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Binding of Hepatitis A Virus to its Cellular Receptor 1 Inhibits T-Regulatory Cell Functions in Humans

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Abstract

Background & Aims—CD4⁺ T regulatory (Treg) cells suppress immune responses and control self-tolerance and immunity to pathogens, cancer, and alloantigens. Most pathogens activate Treg cells to minimize immune-mediated tissue damage and prevent clearance, which promotes chronic infections. However, hepatitis A virus (HAV) temporarily inhibits Treg-cell functions. We investigated whether the interaction of HAV with its cellular receptor 1 (HAVCR1), a T-cell co-stimulatory molecule, inhibits the function of Treg cells to control HAV infection.

Methods—We studied the effects of HAV interaction with HAVCR1 on human T cells using binding, signal transduction, apoptosis, activation, suppression, cytokine production, and confocal microscopy analyses. Cytokines were analyzed in sera from 14 patients with HAV infection using bead arrays.

Results—Human Treg cells constitutively express HAVCR1. Binding of HAV to HAVCR1 blocked phosphorylation of Akt, prevented activation of the T-cell receptor, and inhibited function of Treg cells. At the peak viremia, patients with acute HAV infection had no Treg-cell suppression function, produced low levels of transforming growth factor- β (TGF- β), which limited leukocyte recruitment and survival, and high levels of interleukin-22, which prevented liver damage.

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Author Contributions: Experiments of virus and apoptotic cell binding were performed by M.M. and C.T., signal transduction by J.J., virus PCR and ALT levels by K.K., and confocal microscopy by N.A. J. M. C. prepared the HAVCR1 model. All experiments were designed by G.G.K. in consultation with M.M., J.J., K.K., N.A. and A.P. HAV patient evaluation, collection of blood, and purification of PBMC was done by A.P. and O.P. Monoclonal antibodies and critical constructs were prepared by D.T.U., R.H.D., J.M.C., and G.J.F., who also analyzed and discussed results. G.G.K. wrote the manuscript with help from M.M., J.J., and K.K., and all authors edited and discussed the paper. M.M. and J.J. contributed equally to this work.

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Conclusions—Interaction between HAV and its receptor HAVCR1 inhibits Treg cell function, resulting in an immune imbalance that allows viral expansion with limited hepatocellular damage during early stages of infection—a characteristic of HAV pathogenesis. The mechanism by which HAV is cleared in the absence of Treg-cell function could be used as a model to develop anti-cancer therapies, modulate autoimmune and allergic responses, and prevent transplant rejection.

Keywords

Hepatitis A virus cellular receptor 1; viral clearance; TGF- β ; immune regulation

Introduction

CD4⁺ regulatory T-cells (Tregs) that express CD25⁺ and the FoxP3 transcription factor have a significant role suppressing immune responses to self-antigens to prevent autoimmune disease and controlling the immune response to bacteria, viruses, parasites, and fungi¹. The mechanisms by which Tregs are activated, suppressed, and modulate immune responses are not fully understood. Pathogens have evolved strategies to activate Tregs as a means to limit the immune response and prevent tissue damage, which is advantageous for the expansion and survival of obligate intracellular parasites¹. Treg activation has been extensively studied in chronic infections of pathogens like hepatitis B (HBV) and C (HCV) viruses¹, where it plays a significant role inducing and maintaining chronicity. A variety of functions have been proposed for Tregs in pathogenesis¹ but the mechanisms by which these cells control pathogenesis are unclear. Far less is known about the function of Tregs in acute infection. Hepatitis A virus (HAV), a *Picornaviridae* that causes acute hepatitis in humans², does not result in chronic infection and rarely causes fulminant hepatitis but induces a temporary shut-off of Treg-function³ and elevation of autoantibodies^{4, 5}. The mechanism by which HAV blocks Tregs without enhancing immune-mediated tissue damage or preventing viral growth is unclear. To enter the cell, HAV interacts with its cellular receptor 1, HAVCR1^{6, 7}, a significant allergy and autoimmunity determinant in man^{8, 9}. HAVCR1 is expressed in T-cells where it functions as a phosphatidylserine (PtdSer) receptor and T-cell costimulatory molecule⁸, which suggests that the HAV-HAVCR1 interaction on T-cells is responsible for the long-lasting effect of HAV infection in the immune system.

