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Immunomodulatory role of the hepatocyte during HCV infection: driving CD4+CD25+Foxp3+ Regulatory T cell Development through the Tim-3/Gal-9 Pathway

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Abstract

Hepatitis C virus (HCV) is remarkable at disrupting human immunity to establish chronic infection. Accumulation of regulatory T cells (Tregs) and up-regulation of inhibitory signaling pathways (such as Tim-3/Gal-9) play pivotal roles in suppressing antiviral effector T cells (Teffs) that are essential for viral clearance. While Tim-3/Gal-9 interactions have been shown to negatively regulate Teffs, their role in regulating Tregs is poorly understood. To explore how Tim-3/Gal-9 interactions regulate HCV-mediated Treg development, in this study we provide pilot data showing that HCV-infected hepatocytes express higher levels of Gal-9 and TGF-β, and up-regulate Tim-3 expression and regulatory cytokines TGF-β/IL-10 in co-cultured $CD4^+$ T cells, driving conventional $CD4^+$ T cells into $CD25^+Foxp3^+$ Tregs. Additionally, recombinant Gal-9 protein can transform TCR-activated $CD4^+$ T cells into $Foxp3^+$ Tregs in a dose-dependent manner. Importantly, blocking Tim-3/Gal-9 ligations abrogates HCV-mediated Treg induction by HCV-infected hepatocytes, suggesting that Tim-3/Gal-9 interactions may regulate $Foxp3^+$ Treg development and function during HCV infection.

Keywords

Tim-3; Gal-9; Foxp3; HCV; regulatory T cells; hepatocytes; immune modulation

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Introduction

Hepatitis C virus (HCV) is a global health problem characterized by persistent infection, limited therapeutic options, poor treatment responses, and no available vaccine[1]. Following years of intensive research into the pathogenesis of HCV, it has become evident that this virus is able to modulate host immunity, in particular T cell responses, and by doing so facilitates chronic infection[2]. The mechanisms by which HCV impairs antiviral T cell immunity include blunted T cell activation and proliferation by up-regulating inhibitory pathways, skewed T cell differentiation (Th1 deficiency or Th2 dominance), T cell anergy (antigen-specific hypo-responsiveness or exhaustion), T cell depletion (cell apoptosis or death), and induction of regulatory T cells (Tregs)[2].

Tregs constitute a unique T-cell lineage that suppresses the function of effector T cells (Teffs) and maintains peripheral immune tolerance. Accumulation of Tregs is a common characteristic of most chronic viral infections, and significantly suppresses antiviral $CD4^+$ and $CD8^+$ Teff responses[3]. Natural Tregs are developed intrathymically and represent about 5~15% of total $CD4^+$ T cells, whereas adaptive Tregs are generated extrathymically from naïve $CD4^+$ T cells, by acquiring CD25 and Foxp3 in response to regulatory stimuli under disease conditions[3]. Foxp3 (Forkhead box P3) has been identified as a marker and transcription factor programming $CD4^+CD25^+$ Treg development and function[4–5]. The suppressive function of $CD4^+CD25^+Foxp3^+$ Tregs requires TCR stimulation and additional mechanisms that include cell-cell interaction, regulatory cytokine production (TGF-β/IL-10), and IL-2 trapping[6–7]. Despite intensive studies on the role of Tregs in T cell suppression and in HCV pathogenesis, little is known about how $Foxp3^+$ Tregs are fine-tuned in equilibrium between T-cell-dependent immune protection and immunopathology, potentially contributing to viral persistence in humans[8–9].

In addition to Tregs, the recently described programmed death-1 (PD-1) and T cell immunoglobulin and mucin domain protein-3 (Tim-3) pathways represent other mechanisms that maintain the intricate balance between positive and negative signals to ensure adequate immune responses against pathogens, and yet prevent over-activation of lymphocytes and thus autoimmunity[10–11]. While PD-1 or Tim-3 has been shown to play a central role in Teff dysregulation[12–13], their role in regulation of Treg development and function is poorly explored. Recently, we and others have demonstrated that PD-1 negatively regulates $CD4^+CD25^+Foxp3^+$ Tregs during HCV infection[14–15]. We have also examined Tim-3 expression as well as its role on $CD4^+CD25^+Foxp3^+$ Tregs and $CD4^+CD25^+Foxp3^-$ Teffs, and demonstrated that Tim-3 pathway controls the balance of $Foxp3^-$ Teffs and $Foxp3^+$ Tregs by regulating cell proliferation and apoptosis during chronic HCV infection[16]. Tim-3's natural ligand, galectin-9 (Gal-9), has been shown to be up-regulated during chronic HCV infection, correlating with expansion of $CD4^+CD25^+Foxp3^+$ Tregs, contraction of $CD4^+$ Teffs, and apoptosis of HCV-specific CTLs[17]. To further explore the mechanisms by which Tim-3/Gal-9 interactions regulate HCV-mediated Treg development, in this study we provide pilot data showing that HCV-infected hepatocytes express higher levels of Gal-9 and TGF-β, up-regulating Tim-3 expression and regulatory cytokines TGF-β/IL-10 in co-cultured $CD4^+$ T cells, driving conventional $CD4^+$ T cells into $CD25^+Foxp3^+$ Tregs. Additionally, recombinant Gal-9 protein could transform TCR-activated $CD4^+$ T cells into $Foxp3^+$ Tregs in a dose-dependent manner. Importantly, blocking Tim-3/Gal-9 ligations abrogated HCV-mediated Treg induction, suggesting that Tim-3/Gal-9 interactions may regulate $Foxp3^+$ Treg development and function during HCV infection.

Materials and Methods

Healthy CD4$^+$ T cells co-cultured with HCV$^{+/-}$ Huh-7 hepatocytes

Transfection of Huh-7 hepatocytes (kindly provided by Dr. T.J. Liang, liver section, NIH/NIDDK) with HCV JFH-1 strain (kindly provided by Dr. T. Wakita) was carried out as described previously[18–19]. Immunohistochemical staining for HCV core protein in transfected Huh-7 cells and RT-PCR amplification for HCV core mRNA in the supernatant of transfected Huh-7 cultures were also performed as described[18–19]. For the co-culture experiment, HCV$^{+/-}$ Huh-7 hepatocytes were serum starved for 18h, then activated with rhIFN-γ (0.1μg/ml, R&D Systems) for 48h. Activated hepatocytes were removed from plates by 0.05% trypsin-EDTA, and then plated at 5×10^5 cells/well in a 12 well plate. Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of healthy subjects using Ficoll density gradient centrifugation (Atlanta biological, Lawrenceville, GA). Human CD4$^+$ T cells were purified from PBMCs by magnetic beads with column purification according to the manufacturer's instructions (purity > 95%; Miltenyi Biotec Inc, Auburn CA). Purified healthy CD4$^+$ T cells were subsequently activated with rhIL-2 (10U/ml, R&D Systems) and anti-CD3/CD28 antibodies (1μg/ml each, InvivoGen) for 72h prior to hepatocyte co-culture. Activated CD4$^+$ T cells were added to the adherent hepatocytes in RPMI media containing anti-CD3/CD28 antibody (1 μg/ml each, InvivoGen) and rhIL-2 (10U/ml, R&D Systems), co-cultured for another 48 hours, and stained for surface receptor CD25 as well as intracellular molecules including Foxp3, IL-10, and TGF-β by flow cytometry.

