) and 5'-TGTATTCCGTCTCCTTGGTTC-3' (antisense) for TGF-β; 5'-AGTGCCTGTGTCCTCAATGGTC-3' (sense) and 5'-AGGGCCAGCATAGGTGCAAG-3' (antisense) for Foxp3; 5'-TGTATGGGGTCTCTGTCTGCT-3' (sense) 5'-ACCTGTGGGAAGTTGTACTGG-3' (antisense) for CXCR3; and 5'-ACATCAAGAAGGTGGTGAAGC-3' (sense) and 5'-AAGGTGGAAGAGTGGGAGTTG-3' (antisense) for GAPDH.
Data were normalized to GAPDH expression levels.

Isolation of leukocytes from liver homogenates

Hepatic leukocytes were isolated from liver homogenates by density-gradient centrifugation on 33% Percoll (GE Healthcare Life Sciences, Piscataway, NJ, USA), as previously reported.[14,15]

Flow cytometry analysis

Cells were incubated with the 2.4G2 mAb (anti-cγRIII/II) to block non-specific binding of the primary mAb. The cells were then stained with a combination of the following fluorochrome-conjugated antibodies: anti-CD45 (clone 30-F11; BioLegend, San Diego, CA, USA), anti-CD3 (clone 17A2, BioLegend), anti-CD4 (clone GK1.5, BioLegend), and anti-CD8 (clone 53-6.7, BioLegend), and anti-CD25 (clone PC61, BioLegend), Foxp3 (eBioscience).

Tubes were placed in the dark on ice for 30 minutes. Pellets were washed twice with PBS. For flow cytometric analysis, cells were initially gated on forward-scatter and side-scatter and then gated on CD45$^+$ cells. Cells positive for 7-aminoactinomycin D (BioLegend) were electronically gated from the analysis as dead cells. Samples were measured on a BD FACSVerse (Becton, Dickinson, Franklin Lakes, NJ, USA). The data were analyzed using Kaluza software v1.3 (Beckman Coulter, Brea, CA, USA).[14,15]

Statistical analysis

All results are expressed as the mean $\pm$ SD. All statistical analyses were performed using GraphPad Prism software, version 6.07 (GraphPad Software, La Jolla, CA, USA). Unpaired two-tailed Student's *t*-test was used to compare data between 2 groups, and one-way analysis of variance, followed by Bonferroni's post-hoc test, was used to compare data between multiple groups. P-values of <0.05 were considered statistically significant.

Results

Con A-mediated hepatitis is exacerbated in RAMP1$^{-/-}$ mice

To elucidate the role of RAMP1 signaling during immune-mediated liver injury, we treated WT and RAMP1$^{-/-}$ mice with Con A. Within 6 hours of Con A treatment, ALT levels in WT mice began to increase and were elevated markedly at 24 hours post-treatment (Figure 1A). By contrast, liver injury in RAMP1$^{-/-}$ mice was more severe than that in WT mice, as evidenced by markedly higher ALT levels.

Because T cells are responsible for the development of Con A hepatitis, we next examined the accumulation of T cells in the liver using flow cytometry analysis.

Exposure to Con A for 24 hours caused more CD4$^+$ T cells (CD3^{high}/CD4^{high}/CD8^{low}) in the liver of RAMP1$^{-/-}$ mice than in that of WT mice (Figure 1B). However, there were no significant differences between the genotypes in terms of the number of CD8$^+$ T cells (CD3^{high}/CD4^{low}/CD8^{high}) in the liver (Figure 1B). We observed an increase in Tregs (CD3^{high}/CD4^{high}/CD25^{high}/Foxp3^{high}) in WT livers but a decrease in Tregs in RAMP1$^{-/-}$ livers (Figure 1C).