The poor understanding of the pathogenic process of HAV prompted us to study the consequences of the HAV-HAVCR1 interaction on human Tregs. Here we show that human Tregs constitutively express HAVCR1 and the HAV-HAVCR1 interaction directly inhibits Treg-function. By shutting-off Tregs, HAV overwhelms the immune system with anti-self responses at the same time that limits *de novo* anti-HAV T effector responses by reducing Treg production of TGF- β , which is needed to recruit leukocytes to the inflammation site and promote T cell survival¹⁰. HAV also reduces liver damage by inducing the production of IL-22¹¹. A complete understanding of how HAV controls and disarms Treg-function could lead to the development of therapies to prevent and/or treat chronic infections, cancer, and allergic and autoimmune diseases in which Treg play a significant pathogenic role.

Materials and Methods

Cells, plasmids, and virus

Stable human Embryonic Kidney 293 (HEK293) or acute T-cell leukemia Jurkat E6.1 (Jurkat) cell transfectants expressing human HAVCR1 or its mouse ortholog mHavcr-1, Jurkat cells transiently transfected with plasmids coding for empty vector, HAVCR1, or an HAVCR1 construct containing a Y350A mutation (HAVCR1 Y350A) in the cytoplasmic tail that eliminates HAVCR1 signaling¹², and Jurkat cells cotransfected with the above-mentioned plasmids and luciferase reporters under the transcriptional control of AP-1 or

NFAT/AP-1¹³, and CMV (pRL-CMV, Promega, Corp.) elements were produced as described in Supplementary Materials.

Cell culture adapted strain HM175 of HAV was produced as described⁶. Cell extracts of uninfected cells were used in most experiments as negative control.

Antibodies and reagents

Antibodies and reagents used in ELISA, alanine aminotransferase assay, flow cytometry, binding assays, apoptosis analysis, and confocal microscopy are described in Supplementary Materials. FACSCanto II instrument was used for flow cytometry and analysis was done using FlowJo software.

Human blood and plasma samples

Blood of normal donors (NIH blood Bank, Bethesda, MD) and patients having acute HAV-infection (Hospital D. Cotugno, Naples, Italy) at the time of hospitalization (T0) and 3–4 weeks later after resolution of symptoms and viremia (T1) was obtained in accordance to local ethical committee approvals. Peripheral blood mononuclear cells (PBMC) were purified using Lymphocyte Separation Medium (Mediatech, Inc.). PBMC of nine HAV patients and nine blood donors were cryopreserved for later use. Tregs and T effector (Teff) cells were purified from PBMC using Dynabeads regulatory CD4+CD25+ T cell kit (Invitrogen Corp.) as recommended by the manufacturer. Analyses of plasma samples are described in Supplementary Materials.

Binding Assays

Apoptotic Jurkat cells produced by treatment with etoposide or dexamethasone were stained with 5 μ M CMFDA (Jurkat-CMFDA cells), bound to treated or untreated stable HEK293 transfectants, and analyzed using a fluorescence microscope or plate reader as described in Supplementary Materials.

Quantitative Reverse Transcription Polymerase Chain Reaction

Primers and beacons to quantitate HAV genomes¹⁴ and human *HAVCR1* and *GAPDH* mRNA were obtained from Applied Biosystems (ABI). Sample preparations, reverse transcription (RT), polymerase chain reaction (PCR), and quantitative evaluations were performed as described in Supplementary Materials.

HAVCR1-mediated neutralization of HAV

The interaction of HAV with HAVCR1 was analyzed using a soluble-receptor neutralization assay¹⁵. Construction, expression, and purification of mutants of human and monkey HAVCR1 is described in Supplementary Materials.

xMAP Luminex immunoarray assays

Phosphoantibody-specific and cytokine immunoarray assays were performed as suggested by the manufacturers (Millipore Corp., and Biolegend, Inc.) and as described in Supplementary Materials.

Luciferase assays

Luciferase reporter assays¹⁶ were performed in transiently cotransfected Jurkat cells as described in Supplementary Materials.

Fluorescence confocal microscopy

HAVCR1 and TCR colocalization¹² was analyzed by fluorescence confocal microscopy as described in Supplementary Materials.

In vitro Treg suppression assay

In vitro Treg suppression assays³ using purified Treg and Teff cells were performed as described in Supplementary Materials.

Statistical analysis

A two-tailed unpaired Student's *t*-test was used to determine differences between two means in analyses of cell lines. A two-tailed paired Student's *t*-test was used to determine differences between two means in analyses involving samples from the same blood donors or HAV patients. The paired and unpaired tests were performed using GraphPad software. *P* values of less than 0.05 were considered significant.