Treg induction by recombinant Gal-9 and TGF-β

Purified naïve CD4$^+$ T cells or CD4$^+$CD25$^-$ T cells were cultured with complete RPMI 1640 medium containing anti-CD3/CD28 (1 μg/ml each, InvivoGen) and rhIL-2 (10 U/ml, R&D Systems) in the presence of recombinant human stable-form Gal-9 (rGal-9) or boiled inactive Gal-9 protein (0, 0.25, 0.5, 1.0, 2.0 μg/ml hG9NC, kindly provided by Dr. M. Hirashima from Kagawa University and Dr. T. Niki from GalPharma Co.Ltd., Japan) and/or recombinant human TGF-β (10 ng/ml, eBioscience) for 5 days, and CD4$^+$CD25$^+$Foxp3$^+$ Tregs were analyzed by flow cytometry.

Flow cytometry

Cultured CD4$^+$ T cells were treated with Brefeldin A (BioLegend, San Diego, CA) for the last 6 h to inhibit cytokine secretion before harvesting. Cell surface staining was carried out using APC-CD25 (Miltenyi Biotec), PE-TIM-3 (R&D Systems), followed by intracellular staining for PerCP-Cy5.5-Foxp3 (eBioscience), PE-TGF-β (R&D Systems), or PE-IL-10 (Miltenyi Biotec). The intracellular cytokine staining was carried out using Inside Stain kit (Miltenyi Biotec) per manufacturer's instructions. Intracellular Gal-9 and its surface expression on HCV-infected and non-infected hepatocytes were carried out by immunohistochemical staining and flow cytometric analysis as described[18–19]. Isotype-matched control antibodies (eBioscience) and fluorescence minus one (FMO) controls were used to determine background levels of staining and adjust multicolor compensation as gating strategy. The cells were analyzed on a FACSCalibur flow cytometry (BD, Franklin Lakes, NJ) and CELLQuest or FlowJo software.

IFN-γ assay

Purified healthy CD4$^+$ T cells were co-cultured with HCV$^{+/-}$ Huh-7 hepatocytes as described above, and IFN-γ secretion in the supernatants of the HCV-infected and non-infected cultures was assessed using a C6 flow cytometer (BD Accuri) according to the instructions of the BD™ Cytometric Bead Array (CBA) for human soluble protein assay. In

brief, the supernatants collected at 48h following culture of T cells with hepatocytes were incubated with IFN-γ capture beads; after repeated washing by centrifugation, the beads were immunostained with secondary IFN-γ detection antibody conjugated with PE. The IFN-γ standards were prepared per Human Flex Set System, and assayed the same way as samples. Quantifications for soluble IFN-γ cytokine were acquired on the C6 flow cytometer and the data were analyzed with the FCAP™ array software.

Tim-3 blockade

Co-cultured $CD4^+$ T cells were incubated with anti-TIM3 (10 μg/ml, R&D Systems) or LEAF™ anti-human Tim-3 antibody (10 μg/ml, BioLegend) or control IgG overnight, followed by stimulation with anti-CD3/CD28 antibody (1 μg/ml each, InvivoGen) for 48 h, then subjected for flow cytometric analysis of Foxp3, TGF-β, IL-10 expressions as described above.

Statistical analysis

Study results are summarized for each group and results are expressed as the mean ± standard deviation (SD). Comparison between two groups is performed by SPSS-18 software. Pair wise t-test is used to compare the significance of changes in Tim-3 blocking experiments. Values of $p < 0.05$ (*) and $p < 0.01$(**) or $p < 0.001$ (***) were considered significant or very significant. NS = no significance.

Result

HCV-infected hepatocytes express higher levels of Gal-9 and TGF-β and promote $Foxp3^+$ Treg induction

Up-regulation of Tim-3 and accumulation of $Foxp3^+$ Tregs are characteristics of HCV infection and play pivotal roles in suppressing Teff responses that may be essential for viral clearance. The increased frequency of Tregs during HCV infection might arise from the expansion of thymic-derived natural Tregs or the *de novo* induction from naïve T cells. Having recently characterized the relationship between Tim-3 and Foxp3 expression in differentiated Tregs in patients with chronic HCV infection[16], here we studied the role of Tim-3/Gal-9 interactions in HCV-mediated Treg induction from "naive" $CD4^+$ T cells. Since the primary site of HCV replication is within hepatocytes in the liver, where HCV-infected hepatocytes have close contact with circulating or infiltrating lymphocytes, we employed a novel model involving co-culture of purified healthy $CD4^+$ T cells with HCV-expressing hepatocytes[18–19]. As shown in Fig. 1A–B, Huh-7 cells transfected with the HCV JFH-1 strain express HCV core in cells as well as in the supernatant of the culture, detected by immunohistochemistry staining and RT-PCR, but not in the mock-transfected controls.

In addition to expressing HCV proteins, HCV^+ hepatocytes also express Gal-9 protein, as detected by immunohistochemical staining intracellularly (Fig. 1C) and by flow cytometric analysis on the surface of infected hepatocytes (Fig. 1D). The amount of Gal-9 expressed by HCV-infected hepatocyte is significantly increased not only in the percentage of Gal-9 positive cell frequency, but also in the MFI of Gal-9 expression level on the cell surface, when compared to non-infected hepatocytes. TGF-β has been shown to be essential for Treg induction[20], and here we also show that HCV-infected hepatocytes express higher amounts of TGF-β than non-infected controls (Fig. 1E). Importantly, a significant increase of $CD25^+Foxp3^+$ Tregs (Fig. 1F) is detected in $CD4^+$ T cells co-cultured with HCV^+ versus HCV^- hepatocytes, indicating that HCV-infected hepatocytes drive Treg development.

Tim-3 expressed on $CD4^+CD25^+Foxp3^+$ Tregs negatively controls their development and functions

To recapitulate what we recently discovered involving the role of Tim-3 in controlling the cellular balance of $Foxp3^+$ Treg and $Foxp3^-$ Teff in HCV-infected patients *in vivo*[16], we evaluated Tim-3 expression and its role in the development and function of Tregs generated by incubation of purified $CD4^+$ T cells with HCV expressing hepatocytes *in vitro*. As shown in Fig. 2A, Tim-3 expression is found to be significantly up-regulated on $CD4^+CD25^+Foxp3^+$ Tregs. Further characterization of the newly generated $CD4^+CD25^+Foxp3^+$ Tregs supported the notion that these were regulatory T cells as they also highly express regulatory cytokines such as TGF-β and IL-10 (Fig. 2B). Interestingly, blocking Tim-3/Gal-9 ligations during the course of co-culturing $CD4^+$ T cells with HCV^+ Huh-7 cells further increases TGF-β and IL-10 productions by $CD4^+CD25^+Foxp3^+$ Tregs in the presence of HCV^+ hepatocytes (Fig. 2B). Moreover, blocking Tim-3/Gal-9 interactions abrogates HCV-mediated $Foxp3^+$ Treg induction (Fig. 2C). These results suggest that Tim-3, a marker for T cell exhaustion, is associated with $Foxp3^+$ Treg induction but can feedback to regulate Treg development and functions.