Immunofluorescence analysis revealed that, at 24 hours after Con A treatment, accumulation of CD4$^+$ cells (T cells) in RAMP1$^{-/-}$ livers was higher than that in WT livers (Figure 1D). By contrast, Foxp3$^+$ Tregs were decreased in RAMP1$^{-/-}$ mice compared with those in WT mice. These results suggest that RAMP1 signaling in Tregs is involved in Con A-mediated hepatitis.

Increased expression of pro-inflammatory cytokines and decreased expression of anti-inflammatory cytokines in RAMP1$^{-/-}$ mice with Con A hepatitis

Because pro-inflammatory cytokines, including TNF-α and IFN-γ contribute to Con A hepatitis, we measured TNF-α and IFN-γ mRNA in the liver of mice with Con A hepatitis. It has been shown that expression of pro-inflammatory cytokines, TNF-α and IFN-γ are enhanced during the early phase of injury (1 hour post-Con A treatment). Consistent with this, real-time RT-PCR analysis revealed that WT and RAMP1$^{-/-}$ mice showed an enhanced expression of TNF-α and IFN-γ 1 hour after Con A administration (Figure 2A, B). RAMP1$^{-/-}$ mice showed a higher expression of TNF-α and IFN-γ in the liver than did WT mice.

To clarify the involvement of Tregs in susceptibility of Con A-mediated hepatitis in RAMP1$^{-/-}$ mice, we determined Foxp3 mRNA levels in the liver. The levels of Foxp3 in WT mice were increased compared with those in WT mice (Figure 2C). Correspondingly, mRNA levels of CXCR3, a chemokine receptor for the recruitment of Tregs,[16] were reduced in RAMP1$^{-/-}$ livers as compared with WT livers (Figure 2D). The levels of cytokine IL-10, which is involved in suppression of immune-mediated hepatitis, were higher in RAMP1$^{-/-}$ mice than those in WT mice (Figure 2E). TGF-β is involved in expansion of Tregs, and mRNA levels of TGF-β in RAMP1$^{-/-}$ mice were suppressed compared with those in WT mice (Figure 2F). These results indicate that RAMP1 signaling attenuates Con A-mediated hepatitis with accumulation of Tregs accompanied with IL-10 and TGF-β enhancement.