Results

HAV blocks the PtdSer-receptor function of HAVCR1

There is no evidence that HAV can infect T-cells, and wild type HAV did not infect human T-cells or a Jurkat T cell line ectopically expressing HAVCR1. Nevertheless, virus binding might affect the natural function of HAVCR1 as a pattern recognition receptor that binds PtdSer on apoptotic cells⁸. Here we show that apoptotic cells (Supplementary Figure 1) stained with CMFDA bound to stable HEK293 cell transfectants expressing HAVCR1 at the cell surface (HAVCR1-HEK293) (Figure 1A) but not vector transfected HEK293 (Vector-HEK293) cells (Figure 1B). The binding of apoptotic cells was due to the HAVCR1-PtdSer interaction because anti-HAVCR1 mAb 3D1 (Figure 1B) and annexin V (Supplementary Figure 2), which binds to PtdSer, blocked binding of apoptotic cells to HAVCR1-HEK293 cells. HAV blocked binding of apoptotic cells to HAVCR1-HEK293 cells in a dose-dependent manner (Figure 1C). Cell extracts of uninfected cells had no effect in apoptotic-cell binding to cells expressing HAVCR1, and were used as negative control in most experiments described herein. HAV had no effect on binding of apoptotic cells to mHavcr1-HEK293 cells (Figure 1D), a stable cell line that expresses mHavcr1 (Supplementary Figure 3), the mouse ortholog of HAVCR1. Because mHavcr1 is a receptor for PtdSer⁸ but not for HAV (Figure 1E), these data confirmed that HAV specifically prevents binding of PtdSer to HAVCR1. Using HAVCR1 mutants and a soluble-receptor neutralization assay that mimics the interaction of HAV with HAVCR1 at the cell surface¹⁵, we determined that HAV interacts with residues W92 and F93 but not N94 of the FG-loop of the IgV domain of HAVCR1 (Figure 1F). Control poliovirus receptor (PVR-Fc) did not neutralize HAV. Mutations in HAVCR1 residues A21, D62, and R65 that are distant from the FG-loop did not affect HAV-receptor function (Supplementary Figure 4). Since W92 and F93 form one side of the HAVCR1 PtdSer-binding pocket¹⁷, the virus- and PtdSer-binding sites overlap providing a mechanism for how HAV blocks binding of apoptotic cells to HAVCR1.

Binding of HAV to HAVCR1 reduces activation of Akt and increases FasL-induced cell death

We first analyzed whether HAVCR1 signaling could activate the PI3K/Akt pathway as reported for the mouse ortholog¹³. Treatment of Jurkat cell stable transfectants expressing HAVCR1 at the cell surface (HAVCR1-Jurkat, Supplementary Figure 5) with apoptotic cells induced a peak of Akt phosphorylation at 30 min post-treatment compared to vector-transfected (Vector-Jurkat) cells (Figure 2A). Anti-HAVCR1 mAb 3D1 blocked this Akt activation (Figure 2B) confirming that HAVCR1 signaling also activate the PI3K/Akt

pathway. Interestingly, HAV reduced the basal level of Akt phosphorylation in HAVCR1-Jurkat cells and blocked activation of Akt by apoptotic cells compared with Vector-Jurkat cells (Figure 2C). This effect was not due to a shift in the kinetics of Akt phosphorylation (Figure 2D) suggesting that HAV induces a negative signal that reduces phosphorylation of Akt. Moreover, HAVCR1-Jurkat cells treated with HAV showed increased cell death in response to Fas crosslinking compared to Vector-Jurkat cells (Figure 2E). HAV did not affect the viability of HAVCR1-Jurkat cells (Supplementary Figure 6). Taken together, these data clearly show that binding of HAV to HAVCR1 blocks the activation of the PI3K/Akt pathway in T-cells and enhances Fas-mediated cell death.