Gal-9, synergizing with TGF-β, induces $CD4^+CD25^+Foxp3^+$ Tregs

A recent report demonstrated a role for Gal-9 in inducing Tregs during HCV infection; this was described as primarily occurring through Kuppfer cells as the source for Gal-9[17]. Here we show that HCV-infected hepatocytes express higher level of Gal-9 protein. To further elucidate the role of Tim-3/Gal-9 interactions in $Foxp3^+$ Treg induction, we incubated purified $CD4^+$ T cells with complete RPMI1640 medium containing varying concentrations of recombinant Gal-9 protein, inactivated Gal-9 protein, or control medium for 5 d, followed by flow cytometric analysis of CD25 and Foxp3 expressions in treated $CD4^+$ T cells. As shown in Fig. 3, representative Zebra plots and summary data from three independent experiments, $Foxp3^+$ Tregs were induced by recombinant Gal-9 in a dose-dependent manner, but not by inactivated Gal-9 protein or medium controls.

Since the isolated $CD4^+$ T cells may contain $CD25^+Foxp3^+$ T cells, one may wonder whether Gal-9 is converting naive $CD25^-$ $Foxp3^-$ cells to $CD25^+$ $Foxp3^+$ Treg, or just expanding the existing $Foxp3^+$ T cells present in the $CD4^+$ T cell populations. To clarify this concern, we further purified T cells using a Miltenyi Treg isolation kit and used the $CD4^+$ $CD25^-$ T cells in culture with Gal-9. As shown in Fig. 4A, Gal-9 can drive $CD4^+$ $CD25^-$ T cells differentially into $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs. The data are reproducible using $CD4^+$ $CD25^-$ T cells purified from three healthy subjects. Since we have also shown that HCV-infected hepatocytes can express higher amounts of TGF-β as well as Gal-9 proteins (Fig. 1C–E), and TGF-β has been shown to be essential in induction of $Foxp3^+$ Tregs[20], we next sought to use recombinant TGF-β (10 ng/ml) as a control to compare its (synergistic) effect on induction of $Foxp3^+$ Tregs with rGal-9 protein (1 μg/ml). As shown in Fig. 4B, $Foxp3^+$ Tregs can be induced by incubating $CD4^+$ T cells with recombinant Gal-9 as well as TGF-β alone, and this effect is almost doubled when incubating the cells with the same amount of both proteins. Notably, the same amount of Gal-9 protein (1 μg/ml) induces more $Foxp3^+$ Tregs with bulk $CD4^+$ T cells than $CD4^+CD25^-$ T cells (Fig. 4A and 4B), indicating a transformation plus expansion effect by Gal-9 in mixed $CD4^+$ T cell populations. These data are in line with studies by the Rouse laboratory in which addition of Gal-9 led to up-regulation of regulatory T cells that were able to limit chronic HSV immunopathology in a murine model[20,21]. Additional support comes from studies in which Gal-9 promoted Treg development in a murine model of arthritis[22].

Apoptosis of $CD4^+CD25^+Foxp3^-$ Teffs and inhibition of IFN-γ secretion by $CD4^+$ T cells incubated with HCV^+ hepatocytes

We have previously shown that HCV infection inhibits T cell responses by promoting $Foxp3^+$ Treg proliferation and $Foxp3^-$ Teff apoptosis through the Tim-3/Gal-9 interactions. In this report, we further examined whether the Gal-9 expressing hepatocytes cause contraction of $CD4^+CD25^+Foxp3^-$ Teffs through apoptosis in the HCV co-culture system. As shown in Fig. 5A, purified $CD4^+$ T cells co-cultured with HCV^+ hepatocytes exhibit an increased Annexin V expression on $CD4^+CD25^+Foxp3^-$ Teffs compared to those cultured with HCV^- hepatocytes, suggesting that HCV-infected hepatocytes have an immunomodulatory role by promoting $Foxp3^+$ Treg induction but $Foxp3^-$ Teff apoptosis during infection. In conjunction with this finding, we also examined the levels of IFN-γ secretion in the supernatants of $CD4^+$ T cells co-cultured with $HCV^{+/-}$ hepatocytes by cytometric bead array. Consistent with the increase of suppressive $Foxp3^+$ Tregs and apoptosis of $Foxp3^-$ Teffs, IFN-γ secretion is significantly decreased in the supernatant of $CD4^+$ T cells co-cultured with HCV^+ hepatocytes versus HCV^- hepatocytes (Fig. 5B). Taken together, it appears that HCV-infected hepatocytes may drive "naïve" $CD4^+$ T cells differentiation into suppressive $Foxp3^+$ Tregs and promote contraction of inflammatory $Foxp3^-$ Teffs through the Tim-3/Gal-9 pathway.

Discussion

Both Tim-3/Gal-9 interactions and $Foxp3^+$ Tregs control the balance between an adequate protective immune response and suppression of T cell-dependent immunopathology that may contribute to viral persistence. However, it remains unclear how Treg development and function are regulated to fine-tune this balance, allowing control of excessive T cell-mediated injuries without completely suppressing antiviral T cell responses. We have recently found contraction of $CD4^+CD25^+Foxp3^-$ Teffs coincided with Tim-3 expression on $CD4^+CD25^+Foxp3^+$ Tregs that accumulate during chronic HCV infection[16]. Tim-3 expression on $Foxp3^+$ Tregs positively correlated with their Ki67 expression, but was inversely associated with expansion of IL-2-producing Teffs[16]. In this study, we provide additional data showing that HCV-infected hepatocytes express higher levels of Gal-9 and TGF-β, and up-regulate Tim-3 expression and regulatory cytokines TGF-β/IL-10 by co-cultured $CD4^+$ T cells, driving conventional $CD4^+$ T cells into $CD25^+Foxp3^+$ Tregs. Recombinant Gal-9 protein, by synergizing with TGF-β, also transforms TCR-activated $CD4^+$ T cells into $Foxp3^+$ Tregs in a dose-dependent manner. Importantly, blocking Tim-3/Gal-9 interactions abrogated HCV-mediated Treg induction *in vitro*. Blocking the Tim-3/Gal-9 interactions *ex vivo* also corrected the imbalance of $Foxp3^+$ Treg/$Foxp3^-$ Teff ratio developed *in vivo* during chronic viral infection[16]. Based on these findings, we propose a model (Fig. 6) in which HCV-infected hepatocytes drive conventional $CD4^+$ T cells toward inhibitory $Foxp3^+$ Tregs and induce apoptosis of inflammatory $Foxp3^-$ Teffs through the Tim-3/Gal-9 pathway, representing a novel mechanism that may contribute to dysregulated immune responses and may facilitate chronic viral infection.

Following initial T cell receptor (TCR) activation and interaction with HCV-infected hepatocytes, which express higher levels of Gal-9 and TGF-β, naïve $CD4^+$ T cells are activated and express CD25 and Tim-3. While the majority of activated T cells differentiate into $CD4^+CD25^+Foxp3^-$ Teffs (with relatively less IL-2 producing Teffs in cultures of HCV^+ hepatocytes versus HCV^- cultures, data not shown), a proportion of these T cells developed a regulatory phenotype characterized by increasing expression of the Treg marker (Foxp3) and regulatory cytokines (TGF-β and IL-10). Notably, expression of the inhibitory receptor Tim-3 was up-regulated in the setting of TCR activation and HCV infection, a setting that may contribute to Tim-3 and Foxp3 up-regulation; however, this up-regulation of Tim-3 may represent a feedback mechanism to regulate Treg development and function,

and thus fine-tune their inhibitory effects on Teff responses. Upon blockade of Tim-3/Gal-9 interactions during the incubation of $CD4^+$ T cells with HCV-expressing hepatocytes, TGF-β/IL-10 expressions by $CD4^+CD25^+Foxp3^+$ Tregs were further boosted. These data is in line with our finding that Tim-3 expression positively correlates with Ki67 expression in $CD4^+CD25^+Foxp3^+$ Tregs isolated from HCV-infected patients, and blocking Tim-3 signaling enhances $CD4^+CD25^+Foxp3^+$ Treg proliferation[16], suggesting that the Tim-3 pathway negatively controls Treg development and function.

