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Hepatocyte-derived exosomes promote T follicular regulatory cell expansion during HCV infection

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Abstract

Hepatitis C virus (HCV) is a global health concern that can cause severe liver disease such as cirrhosis and hepatocellular carcinoma. Control of HCV requires vigorous T cell responses, yet CD4⁺ T cells in chronic HCV patients are dysfunctional. T follicular regulatory (Tfr) cells are a subset of regulatory T cells that suppress T follicular helper (Tfh) cells and the generation of high affinity antibody-producing B cells. In this study, we examined the accumulation of Tfr cells in the liver compartment during chronic HCV infection and defined the cellular and molecular mechanisms underlying their expansion. Our analysis revealed a substantial population of Tfr cells in the livers of chronic HCV patients that is absent in liver tissues from non-viral hepatitis or healthy subjects. Co-culture of PBMCs from healthy subjects with HCV-infected hepatoma cells resulted in preferential expansion of circulating Tfr cells leading to the suppression of Tfh cells. Additionally, co-culture of tonsillar cells with infected hepatoma cells lead to an expansion of germinal center Tfr. Notably, expansion was mediated by TGF- β -containing exosomes released from HCV-infected hepatocytes as blockade of exosome-associated TGF- β or inhibition of exosome release abrogated Tfr expansion.

Conclusion—These results show that liver-derived exosomes play a pivotal role in the accumulation of Tfr cells, likely leading to suppression of Tfh responses in HCV-infected patients. Our study identifies a novel pathway in which HCV infection in hepatocytes exacerbates Tfr cell responses to subvert antiviral immunity.

Keywords

Liver; CD4⁺ T cells; T follicular helper cells; extracellular vesicles; TGF- β

HCV has significant implications for global health as an estimated 180 million individuals are infected.⁽¹⁾ The majority of those (~80%) become persistently infected and have an increased risk of developing liver disease including fibrosis, cirrhosis, and hepatocellular carcinoma.⁽²⁾ The propensity of HCV to establish persistent infection stems from multiple

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strategies to evade the immune system.⁽³⁾ The liver is a primary site of replication and thereby recruited immune cells are subjected to the tolerogenic microenvironment and the immune-regulatory processes accompanied by HCV. Immunity during HCV infection is characterized by dysfunctional CD4⁺ T help^(4,5), impaired CD8⁺ T cells⁽⁶⁾, and deficient humoral immunity⁽⁷⁾. A better understanding of HCV-mediated immune modulation will improve our understanding of how chronic infection is established and facilitate vaccine development.

Increased regulatory T cell responses are a prominent feature in HCV infection, such that Tregs are increased in both number and function in chronic HCV patients and are positively correlated with viral load.^(8,9) Furthermore, abundant Tregs are found in the livers of chronic HCV patients.^(10,11) A subset of Tregs, called T follicular regulatory (Tfr) cells, has been identified for functional regulation of germinal center (GC) responses by limiting T follicular helper (Tfh) cells and B cells.^(12–14) Tfr cells are reported to be increased in the circulation of chronic HCV patients.⁽¹⁵⁾ Tfr are identified by expression of follicular markers CXCR5 and PD-1 and regulatory markers CD25 and Foxp3. This allows Tfr cells to co-migrate with Tfh to control GC responses.⁽¹⁶⁾ Interestingly, lymphoid follicles, containing T and B cells, are commonly observed in livers of HCV patients and exhibit signs of GC-like architecture.^(17,18) Recent studies have identified the presence of Tfh within the livers of HCV patients.^(18,19) However, whether Tfr are present in HCV-infected liver and their role and functionality have not been investigated.

In this study, we identified an unrecognized population of Tfr cells present within the livers of chronically-infected HCV patients. Using an *in vitro* co-culture system, we demonstrate that Tfr from healthy subjects undergo expansion following exposure to infected hepatocytes. The expansion of Tfr cells was accompanied by the acquisition of an enhanced regulatory phenotype and leads to the functional suppression of Tfh cells. Increases in Tfr responses were driven by a novel pathway involving release of TGF- β -containing exosomes from HCV-infected hepatocytes. These findings highlight the accumulation of Tfr in the livers of HCV patients, potentially inhibiting protective Tfh and B cell responses at the site of infection, and contributing to viral persistence.