Deletion of Tregs aggravates Con A-mediated hepatitis

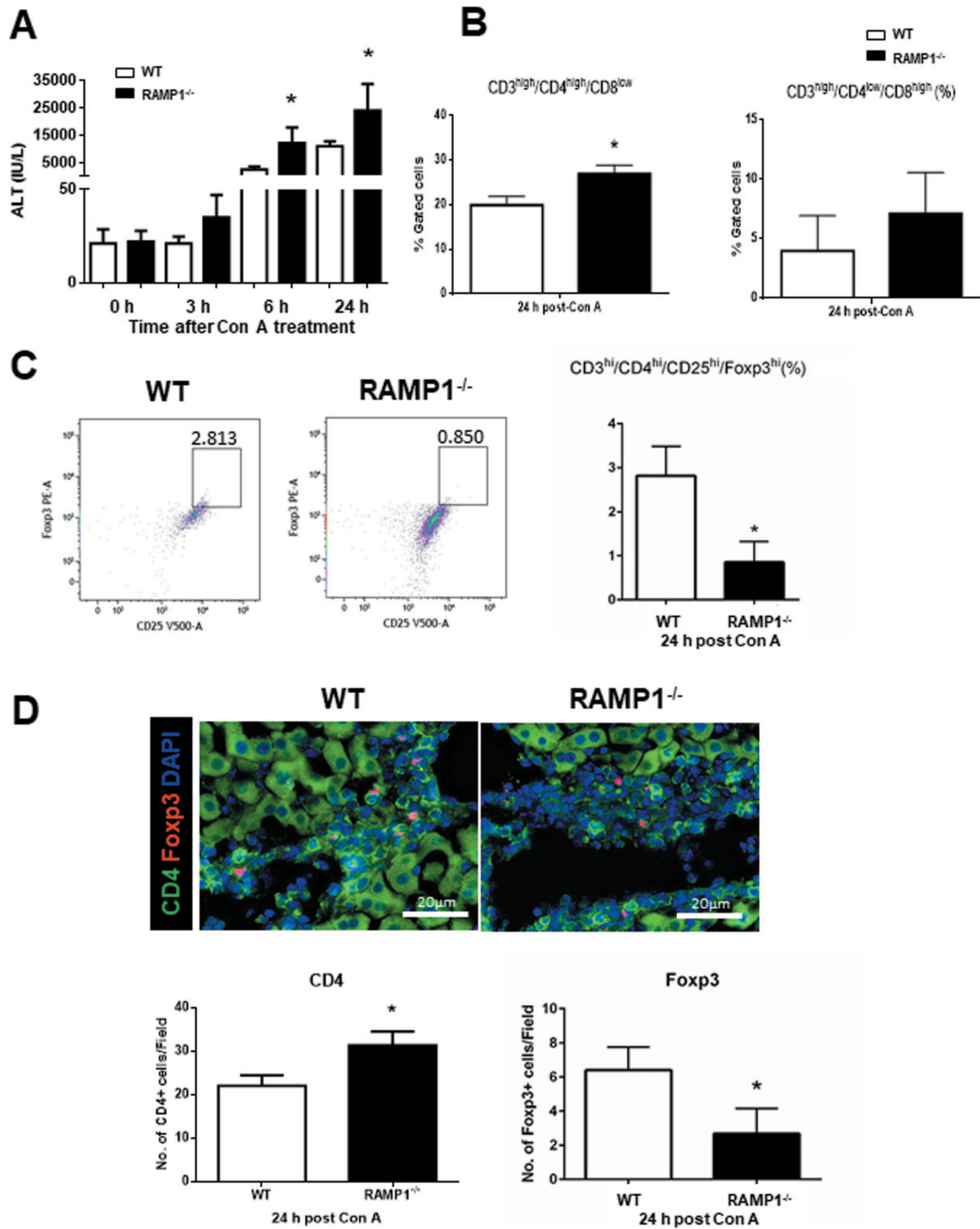To further clarify the role of Tregs in Con A hepatitis, we

Figure 1. Deficient RAMP1 signaling exacerbates Con A-induced liver injury, associated with reduced infiltration of Tregs

RAMP1^{-/-} and WT mice were administered ConA (20 mg/kg) by intraperitoneal injection. **A.** Time course of changes in ALT levels after Con A administration. Data are expressed as the mean $\pm$ SD (n = 6 mice per group). *P < 0.05 vs. WT mice. **B.** Flow cytometry analysis of the percentage of CD3^{high}/CD4^{high}/CD8^{low} cells and CD3^{high}/CD4^{low}/CD8^{high} cells within the CD45^{+} cell population in the liver at 24 hours after Con A treatment. Data are expressed as the mean $\pm$ SD of 6 mice per group. *P < 0.05 vs. WT mice. **C.** Representative dot plot analyses of CD25^{high}/Foxp3^{high} cells and quantification in WT and RAMP1^{-/-} mice at 24 hours after Con A treatment. Data are expressed as the mean $\pm$ SD of 6 mice per group. *P < 0.05 vs. WT mice. **D.** Representative dual immunofluorescence for CD4 (green) and Foxp3 (red) in liver sections from WT and RAMP1^{-/-} mice. The numbers of positive cells for CD4 and Foxp3 at 24 hours post Con A administration. Data are expressed as the mean $\pm$ SD of 6 mice per group. *P < 0.05 vs. WT mice

treated WT mice with an anti-FR4 antibody 24 hours before the Con A treatment. The anti-FR4 antibody exacerbated Con A-induced hepatitis at 24 hours post-Con A administration as indicated by higher levels of ALT (Figure 3A). Deletion of Tregs by FR4 antibody treatment reduced the infiltration of Foxp3+ cells (Figure 3B). The enhanced Con A-mediated hepatitis in mice treated with an anti-FR4 antibody was associated with downregulated mRNA levels of Foxp3, CXCR3, IL-10, and TGF-β (Figure 3C-F).