Tim-3 and PD-1 were originally identified as T cell exhaustion markers[10–13], but recent studies also implicate Tim-3 and PD-1 as T cell activation markers (personal communications). These contradictory observations might result from the status of T cell activation or differentiation. More specifically, Tim-3 and PD-1 are barely expressed on naïve or resting T cells. Following TCR stimulation, cell activation and differentiation occurs, accompanied by expression of negative inhibitory molecules such as Tim-3 and PD-1. Therefore, although Tim-3 and PD-1 may be regarded by some investigators as activation markers in the early phase of cell activation, they appear to be exhaustion markers by function; i.e., they deliver negative feedback to inhibit TCR signaling pathways upon interacting with their ligands (Gal-9, PD-L1), preventing cell over-activation and leading to apoptosis. Notably, the understanding of the expression and function of these inhibitory receptors is still developing. For example, a recent report by Gupta et al[23] demonstrating that $Foxp3^+$ $Tim\text{-}3^+$ cells are susceptible to Gal-9 induced apoptosis is somewhat contradictory to our recent report regarding $Foxp3^+$ Tregs, which were resistant to TCR-over-activation-induced cell apoptosis[16]. Given the fact that the presence of a Gal-9-sensitive $CD4^+Foxp3^+Tim\text{-}3^+$ population of T cells arose from $CD4^+Foxp3^+TIM\text{-}3^-$ proliferating T cells and were often $PD\text{-}1^+$ in Gupta's report, we suspect that the difference in our observations might come from the different status of the cell activation or differentiation. $Tim\text{-}3^+$ T cells are generally over-stimulated, terminally differentiated, dying T cells, and thus are sensitive to Gal-9-induced apoptosis. Additionally, as we demonstrated in this report, Gal-9 mediated cell biology is dose-dependent, with cell apoptosis occurring at higher concentrations of Gal-9. We recently also demonstrated that Tim-3's effect on cells is dependent upon the compartmentalization of Gal-9, in that extracellular Gal-9 (trans-association with Tim-3 expressed on target cells) delivers an inhibitory effect, whereas intracellular Gal-9 (cis-association with Tim-3 on the same cell) exhibits an activating effect on target cells (unpublished data). Therefore, we believe these paradoxical observations may be related to different cell activation/differentiation statuses, ligand/receptor cell compartments, and various models/stimuli and methods used by investigators.

HCV-infected hepatocytes may be key to what has been observed in Treg regulation. In HCV infection, the primary site of HCV replication is within hepatocytes, where there is ample opportunity to contact circulating or infiltrating immune cells due to a lack of basal membranes and low velocity blood flow in the fenestrated structure of hepatic sinusoids. In the last two decades, while efforts to define the mechanisms of HCV persistence have focused primarily on $CD4^+$ and $CD8^+$ Teff responses against virus-infected hepatocytes, the role of HCV-infected hepatocytes in regulation of antiviral immunity has received little attention. Although hepatocytes are not considered immune cells, they do have the ability to express co-stimulatory molecules on the cell surface, such as the ligands, PD-L1 and Gal-9, for the immunoreceptors, PD-1 and Tim-3, respectively; and secrete immunoregulatory cytokines, such as IL-10 and TGF-β, which are known to regulate the development and function of $Foxp3^+$ Tregs, including those generated during HCV infection[24–27]. A Kupffer cell-derived, soluble-form Gal-9 protein has been reported to be increased in the serum of HCV-infected patients, playing a crucial role in regulation of T cell immune responses[17]. Here, we further demonstrate that greater Gal-9 expression is observed on the surface of HCV-infected hepatocytes, implying that cell-cell contact between hepatocytes and $CD4^+$ T

cells plays a critical role in Treg-cell formation. Our studies identify an enhancement of TGF-β and Gal-9 production by HCV-expressing hepatocytes, in conjunction with an increase of Tim-3 and Foxp3 expression by $CD4^+CD25^+$ T cells, suggesting that the HCV-infected liver itself may provide a milieu for Treg induction as conventional T cells sequester within infected liver, where sustained HCV antigens are secreted at the site of infection. HCV-mediated Tim-3/Gal-9 interactions in the liver may thus play an important role in the development of $Foxp3^+$ Tregs. This development may be a consequence of expansion of preexisting $Foxp3^+$ T cells or conversion of $CD4^+$ $CD25^-$ $Foxp3^-$ T cells toward $Foxp3^+$ Tregs,

Interestingly, inflammatory IL-2 (data not shown) and IFN-γ secretion (Fig. 5B) are inhibited, whereas TGF-β and IL-10 productions are enhanced, in activated $CD4^+$ T cells co-cultured with HCV^+ hepatocytes; blockade of Tim-3 signaling in our system, however, boosted not only IL-2 and IFN-γ but also TGF-β and IL-10 expressions. This paradoxical effect on cytokine production may be due to the differential regulation of pro- and anti-inflammatory cytokine expressions by HCV-mediated Tim-3/Gal-9 interactions in this model. As we have observed both *in vitro* and *ex vivo*, Tregs are induced and Teffs suppressed through their altered ability to proliferate and their susceptibility to apoptosis[16], consistent with inhibited IL-2/IFN-γ and increased TGF-β/IL-10 production during HCV infection. With blockade of Tim-3 signaling in $CD4^+CD25^+$ T cells, both Tregs and Teffs exhibited improved cell proliferation, perhaps explaining the increased IL-2/IFN-γ and TGF-β/IL-10 production in our system. This is in line with our recent finding that Tim-3 regulates pro- and anti-inflammatory cytokines during innate immune responses in that blockade of Tim-3 ligation by antibodies or silencing Tim-3 expression by siRNAs enhances both IL-12 and IL-10 productions by monocytes[28]. A change in the balance of the pro- and anti-inflammatory cytokine milieu (such as IL-12/IL-23) may contribute to $CD4^+$ and $CD8^+$ T cell differentiation into different phenotypes (such as Th-17, manuscript submitted) that are regulated by Tim-3/Gal-9 interactions through cell differentiation, expansion and apoptosis.

Tim-3/Gal-9 interactions might represent a physiological means by which Teff/Treg responses are fine-tuned to balance immune protection and immune injury during infection. HCV or other chronic pathogens may capitalize on this inhibitory pathway by up-regulating Tim-3 and Gal-9 expressions and in doing so facilitate persistent infection. Tim-3/Gal-9 interactions have been shown to be able to constrain $CD8^+$ T cell immunity to herpes simplex virus (HSV) infection by direct inhibitory effects on $TIM\text{-}3^+$ $CD8^+$ T effector cells as well as the promotion of $Foxp3^+$ regulatory T cell activity[20–21]. We demonstrate the same mechanism for Tim-3/Gal-9 interactions in T cell regulation during HCV infection, suggesting that manipulating galectin signals (as can be achieved using appropriate sugars by injecting alpha-lactose that binds to the carbohydrate-binding domain of Gal-9 and limits its engagement of Tim-3) may represent a convenient and inexpensive approach to enhance acute and memory responses to viral infection. However, it should be pointed out that T-cell-dependent immune responses represent a double-edged sword, inducing tissue damage by inflammatory responses in the process of eradicating viral infection. Therefore, a balance of Teffs/Tregs regulated by Tim-3/Gal-9 pathway may be critical in control of immune protection and immune pathology during viral infection, and must be taken into consideration when contemplating immunotherapy. To our knowledge, this is the first report focusing on HCV-infected hepatocytes regulation of $Foxp3^+$ Treg development and function through Tim-3/Gal-9 interactions. This study contributes to our understanding of the mechanisms by which the balance of Tregs and Teffs is fine-turned through the Tim-3 pathway during HCV-host interactions.