Binding of HAV to HAVCR1 prevents TCR activation

In addition to the activation of the PI3K/Akt pathway, crosslinking of HAVCR1 with agonistic mAbs induces association of HAVCR1 with the TCR complex and upregulation of T-cell activation¹². Therefore, it was of interest to study the effect of HAV binding on T-cell activation. We used anti-HAVCR1 mAb 1D12 because it protects cells against HAV infection (Figure 2F) and blocks PtdSer binding but does not affect T cell activation (Figure 3A, B, D, E). Binding of HAV to HAVCR1-Jurkat cells prevented the activation of TCR by CD3/CD28 ligation as assessed by the phosphorylation of ZAP-70 (Figure 3A) and LAT (Figure 3B), two key kinases in the TCR activation pathway¹⁸. However, HAV could not block phosphorylation of already activated T-cells (Figure 3C). Treatment with anti-HAVCR1 mAb 1D12 restored phosphorylation of ZAP-70 and LAT. Neither HAV nor anti-HAVCR1 mAb affected activation of ZAP70 or LAT in Vector-Jurkat cells (Figure 3A,B). HAV did not affect phosphorylation of Syk (Supplementary Figure 7A), a kinase involved in the B cell receptor pathway, and transcription from a luciferase reporter under the control of a CMV promoter (Supplementary Figure 7B) indicating that HAV has no global effect on phosphorylation or transcription in T-cells. Taken together, these data indicate that HAV prevents TCR activation in T-cells expressing HAVCR1. Since mHavcr1 costimulation increases transcription of genes under the control of activator protein 1 (AP-1) elements¹³, we analyzed the effect of the HAV-HAVCR1 interaction in CD3/CD28-mediated activation of T-cells using an AP-1- luciferase reporter¹³ (Figure 3D). Jurkat cells cotransfected with plasmids coding for HAVCR1 and the reporter gene expressed HAVCR1 at the cell surface (Supplementary Figure 8). CD3/CD28 ligation significantly increased AP-1 transcription in cells that expressed HAVCR1 compared to an HAVCR1 mutant containing a Y350A change in the cytoplasmic tail (HAVCR1 Y350A) that prevents signaling¹² or to vector transfected cells ($P<0.001$). HAV completely blocked the CD3/CD28-mediated activation of AP-1-transcription in cells expressing HAVCR1, but had no significant effect in cells expressing HAVCR1 Y350A or vector-transfected cells. Moreover, HAVCR1 blockade with mAb 1D12 restored AP-1 transcription. We also analyzed transcription from a nuclear factor of activated T-cells (NFAT)/AP-1 composite element¹³, which is regulated by calcium and is present in many genes transcribed in immune cells¹⁹. Stimulation with anti-CD3/CD28 antibodies enhanced NFAT/AP-1 reporter activity in cells expressing HAVCR1 compared to HAVCR1 Y350A or vector transfected cells (Figure 3E), which confirmed that HAVCR1 enhanced NFAT/AP-1-mediated transcription. Again, HAV significantly inhibited the CD3/CD28 activated NFAT/AP-1 transcription in HAVCR1 but not in HAVCR1 Y350A or vector-transfected cells, and blockade of HAVCR1 with mAb 1D12 prevented the HAV-mediated inhibition. These data on the activation of kinases and transcription from AP-1 and NFAT/AP-1 elements clearly show that HAV not only blocks HAVCR1-mediated costimulation but also prevents TCR activation.

We examined the association of HAVCR1 with TCR by fluorescence confocal microscopy¹². Binding of HAV to HAVCR1-Jurkat cells (Supplementary Figure 9) does not prevent colocalization of HAVCR1 with TCR (Figure 3F) in anti-CD3 mAb activated cells.

Since anti-HAVCR1 Ab 1750 binds specifically to HAVCR1 (Supplementary Figure 10) but does not itself induce TCR colocalization (Supplementary Figure 11) and anti-CD3 mAb activates T-cells inducing colocalization of HAVCR1 and TCR in the absence of HAV, our data indicated that HAV induces a negative signal that does not affect colocalization of HAVCR1 with TCR.