Discussion

In the present study, we analyzed the role of RAMP1, in a murine model of immune-mediated liver injury caused by Con A administration. We demonstrated that RAMP1 signaling plays an immunosuppressive role in Con A-mediated hepatitis by the accumulation of Tregs. These findings indicate that RAMP1 is a good therapeutic target for immune-mediated hepatitis.

We observed the exacerbation of Con A-mediated hepatitis in RAMP1$^{-/-}$ mice, which was associated with enhancement of CD4+ T cells with the pro-inflammatory cytokines, TNF-α and IFN-γ. Consistent with our results, RAMP1 signaling down-regulates inflammatory cytokines from isolated CD4+ cells and dendritic cells.[12,17] These observations indicate that RAMP1 signaling attenuates Con A hepatitis by suppressing CD4+ T cells with pro-inflammatory cytokines.

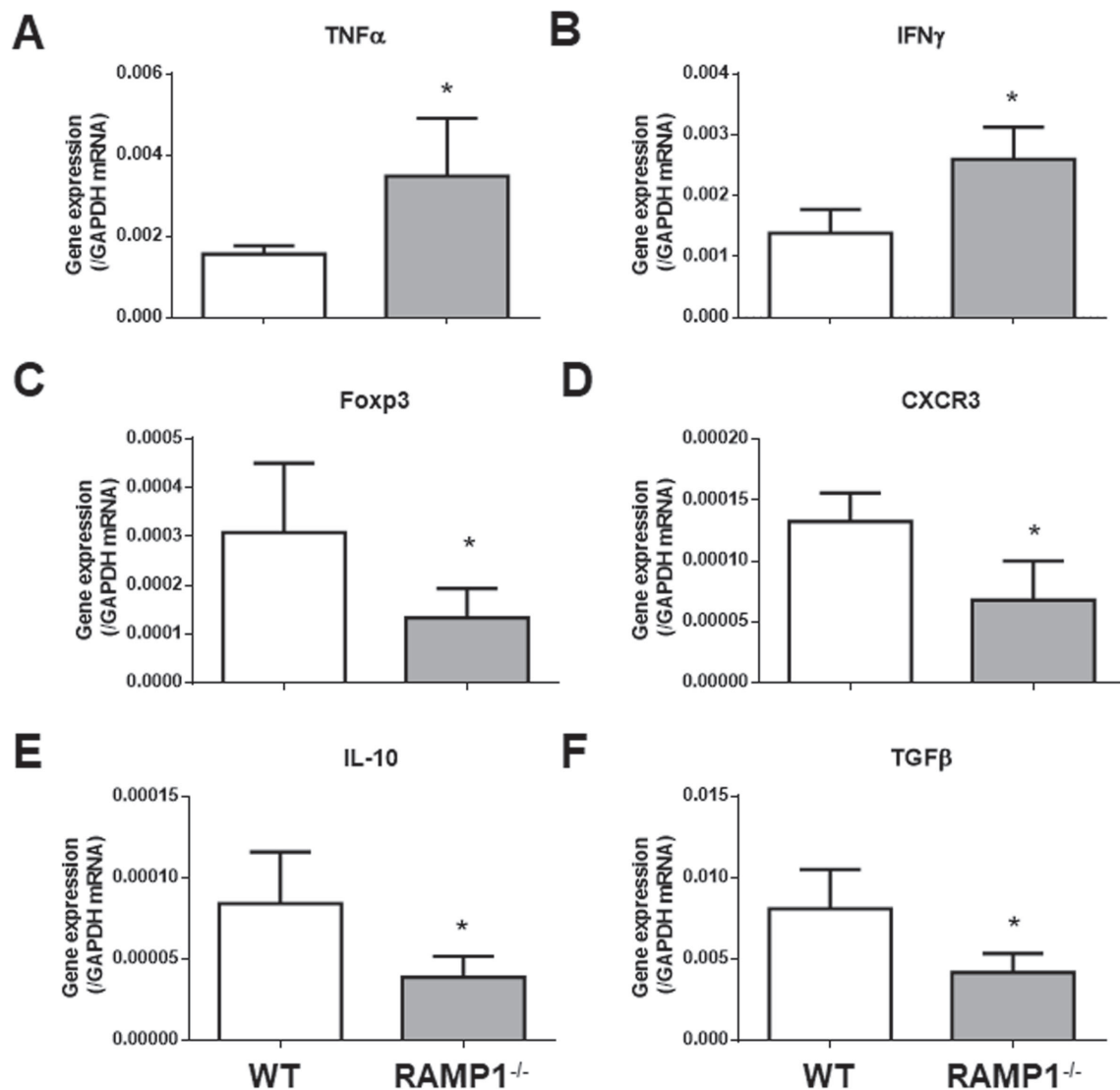AIH is characterized by high frequencies of both Tregs