Materials and Methods

Human Subjects

Intrahepatic leukocytes from explanted liver tissue of HCV-infected patients (n=8), non-viral hepatitis patients (n=6; non-alcoholic steatohepatitis, alcoholic liver disease, autoimmune hepatitis), and healthy control subjects (n=7) and sera were provided by Dr. Hugo Rosen (University of Colorado). Briefly, intrahepatic leukocytes were isolated from liver tissues by first dissecting tissues into small fragments and then incubating with collagenase type IV as previously described.⁽³⁷⁾ Intrahepatic leukocytes were then cryopreserved and shipped from the University of Colorado. All participants of this study provided written informed consent and IRB protocol 06-0566 was approved by the Colorado Multiple Institutional Review Board.

Hepatocytes, HCV, and PBMC co-cultures

The human hepatoma cell line Huh7.5.1 was maintained in complete DMEM. One day following seeding of hepatocytes, cells were infected with HCV (JFH-1 strain, genotype 2a) at a multiplicity of infection (MOI) of 0.1. JFH-1 was kindly provided by Dr. Wakita (Tokyo Metropolitan Institute). For co-culture, cryopreserved PBMCs previously isolated from the buffy coats of healthy subjects (Virginia Blood Services, Richmond, VA) were re-suspended in complete RPMI and added to uninfected or HCV-infected hepatoma cells on day 4 post-infection or cultured alone for 4 days. In some experiments, tonsillar MNCs were co-cultured with uninfected or infected hepatocytes. Cryopreserved primary human hepatocytes (PHHs; Thermo Fisher Scientific) were cultured with Williams' Medium E and Hepatocyte Maintenance Supplement Pack on Collagen I-coated plates according to manufacturer protocols (Thermo Fisher Scientific) and were inoculated with HCV-infected patient sera. PHHs and PBMCs exhibited greater than >80% viability following cryopreservation and during experimentation.

Flow cytometry and T cell isolations

Cells were stained with Zombie Aqua Fixable Viability dye (Biolegend). For identification of liver Tfr cells, surface staining was performed with the following antibodies: CD45-PerCP (Tonbo;2D1), CD4-APC/Cy7 (Tonbo;RPA-T4), CD14-APC (eBioscience;61D3), CD56-APC (eBioscience; CMSSB), CD11b-APC (Biolegend;ICRF44), CXCR5-BV421 (Biolegend;J252D4), PD-1-PE/Cy7 (Biolegend;EH12.2H7), and CD25-FITC (BD Biosciences;BC96). Following fixation with the Fcγ3/Transcription Factor Fixation/Permeabilization Kit (eBioscience), cells were stained with Foxp3-PE (eBioscience;236A/E7). For intracellular cytokine analysis, co-cultures were stimulated with 0.1 μg/mL PMA and 0.5 μg/mL ionomycin (Sigma) in the presence of GolgiPlug (BD Biosciences) for 4-6 hours. Cells were then surface stained with the following antibodies: CD4-APC/Cy7, CXCR5-BV421, and PD-1-PE/Cy7. Following fixation with CytoFix/CytoPerm (BD Bioscience), cells were stained with IFN-γ-FITC (Biolegend;4S.B3), IL-21-PE (eBioscience;eBio3A3-N2), or IL-17-PerCPeFluor710 (eBioscience;BL168). Intracellular staining of Tfr cells was performed by staining with Foxp3-APC (eBioscience;236A/E7), IL-10-PE/Cy7 (Biolegend;JES3-9D7), and CTLA-4-PE (eBioscience;14D3). CD4 T cell isolations were performed by depleting CD14⁺ monocytes using CD14 microbeads (Miltenyi) followed by positive selection with CD4 microbeads (Miltenyi). For depletion or sorting of Tfr, enriched CD4 T cells were stained with CD4-APC/Cy7, CXCR5-BV421, CD25-FITC or CD25-PE (Biolegend;BC96), and CD127-APC (eBioscience;eBioRDR5). CD4 T cell isolations and Tfr/Tfh sorting procedures always yielded cell purities of at least 97%.