The interaction of HAV with HAVCR1 shuts-off Treg-function

During HAV acute infection, most patients develop a transient impairment of T regulatory cells (Treg) function³ and an increase in the production of autoantibodies^{4, 5}. HAV infection can trigger autoimmune hepatitis²⁰ and is associated with other autoimmune manifestations such as lupus-like syndrome²¹. Therefore, we analyzed HAVCR1 expression on Treg cells. Expression levels of CD4 and CD25 vary in the human population with two typical patterns in our blood donors (Figures 4A and C). Purified CD4+CD25+ T-cells from healthy blood donors (Figure 4A) express forkhead box protein 3 (FoxP3) intracellularly and HAVCR1 at the cell surface (Figure 4B) whereas CD4+CD25- T effector cells (Teff) (Figure 4C) lack expression of both proteins (Figure 4D). The proportion of cells that express HAVCR1 and FoxP3 is similar in both donors (Figures 4B and D). Expression of HAVCR1 was also verified by qRT-PCR analysis, which showed lower cycle threshold (Ct) values for HAVCR1 in Tregs than in Teff (Supplementary Figure 12) corresponding to approximately 6-fold higher expression levels in Tregs (Figure 4E). These CD4+CD25+ T-cells showed Treg-function, as they inhibited the proliferation of CD4+CD25- Teff activated with anti-CD3/CD28 antibodies (Figure 5A). Importantly, treatment with HAV turned-off the Treg-function, and this effect was prevented by HAVCR1 blockade with mAb 1D12. HAV and extracts of uninfected cells did not affect activation of Teff ruling out the possibility of a toxic effect on Teff. HAV also reduced production of TGF- β and IL-10 (Figure 5B), two inhibitory cytokines produced by activated Tregs. HAV blocked phosphorylation of Akt and ZAP-70 in Treg treated with anti-CD3/CD28 mAbs (Figure 5C), and phosphorylation was restored by treatment with anti-HAVCR1 mAb 1D12. These data clearly show that the HAV-HAVCR1 interaction blocks Akt and TCR activation, which are required for Treg-function²². We also studied proliferation of PBMC activated with a mixture of 27 peptides (PepMix) containing Cytomegalovirus, Epstein-Barr virus, Influenza virus, and *Clostridium tetani* restricted HLA class I and II restricted T-cell epitopes (Figure 5D). Depletion of CD25+ cells increased PBMC proliferation. Supplementation of CD25-depleted PBMC with purified autologous CD4+CD25+ Treg treated with cell extracts reduced proliferation whereas treatment with HAV blocked Treg-function increasing PBMC proliferation, which is consistent with our suppression assays results using purified Teff and Treg (Figure 5A).

Acute HAV infection shuts-off Treg-function

To study Treg in acute HAV patients, we purified PBMC from blood of nine HAV patients having clinical signs of hepatitis at the time of hospitalization (T0) and 3–4 weeks later after resolution of symptoms and viremia (T1). Increased ALT levels and HAV RNA were detected in the plasma from these HAV patients (Supplementary Figure 13). PBMC from nine HAV-seronegative blood donors were used as control. HAV patients and donors had comparable numbers of CD4+CD25+ Treg, which expressed similar levels of FoxP3 (Supplementary Figure 14). Activation with PepMix induced a higher level of PBMC proliferation at T0 than at T1 and donors (Figure 6A). Interestingly, HAV did not induce PBMC proliferation in patients and donors, which agrees with the lack of a strong HAV-specific Teff response during the early phase of HAV acute infection². In a suppression assay, Treg from T0 did not suppress proliferation of autologous Teff (Figure 6B, left panel) compared to Treg from donors, which were blocked by treatment with HAV (Figure 6C). The suppression function of Tregs was restored in four patients at T1 (Figure 6B, right panel) but did not reach the levels observed in the donors (Figure 6C), which suggested that

the complete restoration of Treg-function in HAV patients requires more time beyond T1. Taken together, these data show that Treg-function was shut-off at T0 in all HAV patients confirming previous reports³.

Patients with acute hepatitis A virus infection have low levels of TGF- β and produce IL-22 during peak viremia

Treg are a major source of regulatory cytokines such as IL-10 and TGF- β , which have a very significant role in pathogenesis. Since we showed that HAV blocks Treg-function and cytokine secretion, it was of great interest to study the cytokine pattern during acute hepatitis A. Typically, after a window period where HAV remains undetected by the immune system, patients develop a peak of anti-HAV IgM Ab early in infection followed by a steady increase of anti-HAV IgG Ab that precedes virus clearance²(Figure 6D). Analysis of the cytokines present in plasma samples from an HAV-infected patient, including IFN- γ , IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, IL-22, and TGF- β , taken at different times after the detection of HAV RNA by PCR revealed a significant increase in only two cytokines in plasma: a peak of IL-22 early in infection followed by a peak of TGF- β after resolution of symptoms as indicated by normal ALT levels (Figure 6D). The increase in IL-22 coincided with the peak viremia whereas the increase of TGF- β occurred at the end of the viremia when low or undetectable levels of virus are present in the blood. Based on the presence of viral RNA and the levels of anti-HAV IgM and IgG Ab, we classified the plasma samples into 4 stages: HAV RNA and no Ab in stage 1; HAV RNA, high IgM, and low IgG in stage 2; HAV RNA, low IgM, and high IgG in stage 3; and no HAV RNA, low IgM, and high IgG in stage 4 (Figure 6D). Plasma samples from thirteen additional HAV-infected patients were grouped according to the above-mentioned parameters. Analysis of IL-22 and TGF- β levels in plasma samples from all the patients, including samples from the patient in Figure 6D, showed that patients in stage 2 had high levels of IL-22 and low levels of TGF- β whereas patients in stage 3 had undetectable levels of IL-22 and high levels of TGF- β compared to low or undetectable levels of both cytokines in stages 1 and 4 (Figure 6E). Taken together, these data indicate that patients with acute HAV infection produce IL-22 during peak viremia in the absence of Treg control and low levels of TGF- β . Further experimentation will be required to confirm that IL-22 protects the liver from the excessive immunopathogenic damage during HAV infection in the absence of Treg-function.