Figure 2. Increased expression of pro-inflammatory cytokines in Ramp1$^{-/-}$ mice with Con A hepatitis

A, B. The mRNA levels of TNF-α and IFN-γ in the liver of WT and RAMP1$^{-/-}$ mice at 1 hour after Con A treatment. **C-F**. The mRNA levels of Foxp3, CXCR3, IL-10, and TGF-β in liver cells of WT and RAMP1$^{-/-}$ mice at 24 hours after Con A treatment. Data are expressed as the mean $\pm$ SD from 6 mice per group. *P < 0.05 vs. WT mice
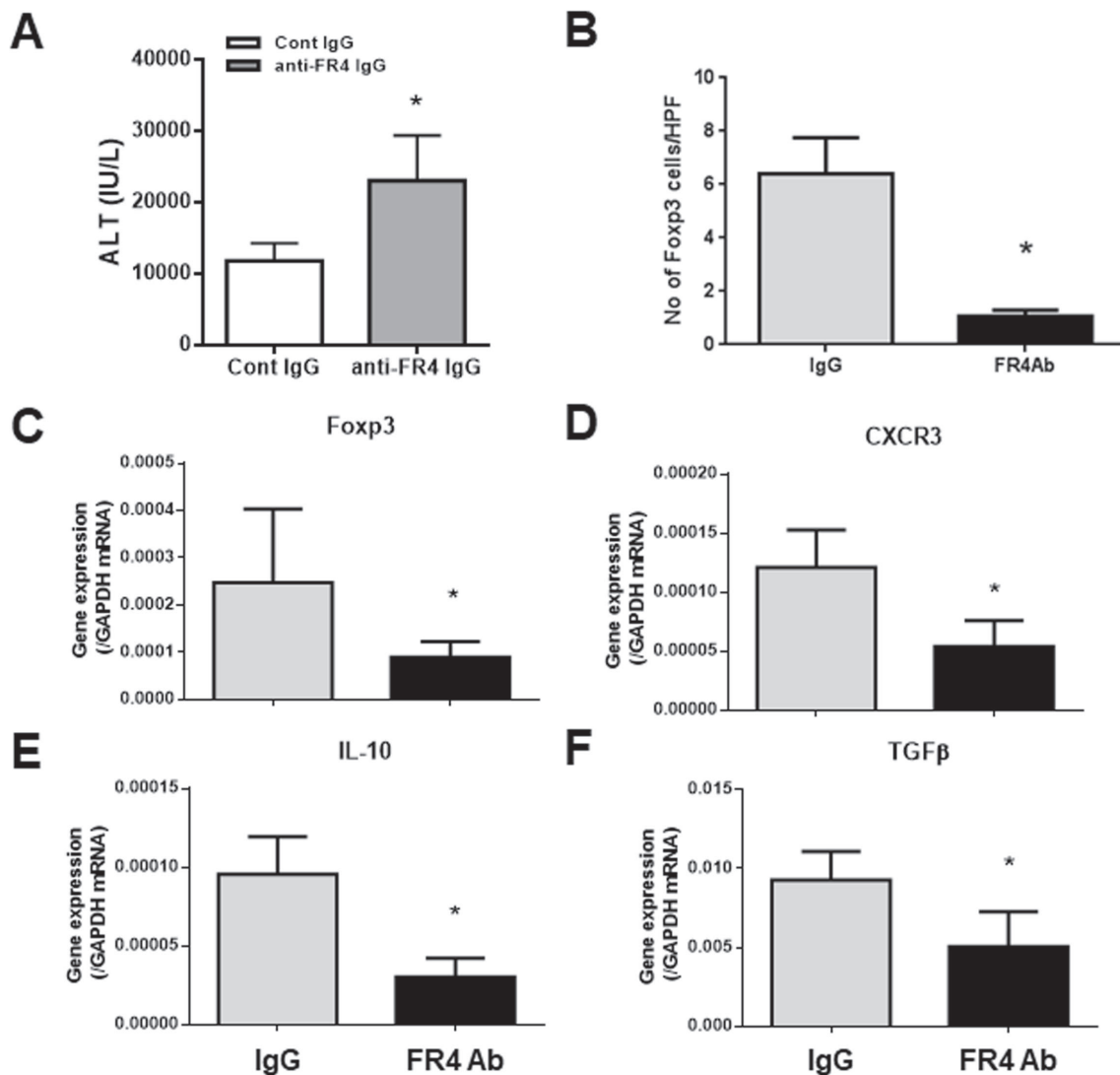
Figure 3. Deleting Tregs aggravates Con A-induced liver injury and decreases production of anti-inflammatory cytokines.