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Abbreviations

Tim-3	T cell immunoglobulin and mucin domain-3
Gal-9	galectin-9
PD-1	programmed death-1
PD-L1	programmed death-ligand 1
M/M$_\Phi$	monocyte/macrophages
Teff	effector T cells
TLR	Toll-like receptor
APC	antigen presenting cells
Treg	regulatory T cells
VA	Veteran’s Administration
IRB	Institutional Review Board
PBMC	peripheral blood mononuclear cells
LPS	Lipopolysaccharide
DC	dendritic cells
MDDC	monocyte-derived dendritic cells
HCV	hepatitis C virus
LEAF	low endotoxin, azide-free
Foxp3	Forkhead box P3
TGF-β	tumor growth factor-β
IFN-γ	interferon-γ
TCR	T cell receptor

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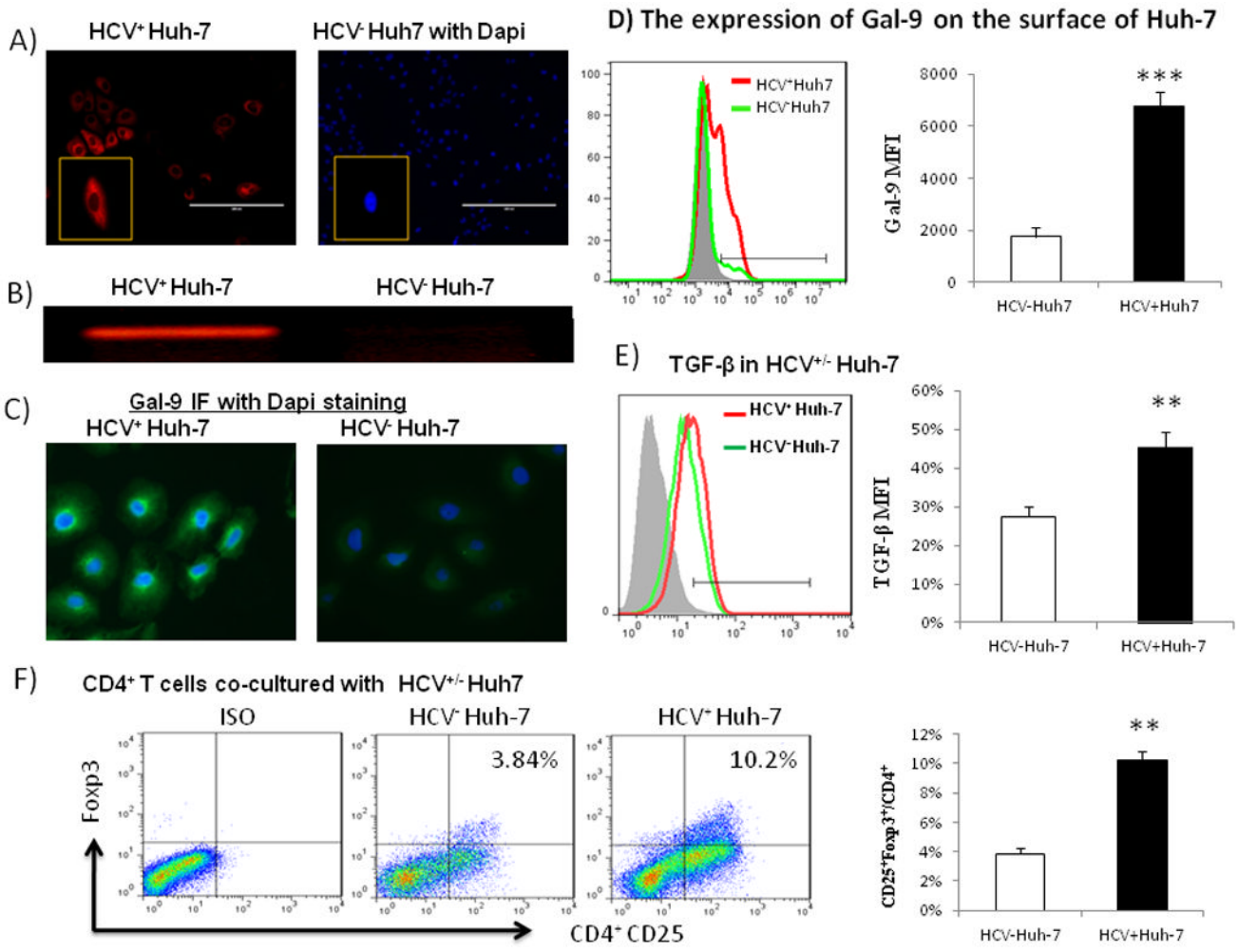

Fig. 1. Foxp3$^+$ Treg induction in CD4$^+$ T cells co-cultured with HCV-infected hepatocytes that express Gal-9 and TGF-β

A) HCV core protein is detected positive by immunofluorescence staining in HCV JFH-1-transfected Huh-7 cells, but not in the mock-transfected Huh-7 cells that are double stained with Dapi. x 40 magnificence imaging is inserted in the left lower corner. B) HCV core mRNA is detected by RT-PCR in the supernatant of HCV JFH-1-transfected Huh-7 cells, but not in the mock-transfected Huh-7 cells. C) Gal-9 expression is up-regulated in HCV$^+$ Huh-7 cells compared with HCV$^-$ Huh-7 cells, double stained with Dapi. D) The percentage of Gal-9 positive cell frequency is up-regulated on the surface of HCV$^+$ Huh-7 cells (red line) compared with HCV$^-$ Huh-7 cells (green line). Isotype control staining is shown as a grey-filled area. The summarized MFI of Gal-9 expression level on HCV$^{+/-}$ hepatocytes from three independent experiments is shown on the right. ***P<0.001. E) TGF-β expression is up-regulated in HCV$^+$ Huh-7 cells (red line) versus HCV$^-$ Huh-7 cells (green line). Isotype control staining is shown as grey-filled area. Summary data of TGF-β expression level in HCV$^{+/-}$ hepatocytes from three independent experiments is shown in the right, **P<0.01. F) CD25$^+$Foxp3$^+$ Treg induction following co-culture of purified CD4$^+$ T cells with HCV$^+$ Huh-7 or HCV$^-$ Huh-7 hepatocytes, as described in Materials and Methods. Isotype-matched antibodies and fluorescence minus one (FMO) controls were used to determine background levels of staining and adjust multicolor compensation as a

gating strategy. Summary data using $CD4^+$ T cells isolated from 8 healthy subjects is shown in the right panel. **P<0.01.