Discussion

Analysis of the pathogenic process of HAV demonstrated that its receptor, HAVCR1, controls Treg-function. Our results show that HAV blocks binding of apoptotic cells to HAVCR1 and prevents activation of Akt and TCR in T-cells that express HAVCR1. Most interestingly, we found that CD4⁺CD25⁺Foxp3⁺ Tregs express HAVCR1 at the cell surface and that the HAV-HAVCR1 interaction shuts-off the function of Treg and reduces the production of IL-10 and TGF- β , two main regulatory cytokines. Our study of Treg-function in HAV patients confirmed that Treg are transiently impaired during viremia, which is supported by our *in vitro* and *ex-vivo* studies with normal donors showing that the HAV-HAVCR1 interaction blocks TCR and shuts-off Treg-function. Most viruses induce the activation of Akt²³ to increase cellular metabolism and suppress apoptosis, and also activate Treg¹ to limit immune responses. Surprisingly, HAV has a very different approach to infection and blocks both pathways.

HAV-infection induces a poorly understood immunopathogenic process² resulting in limited liver damage and the permanent modulation of allergic^{24–26} and autoimmune^{20, 21, 24} responses. The association between allelic forms of HAVCR1 and protection against the development of atopy in HAV seropositive individuals suggested that the HAV receptor is involved in the modulation of allergic diseases²⁶. However, the mechanism by which HAV

modulates autoimmune diseases has not been explored in detail. In man, HAVCR1 has been implicated in susceptibility to rheumatoid arthritis^{9, 27}. In mouse, Treg do not express mHavcr1 but it is constitutively expressed in dendritic cells and indirectly modulates Treg-function²⁸. Our finding that human Treg express HAVCR1 and that HAV shuts-off Treg-function point to a direct link between HAV infection and autoimmunity via interaction with HAVCR1. This transitory block of Treg-function may allow clonal expansion and activation-induced cell death²⁹ leading to the deletion of pathogenic T-cells, which could be responsible for the protective effect of HAV infection in the development of autoimmune and allergic diseases²⁴.

Given the current interest in the role of Treg-function in pathogenesis¹, the HAV-mediated shut-off of Treg described here could be used as an experimental model to understand how Treg control immune responses and develop therapeutics to prevent immune diseases and transplant rejections. The fine balance of turning-off Treg-function under low levels of TGF- β , which limit T-cell effector-function^{30, 31}, and in the presence of IL-22, a cytokine produced by T, NK, and NKT cells that prevents tissue damage, could be responsible for the limited liver damage and successful clearance of HAV that is not achieved in HBV and HCV chronic infections. The strong anti-HAV humoral response that reduces HAV viremia prevents binding of viral particles to HAVCR1³² and shutting-off Treg-function. A late onset of CTL completes viral clearance². It is possible that the transitory Treg/IL-22/TGF- β environment induced by the HAV infection has a permanent affect in allergic and autoimmune responses. Imbalances in this system may be responsible for the rare fulminant hepatitis events triggered by HAV infection. We propose that the HAV-mediated shut-off of Treg-function under low levels of TGF- β in the presence of IL-22 is responsible for the long-lasting modulation of the immune response in HAV-infected individuals, and could serve as a model to develop therapeutic strategies to treat cancer, modulate autoimmune and allergic responses, and prevent transplant rejections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HAV	Hepatitis A Virus
HAVCR1	HAV cellular receptor 1
TCR	T-cell receptor
Treg	CD4+CD25+ regulatory T-cells
Teff	CD4+CD25- effector T-cells

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Figure 1. HAV blocks binding of apoptotic cells to HAVCR1