Suppression assays

Tfr cells (CD4⁺CXCR5⁺PD-1⁺CD25^{Hi}CD127^{Low}) were sorted from hepatoma cell/tonsillar MNC co-cultures on day 4. Autologous Tfh cells (CD4⁺CXCR5⁺PD-1⁺CD25⁻), not exposed to HCV, were sorted and labeled with CFSE. 2.5×10⁴ Tfh were cultured alone, with 2.5×10⁴ Tfr (1:1), or with 2,500 Tfr (1:10) in the presence of 1×10⁵ autologous irradiated tonsillar MNCs in a 96-well round-bottom plate with 1 μg/ml plate-bound anti-CD3

(Biolegend;OKT3), 0.5µg/ml soluble anti-CD28 (Biolegend;CD28.2), and 10ng/ml of rhIL-2 for 2 or 5 days.

Hepatocyte exosome isolation and PBMC culture

Culture media from hepatoma cells or primary human hepatocytes cultured with exosome-depleted serum was collected on day 4 or day 12 post-infection, respectively, and centrifuged at 1500rpm for 5 minutes. Supernatants were centrifuged at 2500rpm for 15 minutes. Media was then passed through 0.4µm and 0.2µm filters. Exosomes were precipitated by mixing with ExoQuick-TC (System Biosciences) and incubated overnight. Exosomes were pelleted and re-suspended in PBS. Exosomes were indirectly quantified by measuring total protein by BCA assay and concentrations were standardized prior to treating PBMC or CD4 T cells. For exosome treatments, PBMC or CD4 T cells were activated with anti-CD3/anti-CD28 (0.1µg/ml each) for 48 hours. Isolated exosomes (50µg) were added and cultures were incubated for 4 days. In some experiments, exosomes were further purified by incubating overnight with CD63-FITC (Biolegend;H5C6) followed by anti-FITC selection and magnetic particles (StemCell Technologies). For flow cytometry, Exosome-Human CD63 Isolation/Detection kit (Invitrogen) was used to capture exosomes, followed by staining with CD63-FITC and TGF-β-PE (IQ Products;TB21).

Statistics

Flow cytometry FCS files were analyzed in FlowJo v10. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA). Mann-Whitney tests were performed to compare groups of unpaired, non-parametric values. Two-tailed paired *t*-tests were used to compare different experimental conditions of paired, parametric values or Wilcoxon tests for paired, nonparametric values. A *P* value of <0.05 was considered significant.

Results

T follicular regulatory cells are increased in the liver during chronic HCV infection

Profoundly impaired CD4⁺ T cell responses are associated with chronic HCV infection. Interestingly, Tfh cells in the livers of chronically-infected patients appear to be engaged in GC-like lymphoid follicles.^(18,19) However, the function of Tfh in regulating anti-viral immune responses to HCV within liver GCs is unclear. As Tfr cells are critical regulators of Tfh/GC reactions, we hypothesize that HCV infection may trigger the induction and/or expansion of Tfr in the liver microenvironment. We sought to determine the presence of Tfr in the livers of chronically-infected HCV patients. Flow cytometry was performed to analyze Tfh and Tfr populations within liver mononuclear cell preparations, as shown in Fig. 1A and Supplemental Fig. 1. As expected, the frequency of CD4⁺ T cells was significantly increased in HCV and non-viral hepatitis patients compared to healthy subjects (Fig. 1B). Consistent with recent reports^(18, 19), we observed CXCR5⁺PD-1⁺ Tfh present in HCV-infected livers (Fig. 1A,C). Within the CXCR5⁺PD-1⁺ population, HCV-infected patients exhibited a population of CD25⁺Foxp3⁺ Tfr cells, whereas there were little to no Tfr cells detectable in non-viral hepatitis and healthy subject controls (Fig. 1A,D). These results suggest that Tfr

may play a pivotal role in suppression of host immune responses to HCV infection, particularly Tfh responses in the liver microenvironment.