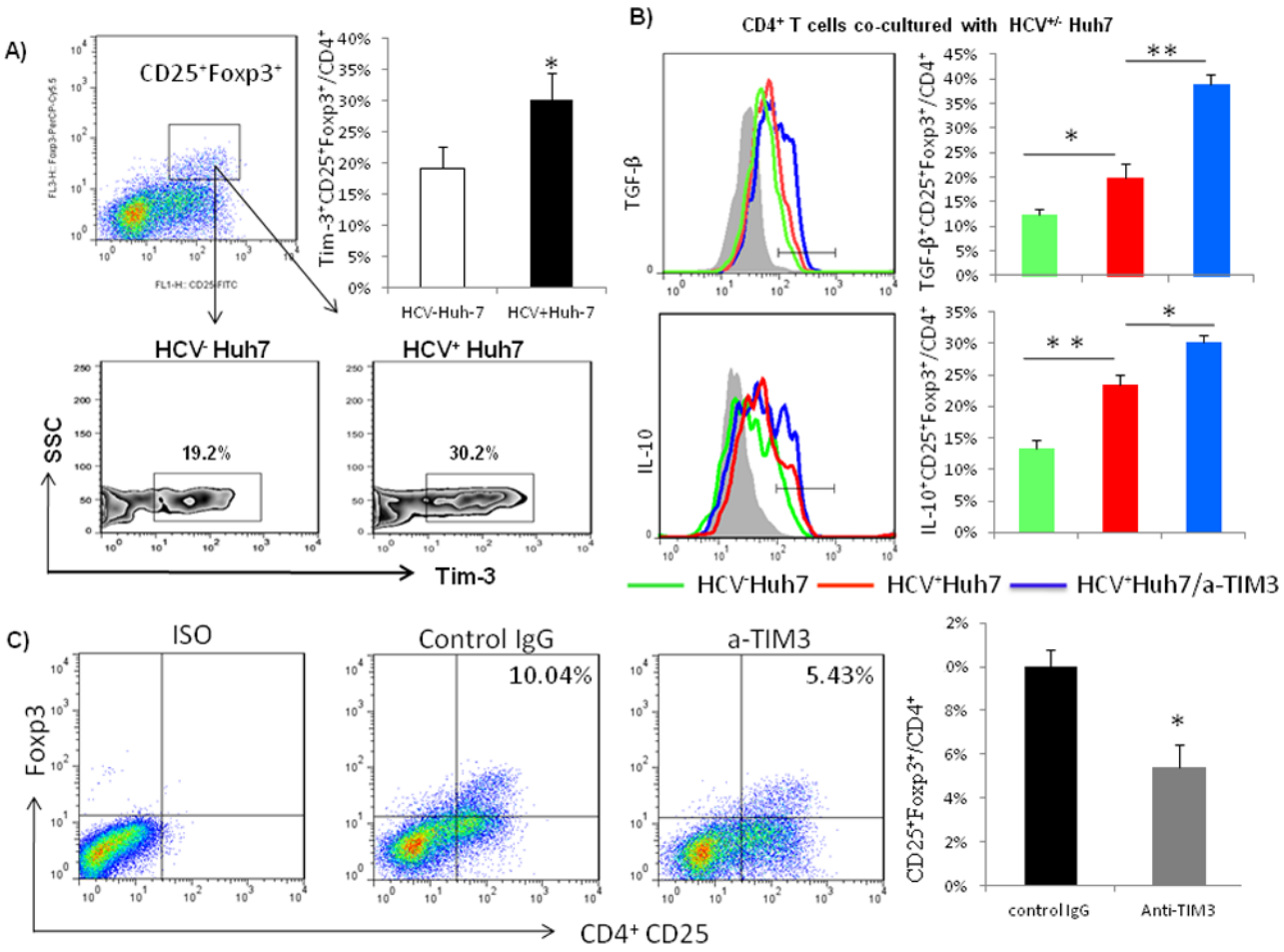

Fig. 2. Tim-3 pathway is involved in the Foxp3$^+$ Treg induction by incubation of CD4$^+$ T cells with HCV-infected hepatocytes

A) Tim-3 expression is up-regulated on CD4$^+$CD25$^+$Foxp3$^+$ Tregs following co-culture of purified CD4$^+$ T cells with HCV$^+$ Huh-7 compared with those HCV$^-$ Huh-7 hepatocytes. Representative plots show gating strategy of Tim-3 expression on CD25$^+$Foxp3$^+$ Tregs in CD4$^+$ T cells co-cultured with HCV$^{+/-}$ Huh-7 hepatocytes, per isotype and FMO controls, and summary data of Tim-3 expression on CD4$^+$CD25$^+$Foxp3$^+$ Tregs is shown in the upper right panel. B) Left panels show representative flow histogram analysis of intracellular TGF-β and IL-10 detected in CD25$^+$Foxp3$^+$ Tregs following co-culture of CD4$^+$ T cells with HCV$^-$ Huh-7 (green line), HCV$^+$ huh-7 (red line), and HCV$^+$ Huh-7 in the presence of anti-Tim-3 (blue line). Data is reproducible in three repeated experiments, which is summarized in the right panel. *P<0.05, **P<0.01. C) Tim-3 blockade abrogates the CD4$^+$CD25$^+$Foxp3$^+$ Treg induction during co-culture of CD4$^+$ T cells with HCV$^+$ hepatocytes. Representative dot plots of CD25 and Foxp3 expressions in isotype staining, IgG control, and anti-Tim-3 treatment are shown in the left, and summary data using CD4$^+$ T cells from 8 healthy subjects is shown in the right. *P<0.05.