A. ALT levels at 24 hours after Con A administration in WT mice treated with an anti-FR4 antibody or control IgG. Data are expressed as the mean $\pm$ SD from 6 mice per group. **P < 0.05 vs. WT mice. **B-E**. Amounts of mRNA for Foxp3, CXCR3, IL-10, and TGF-β in WT mice treated with an anti-FR4 antibody or control IgG. Data are expressed as the mean $\pm$ SD from 6 mice per group. **P < 0.05 vs. WT mice

and effector T cells in the inflamed liver.[18] In Con A-induced hepatitis, the administration of Con A in mice increases the numbers of activated T cells and Tregs[5] in the liver, which is consistent with our results. Furthermore, Tregs protect the liver from Con A hepatitis.[5,19] Our data show that enhanced Con A-mediated hepatitis in RAMP1^{-/-} mice was associated with reduction in Tregs. We also observed that deletion of Tregs by FR4 antibody exacerbated Con A hepatitis in WT mice, suggesting that Tregs are important for suppression of immune-mediated hepatitis. Furthermore, it has been shown that CGRP induces Tregs.[11] Taken together, RAMP1 signaling attenuates Con A-mediated hepatitis via the enhancement of Tregs.

The current studies showed that decreased Tregs were associated with reduced hepatic expression of IL-10 and TGF-β in RAMP1^{-/-} mice. These results suggest that RAMP1 signaling induces Tregs and stimulates IL-10 production, leading to the suppression of the development of Con A hepatitis. Moreover, TGF-β plays a critical role in the differentiation of Tregs[20] to produce IL-10. We also found that treatment of FR4 antibody reduced the levels of TGF-β and IL-10, indicating that Tregs produce TGF-β and IL-10 to attenuate immune-mediated hepatitis.

These results suggest that RAMP1 signaling regulates immune-mediated hepatitis by enhancing TGF-β and IL-10 from Tregs. Further studies are warranted to

elucidate the action of RAMP1 signaling in Tregs as the immune-regulator in AIH.