(A) HAVCR1- or Vector-HEK293 cells stained with PE-labeled anti-HAVCR1 mAb (blue) or isotype (grey) and analyzed by flow cytometry. (B) Apoptotic Jurkat cells stained with CMFDA (Jurkat-CMFDA, green) were bound to Vector- and HAVCR1-HEK293 cell monolayers pretreated with anti-FLAG mAb or anti-HAVCR1 mAb 3D1. Monolayers analyzed by epifluorescence and micrographs taken with a Zeiss Axioscope inverted microscope at 200 X using FITC filters. (C) Binding of apoptotic Jurkat-CMFDA cells to HAVCR1-HEK293 cell monolayers treated with uninfected cell extracts or different amounts of HAV. Monolayers analyzed and micrographs taken as in (B). (D) Apoptotic Jurkat-CMFDA cells bound to HAVCR1- or mHavcr1-HEK293 cell monolayers treated with uninfected cell extracts or HAV. Fluorescence associated with the cell monolayers determined in a plate reader. (E) Binding of HAV to HAVCR1-, mHavcr1-, and Vector-HEK293 cells determined by qRT-PCR. (F) Space-filling model of the IgV domain of HAVCR1 based on the crystal structure of mHavcr1 (PDB 2OR8) showing the location of W92, F93, and N94 residues in the FG-loop and A21, D62 and R65 residues in the BED face (left panel). Soluble-receptor neutralization assay using monkey HAVCR1-Fc (mkHAVCR1-Fc) W92A, F93A, and W92A-F93A, and N94A mutants (right panel). Wild type mkHAVCR1-Fc and poliovirus receptor (PVR-Fc) were used as controls. Values in graphs represent means $\pm$ s.d., data representative of 3 different experiments, $n=3$ (D,E) and $n=8$ (F). *, $P=0.001$; **, $P<0.0001$.

Figure 2. HAV blocks HAVCR1 signaling

(A) Kinetics of Akt phosphorylation at Ser473 in extracts of HAVCR1- and Vector-Jurkat cells activated with apoptotic cells determined using phospho-specific antibodies by xMAP Luminex technology. (B) Akt phosphorylation at Ser473 as in (A) but cells treated or not with anti-HAVCR1 mAb 3D1 and activated or not with apoptotic cells for 30 min. (C) Same as (B) but cells pretreated with cell extracts of uninfected cells or HAV and activated with apoptotic cells for 30 min. (D) Same as (A) but HAVCR1-Jurkat cells pretreated with cell extracts of uninfected cells or HAV. (E) HAVCR1- and Vector Jurkat cells treated with medium (grey), 100 ng/ml FasL (green), or FasL plus HAV (red) for 16 h at 37°C, stained with LIVE/DEAD Aqua fluorescent reactive dye that binds preferentially to dead cells, and analyzed by flow cytometry. (F) Protection against HAV infection by anti-HAVCR1 mAb 1D12. Antibody protection assay^{6,7} in African green monkey kidney cells treated or not with 50 µg/ml anti-HAVCR1 mAb 1D12 and infected with HAV or mock-infected for 3 days, fixed, and stained with human anti-HAV and FITC-labeled anti-human antibodies. Micrographs taken as in Figure 1B. The mean fluorescence intensity (MFI) values are means $\pm$ s.d. after normalization by total GAPDH, n=3, data representative of 3 different experiments. *, $P < 0.001$.

Figure 3. Binding of HAV to HAVCR1 blocks TCR activation

(A) Phosphorylation of ZAP-70 determined by multiplex phospho-specific immunoarray assay using xMAP Luminex technology in cell lysates of HAVCR1- and Vector-Jurkat cells treated or not with anti-HAVCR1 mAb 1D12, followed by HAV or uninfected cell extracts, and finally anti-CD3/CD28 beads. (B) Same as (A) but for the detection of activated LAT. (C) Phospho-specific immunoarray assay as (A and B) for the detection of activated ZAP-70 and LAT in cell lysates of HAVCR1-Jurkat cells treated first with anti-CD3/CD28 beads and then with HAV or uninfected cell extracts. (D) Jurkat cells cotransfected with plasmids coding for HAVCR1, HAVCR1 Y350A, or vector and an AP-1 luciferase reporter were treated as in (A,B). Luciferase in cell extracts measured as Relative Light Units (RLU). (E) Jurkat cells cotransfected and analyzed as in (D) but with a plasmid coding for NFAT/AP-1 luciferase reporter instead of AP-1 reporter. (F) Fluorescence confocal microscopy analysis of HAVCR1-jurkat cells treated with HAV, activated with anti-CD3 mAb, and stained with biotin-labeled anti-HAVCR1 Ab 1750, Alexa Fluor 594-labeled streptavidin, and Alexa Fluor 488-labeled anti-CD3 mAb. Micrographs taken at 630 X with an oil immersion objective are representative of two experiments. Values in graphs represent means $\pm$ s.d. after normalization by total GAPDH (A,B) or protein content (D,E), n=3, data representative of 3 different experiments. *, $P < 0.01$; **, $P < 0.001$.