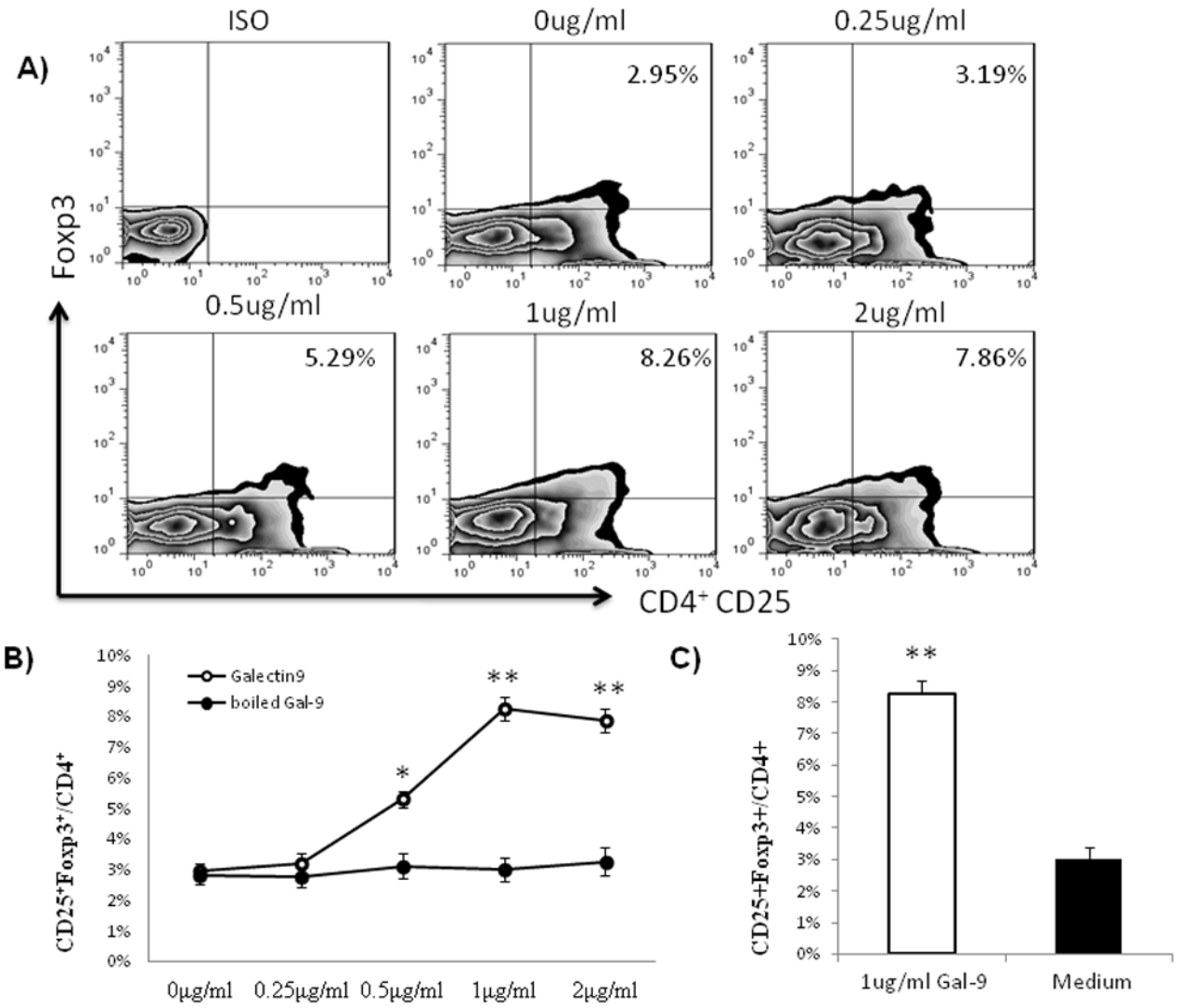

Fig. 3. Induction of CD4$^+$CD25$^+$Foxp3$^+$ Tregs by incubation of purified healthy CD4$^+$ T cells with recombinant Gal-9 protein in a dose-dependent manner

Representative zebra plots of CD25 and Foxp3 expression in purified CD4$^+$ T cells incubated with escalating concentrations of recombinant Gal-9 proteins for 5 days, followed by flow cytometric analysis. Inactive boiled Gal-9 protein or culture medium were used as controls. Summary data using CD4$^+$ T cells from 3 healthy subjects are shown below. *P<0.05; ** P<0.01.

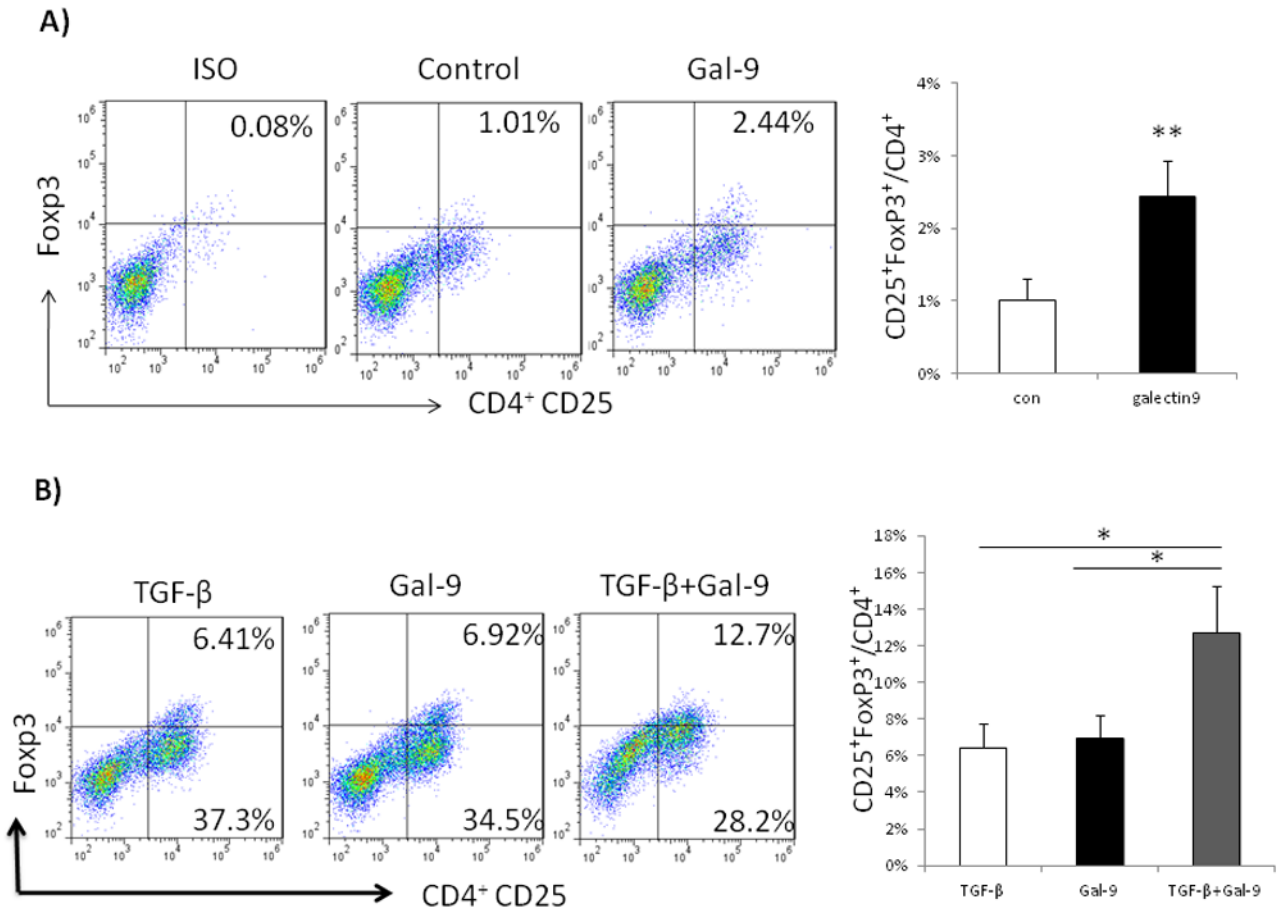

Fig. 4. Induction of $CD4^+CD25^+Foxp3^+$ Tregs by recombinant Gal-9 in $CD4^+$ $CD25^-$ T cells

A) Representative dot plots and summary data of induction of $CD4^+CD25^+Foxp3^+$ Tregs by incubation of $CD4^+$ $CD25^-$ T cells with recombinant Gal-9 protein versus isotype and medium controls. The results are reproducible using cells purified from three subjects. **P<0.01. B) Induction of $CD4^+CD25^+Foxp3^+$ Tregs by recombinant Gal-9 and/or TGF-β in bulk $CD4^+$ T cells. *P<0.05.