Acknowledgements

Conflicts of interest: None

References

1. Manns MP, Lohse AW, Vergani D. Autoimmune hepatitis--Update 2015. *J Hepatol* 2015; 62 (1 Suppl): S100-11.
2. Sakaguchi S, Miyara M, Costantino CM, et al. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol* 2010; 10: 490-500.
3. Liston A, Gray DH. Homeostatic control of regulatory T cell diversity. *Nat Rev Immunol* 2014; 14: 154-65.
4. Tiegs G, Hentschel J, Wendel A. A T cell-dependent experimental liver injury in mice inducible by concanavalin A. *J Clin Invest* 1992; 90: 196-203.
5. Erhardt A, Biburger M, Papadopoulos T, et al. IL-10, regulatory T cells, and Kupffer cells mediate tolerance in concanavalin A-induced liver injury in mice. *Hepatology* 2007; 45: 475-85.
6. Poyner DR, Sexton PM, Marshall I, et al. International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. *Pharmacol Rev* 2002; 54: 233-46.
7. Hay DL, Poyner DR, Quirion R; International Union of Pharmacology. International Union of Pharmacology. LXIX. Status of the calcitonin gene-related peptide subtype 2 receptor. *Pharmacol Rev* 2008; 60: 143-5.
8. Kawashima-Takeda N, Ito Y, Nishizawa N, et al. RAMP1 suppresses mucosal injury from dextran sodium sulfate-induced colitis in mice. *J Gastroenterol Hepatol* 2017; 32: 809-18.
9. Kurashige C, Hosono K, Matsuda H, et al. Roles of receptor activity-modifying protein 1 in angiogenesis and lymphangiogenesis during skin wound healing in mice. *FASEB J* 2014; 28: 1237-47.
10. Mishima T, Ito Y, Nishizawa N, et al. RAMP1 signaling improves lymphedema and promotes lymphangiogenesis in mice. *J Surg Res* 2017; 219: 50-60.
11. Szklany K, Ruiter E, Mian F, et al. Superior cervical ganglia neurons induce Foxp3+ regulatory T cells via calcitonin gene-related peptide. *PLoS One* 2016; 11: e0152443.
12. Tsujikawa K, Yayama K, Hayashi T, et al. Hypertension and dysregulated proinflammatory cytokine production in receptor activity-modifying protein 1-deficient mice. *Proc Natl Acad Sci U S A* 2007; 104: 16702-7.
13. Fujita T, Soontrapa K, Ito Y, et al. Hepatic stellate cells relay inflammation signaling from sinusoids to parenchyma in mouse models of immune-mediated hepatitis. *Hepatology* 2016; 63: 1325-39.
14. Kojo K, Ito Y, Eshima K, et al. BLT1 signalling protects the liver against acetaminophen hepatotoxicity by preventing excessive accumulation of hepatic neutrophils. *Sci Rep* 2016; 6: 29650.
15. Nishizawa N, Ito Y, Eshima K, et al. Inhibition of microsomal prostaglandin E synthase-1 facilitates liver repair after hepatic injury in mice. *J Hepatol* 2018; 69: 110-20.
16. Oo YH, Weston CJ, Lalor PF, et al. Distinct roles for CCR4 and CXCR3 in the recruitment and positioning of regulatory T cells in the inflamed human liver. *J Immunol* 2010; 184: 2886-98.
17. Mikami N, Matsushita H, Kato T, et al. Calcitonin gene-related peptide is an important regulator of cutaneous immunity: effect on dendritic cell and T cell functions. *J Immunol* 2011; 186: 6886-93.
18. Taubert R, Hardtke-Wolenski M, Noyan F, et al. Intrahepatic regulatory T cells in autoimmune hepatitis are associated with treatment response and depleted with current therapies. *J Hepatol* 2014; 61: 1106-14.
19. Horst AK, Wegscheid C, Schaefers C, et al. Carcinoembryonic antigen-related cell adhesion molecule 1 controls IL-2-dependent regulatory T-cell induction in immune-mediated hepatitis in mice. *Hepatology* 2018; 68: 200-14.
20. Chen W, Jin W, Hardegen N, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003; 198: 1875-86.