HCV infection in hepatocytes leads to expansion of T follicular regulatory cells

To understand how HCV infection induces accumulation of Tfr cells in the livers of chronically-infected patients, we assessed the contribution of liver parenchymal cells. As infection and replication primarily occur in hepatocytes, we sought to determine the ability of HCV-infected hepatocytes to promote Tfr accumulation. To address this, we utilized an established co-culture system of PBMCs from healthy individuals with HCV-infected hepatoma cells. Hepatoma cells were infected with HCV for 4 days. PBMCs were co-cultured with uninfected or infected hepatoma cells, or cultured alone for 4 days. Following co-culture, PBMCs were analyzed by flow cytometry and Tfr frequency was determined (Fig. 2A). The frequency of CD4⁺ T cells was similar in all groups (Fig. 2B), while the frequency of CXCR5⁺PD-1⁺ cells in the HCV-infected co-cultures was significantly increased compared to uninfected controls and PBMC cultured alone (Fig. 2B). Moreover, there was a significant increase in the percentage of Tfr following exposure to HCV-infected hepatocytes (Fig. 2A,B) as well as increased expression levels of Foxp3 in Tfr cultured with infected hepatocytes (Fig. 2B). Consistent with previous studies^(20,21), the Treg population (CD4⁺CD25⁺Foxp3⁺) was increased following co-culture with infected hepatocytes.

To confirm the ability of HCV infection to induce Tfr accumulation, we performed experiments using cells obtained from human tonsils which are secondary lymphoid tissue and contain a high proportion of Tfh and Tfr cells. As shown in Fig. 2C, tonsillar CD4⁺ T cells exhibited a traditional Tfh phenotype with high expression of CXCR5 and PD-1. Tonsillar MNCs showed no difference in the percentage of CD4⁺ T cells or Tfh (Fig. 2D). However, the percentage as well as the number of Tfr was significantly increased in the HCV infection co-culture compared to uninfected hepatocytes (Fig. 2C,D). Tfr exposed to infected hepatocytes also up-regulated Foxp3 (Fig. 2D). Thus, similar to PBMCs, tonsillar Tfr underwent expansion following exposure to HCV-infected hepatoma cells. These results demonstrate that HCV infection in hepatocytes may contribute to the increase in Tfr found in the liver tissues of patients chronically-infected with HCV.

Although Tfr cells are primarily thought to originate from thymic-derived Tregs⁽¹⁶⁾, it was recently reported that Tfr can differentiate from non-Treg precursors⁽²²⁾. We tested the possibility of whether the increase in Tfr was due to differentiation from a non-Tfr population (i.e. CXCR5⁻ Tregs or CXCR5⁺PD-1⁺ Tfh) or not by depleting pre-existing Tfr cells (CXCR5⁺CD25⁺CD127⁻) using cell sorting prior to co-culture with hepatocytes. Following co-culture, while the increase in total Tregs was still evident, there was no increase in Tfr frequency in the Tfr-depleted group (Fig. 2E), indicating that HCV infection in hepatocytes likely promotes the expansion of pre-existing Tfr cells.

HCV-induced Tfr cells up-regulate the expression of IL-10 and CTLA-4 and exert suppressor function on Tfh cells

Tfr employ a variety of suppressor mechanisms to regulate T cell function including secretion of IL-10 and TGF- β , and the expression of CTLA-4.^(23–25) We evaluated the

expression of these factors by tonsillar Tfr following co-culture with HCV-infected or uninfected hepatocytes. The percentage of IL-10-producing Tfr was significantly increased following exposure to HCV-infected hepatocytes compared to uninfected hepatocytes (Fig. 3A,B). As expected, Tfr produced minimal levels of IL-10, and was not increased by exposure to infected hepatocytes (Fig. 3C). Additionally, Tfr from HCV-infected hepatocytes up-regulated CTLA-4 compared to Tfr from uninfected hepatocytes (Fig. 3D,E). However, we did not observe any difference in intracellular TGF- β in Tfr (data not shown). These data indicate that HCV infection in hepatocytes leads to the expansion of Tfr cells with increased suppressor function.