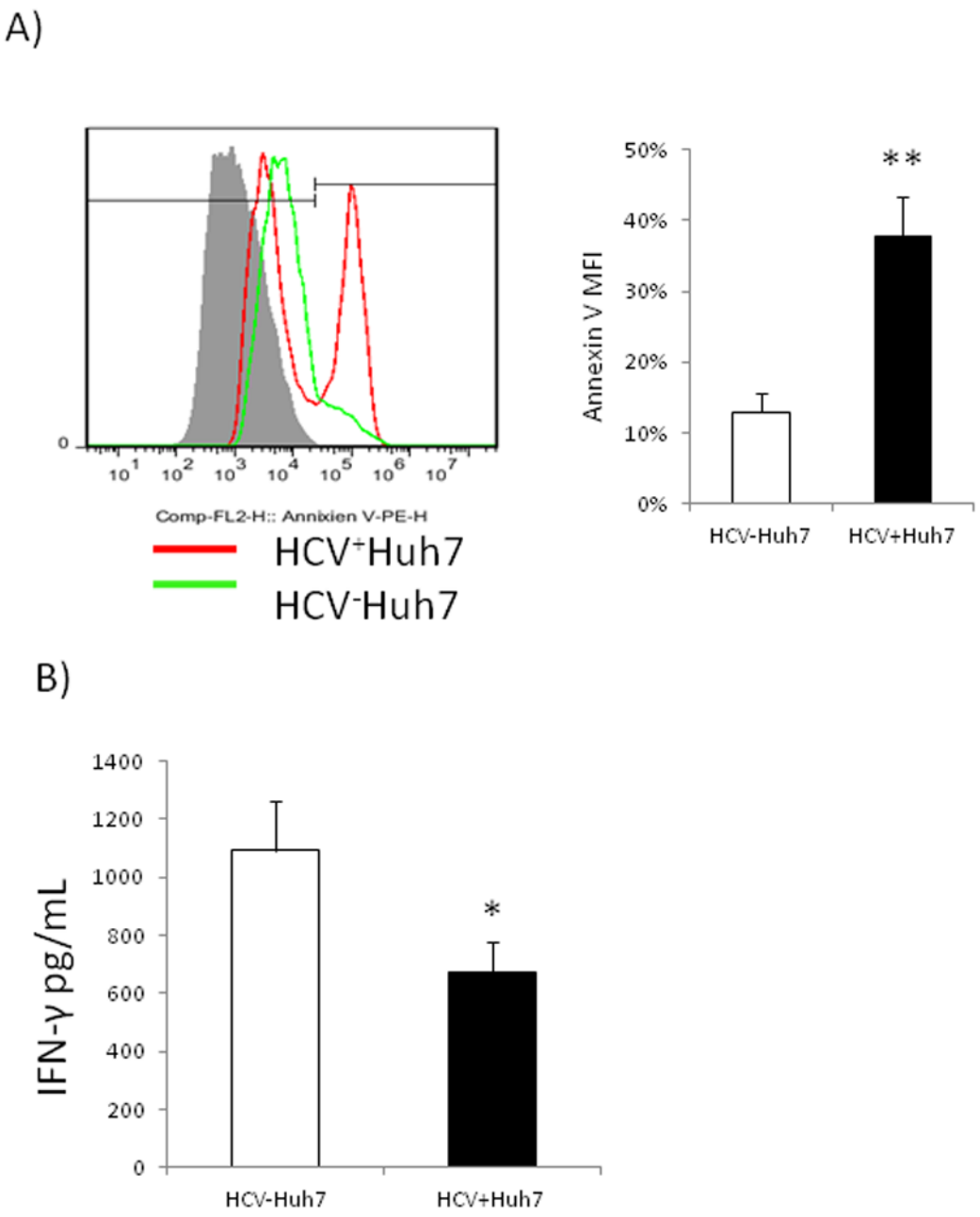

Fig. 5. Induction of $CD4^+CD25^+Foxp3^-$ Teff apoptosis and inhibition of IFN-γ secretion by HCV^+ hepatocytes

A) Annexin V expression on $CD4^+CD25^+Foxp3^-$ Teffs by incubation of $CD4^+$ T cells with $HCV^{+/-}$ hepatocytes. The data are reproducible in three independent experiments, **P<0.01. B) IFN-γ secretion in the supernantant of $CD4^+$ T cells co-cultured with $HCV^{+/-}$ Huh-7 cells, measured by cytometric bead array in three independent experiments. *P<0.05.

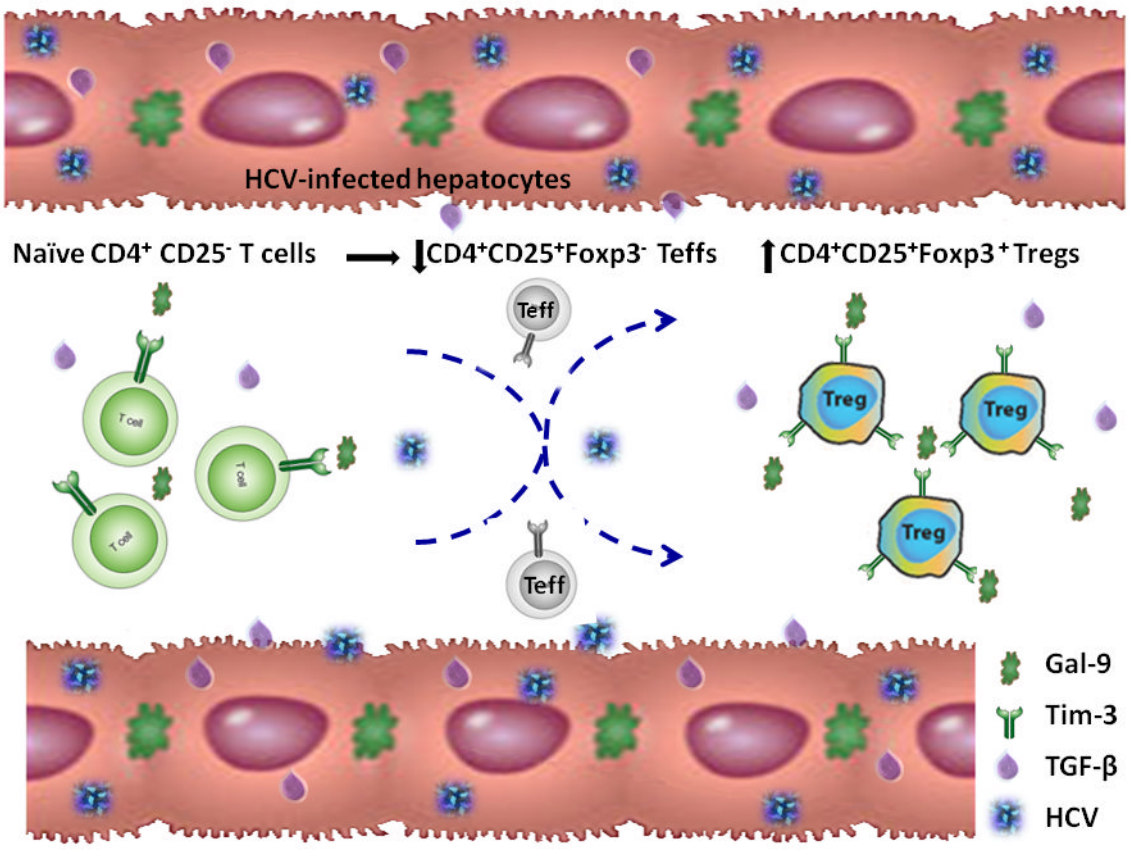

Fig. 6. Model for HCV-infected hepatocyte-driven Foxp3$^+$ regulatory T cell development

HCV-infected hepatocytes express HCV, Gal-9, and TGF-β proteins. CD4$^+$ T cells exposed to this milieu up-regulate Tim-3 expression and are driven toward a CD4$^+$CD25$^+$Foxp3$^+$ regulatory phenotype, but there is apoptosis of CD4$^+$CD25$^+$Foxp3$^-$ effector T cells. This would counter immunopathology but favor viral persistence.