Figure 4. Human Treg express HAVCR1

(A) Purified CD4+CD25⁻ and CD4+CD25⁺ human T-cells stained with anti-CD4-AmCyan and anti-CD25-PE mAbs and analyzed by flow cytometry. (B) CD4+CD25⁺ cells from (A) stained with anti-FoxP3-APC (purple) and anti-HAVCR1-1750-PE (blue line) mAbs or corresponding isotype controls (grey fills) and analyzed by flow cytometry. (C) Purified CD4+CD25⁻ and CD4+CD25⁺ human T-cells from a different donor stained with anti-CD4-AmCyan, anti-CD25-PE, anti-HAVCR1-1D12-APC-Cy7, and anti-FoxP3-APC mAbs and analyzed by flow cytometry. (D) Analysis in the APC-Cy7 and APC channels from (C). (E) qRT-PCR analysis of the expression level of HAVCR1 mRNA in purified CD4+CD25⁻ and CD4+CD25⁺ cells relative to GAPDH mRNA. The mRNA content was calculated according to the $2^{-\Delta\Delta C_t}$ method and expressed in arbitrary units. Data are representative of 3 donors. Values represent means $\pm$ s.d., n=3. *, $P < 0.001$.

Figure 5. Binding of HAV to HAVCR1 blocks Treg-function in T-cells from blood donors
 (A) Purified CD4+CD25- (Teff) or CD4+CD25+ (Treg) T-cells from donors treated or not with HAV or cell extracts of uninfected cells and activated with anti-CD3/CD28 beads. Teff co-cultured with Treg pre-treated with HAV or uninfected cell extracts in the presence of anti-HAVCR1 1D12 mAb or control anti-FLAG mAb and activated with anti-CD3/CD28 beads. Proliferation assessed by ^3H -thymidine incorporation in T-cells. (B) TGF- β 1 and IL-10 concentration in Teff and Treg co-cultivation supernatants in the presence of uninfected cell extracts or HAV as assessed by xMAP Luminex technology. (C) Purified Treg were treated or not with anti-CD3/CD28 activating beads, anti-HAVCR1 mAb 1D12, and HAV or uninfected cell extracts. Phosphorylation of ZAP-70 and Akt in cell lysates determined as in Figure 3A,B. (D) Donor PBMC, CD25-depleted PBMC, CD25-depleted PBMC co-cultivated with purified autologous CD4+CD25+ Treg treated with HAV or cell extracts from uninfected cells were activated with PepMix or not activated (Medium). Proliferation assessed by ^3H -thymidine incorporation in cells. Data representative of 5 (A) or 2 (B and D) different donors. Values in graphs represent means $\pm$ s.d., n=3. *, $P < 0.01$.

Figure 6. Treg-function and cytokine production during the course of acute HAV infection

(A) proliferation of PBMC from nine HAV patients collected at T0 and T1 and nine blood donors treated or not with 0.5 mg/ml of PepMix CEFT or HAV. (B) Suppression assay using purified CD4+CD25+ Treg, CD4+CD25- Teff, or co-culture of Treg and Teff at a 1/20 ratio from nine HAV patients at T0 or T1 and activated with anti-CD3/CD28 beads. (C) Suppression assay as (B) but Treg and Teff purified from blood donors. (D) Analysis of serial plasma samples taken from a patient with acute HAV infection at different days postinfection. Concentration of IL-22 (red bars) and TGF- β 1 (blue bars) determined by xMAP Luminex technology and alanine transaminase (ALT) concentration determined enzymatically (green line). Presence of HAV RNA by RT-PCR (UltraQual, NGI) and levels of anti-HAV IgM (HAVAB M, Abbott Labs) and IgG (HAVAB, Abbott Labs) levels (co/s, cut-off to signal ratio) determined at SeraCare, Inc. Samples were classified into 4 stages according to HAV RNA, IgM, and IgG levels. (E) TGF- β 1 and IL-22 concentrations measured in plasma samples as in (D). Cytokine concentrations in plasma samples from a normal individual (X), thirteen HAV patients (closed symbols), and samples from the patient in (D) (open symbols) were included in the analysis. N is the number of samples per stage. Data is representative of 3 experiments (D and E). Proliferation assessed by ^3H -thymidine incorporation in T-cells (A–C). Values in graphs represent means $\pm$ s.d., n=3 (B–D). Values are readings in each sample (A and E) and are representative of 3 experiments. *, $P < 0.05$; **, $P < 0.01$. N.S., not significant.