Tfr inhibit Tfh responses by suppressing activation, proliferation, and cytokine production.⁽¹⁶⁾ To investigate the impact of increased Tfr responses, the functionality of Tfh was evaluated following exposure to HCV-infected hepatocytes. Circulating or memory Tfh found in PBMC contain subsets that produce IL-21, IFN- γ , or IL-17.⁽²⁶⁾ Therefore, we evaluated the ability of Tfh to produce these cytokines following co-culture with infected hepatocytes. There was a significant decrease in the percentage of IL-21-producing Tfh when exposed to HCV-infected hepatocytes relative to uninfected hepatocytes (Fig. 4A,B). Importantly, the mean fluorescence intensity (MFI) of IL-21 was also significantly reduced in Tfh cultured with HCV-infected hepatocytes (Fig. 4B). The percentage of Tfh producing IFN- γ or IL-17 was not affected (Fig. 4C,D); however, the level of IFN- γ expression per cell was significantly reduced as evidenced by a decrease in MFI (Fig. 4C). These results demonstrate that the ability of Tfh to produce IL-21 and IFN- γ was impaired following exposure to HCV-infected hepatocytes. Despite diminished cytokine production, Tfh exhibited a significant increase in ICOS expression, a costimulatory molecule involved in Tfh activation and functionality, upon co-culture with HCV-infected hepatocytes (Fig. 4E,F).

Next, we sought to demonstrate that impaired Tfh function is due to enhanced Tfr responses driven by HCV infection. To examine the role of HCV-induced Tfr cells in Tfh impairment, Tfr cells (CXCR5⁺CD25⁺CD127⁻) were depleted from PBMCs by sorting prior to co-culture with hepatocytes. Depletion of Tfr resulted in a modest increase in the percentage of IL-21⁺ and IFN- γ ⁺ Tfh cells during HCV-infected hepatocyte co-culture (Fig. 5A,B), although these results were not statistically significant, likely due to the presence of increased CXCR5⁻PD-1⁻ Tregs despite Tfr depletion. We next determined a direct role of HCV-induced Tfr in suppression of Tfh by performing a suppression assay. Following co-culture with infected hepatocytes, tonsillar Tfr were FACS sorted and then cultured at a 1:1 or 1:10 ratio with CFSE-labeled autologous Tfh not previously exposed to HCV. Tfh cultured with Tfr derived from HCV-infected hepatocyte co-cultures proliferated significantly less than Tfh alone (Fig. 5C,D). These results demonstrate that enhanced Tfr responses observed following exposure to HCV-infected hepatocytes can directly suppress Tfh responses.

Exosomes from HCV-infected hepatocytes can promote Tfr expansion and lead to reduced Tfh functionality

Hepatocytes infected with HCV release soluble mediators such as TGF- β that promote the development or enhancement of Treg responses.⁽²⁰⁾ Interestingly, several recent reports have

demonstrated important roles for exosomes, secreted extracellular vesicles (<100nm), in mediating cell to cell transfer of HCV infection between hepatocytes, and in activating myeloid cells through transfer of viral RNA.^(27–29) Likewise, exosomes have a plethora of immuno-modulatory activities, particular tumor cell-derived exosomes, on dampening T cell responses.^(30,31) Thus, we hypothesized that exosomes released from HCV-infected hepatocytes may have the ability to enhance Treg/Tfr responses in our system.

To evaluate the role of hepatic exosomes in promoting Tfr responses, we exposed PBMC to secreted exosomes isolated from uninfected or HCV-infected hepatoma cells. The isolation of exosomes was confirmed by electron microscopy, western blot, and flow cytometry for the exosomal marker CD63 (Fig. 6A,B). Treatment with exosomes from uninfected hepatocytes did not affect the percentage of Tfr compared to the untreated controls. However, exposure of PBMCs to exosomes from HCV-infected hepatocytes led to an increase in the percentage of Tfr cells (Fig. 6C,D). In some experiments, the effect of exosomes on Tfr was confirmed using isolated exosomes that were additionally purified by incorporating an anti-CD63 immuno-isolation step to rule out contamination of free virus within exosome preparations. Importantly, treatment of PBMC and hepatocyte co-cultures with the exosome release inhibitor spiroepoxide resulted in a significant reduction in the percentage of Tfr following exposure to HCV-infected hepatocytes (Fig. 6E). Consistent with inhibition of IL-21 production by Tfh observed in the infected hepatocyte co-cultures (Fig. 4A,B), there was also a decrease in the percentage of IL-21⁺ and IFN- γ ⁺ cells following exosome exposure (Fig. 6F). These results identify a novel pathway by which hepatocytes infected with HCV are able to influence adaptive immunity, via their release of immune-modulatory exosomes, leading to enhanced Tfr responses and diminished functionality in Tfh cells.

Exosomes from HCV-infected hepatocytes promote Tfr responses by acting directly on CD4 T cells in a TGF- β -dependent manner

We next wanted to determine if exosomes interact directly with CD4⁺ T cells to drive expansion of Tfr cells. In order to track interactions with immune cells, exosomes were labeled with CFSE, and incubated overnight with PBMC. Exosomes from uninfected and HCV-infected hepatoma cells readily interacted with CD4⁺ T cells (Fig. 7A). To determine if exosomes directly engaging CD4⁺ T cells would result in an increase in Tfr, exosomes were cultured with pre-activated CD4⁺ T cells for 4 days. Exposure of CD4⁺ T cells to exosomes from HCV-infected hepatocytes led to a significant increase in Tfr, whereas treatment with exosomes from uninfected hepatocytes had no effect on Tfr percentage (Fig. 7B, left). Next, we sought to confirm that primary human hepatocytes also produce exosomes with immunosuppressive effects. Exosomes were isolated from uninfected or HCV-infected primary hepatocytes and cultured with pre-activated CD4⁺ T cells. Exposure to exosomes from HCV-infected primary hepatocytes, but not exosomes from uninfected hepatocytes, led to an increase in Tfr cells (Fig. 7B, right). Given that HCV exosomes have recently been shown induce monocyte differentiation, we wanted to determine that monocytes were not playing a role in Tfr cell responses. Depletion of CD14⁺ monocytes prior to co-culture did not affect the ability of infected hepatoma cells to induce Tfr cell expansion (Supplemental

Fig. 2), demonstrating that monocytes are not required and further supporting our results that exosomes act directly on CD4 T cells to drive Tfr responses.

TGF- β is important for induction and expansion of Tregs.^(32,33) However, the contribution of HCV-infected hepatocyte derived TGF- β on Tfr cells has not been studied. TGF- β can be detected in exosomes of various sources.^(34,35) We hypothesized that upon HCV infection, hepatocytes up-regulate expression of TGF- β in exosomes, which may act on T cells to promote Tfr responses. To determine whether exosomes released by infected hepatocytes contained more TGF- β , exosomes were isolated using CD63 immuno-isolation, and then stained with anti-TGF- β antibodies. Exosomes were analyzed by flow cytometry to evaluate membrane-bound TGF- β . A small proportion of exosomes from uninfected hepatocytes were positive for TGF- β . However, significantly more exosomes from HCV-infected hepatocytes expressed TGF- β on their surface (Fig. 7C,D).

To determine whether exosome-associated TGF- β could induce Tfr expansion following HCV infection, exosomes from uninfected and HCV-infected hepatocytes were first treated with anti-TGF- β blocking antibodies. Exosomes were then washed and incubated with CD4⁺ T cells. As expected, treatment of CD4⁺ T cells with exosomes from infected hepatocytes lead to an increase in Tfr after 4 days (Fig. 7E,F). Importantly, blockade of TGF- β on exosomes prevented the increase in Tfr (Fig. 7E,F). These results demonstrate that the increased TGF- β -containing exosomes derived from HCV-infected hepatocytes can drive the expansion of Tfr by directly acting on T cells. These results highlight a novel mechanism by which HCV-infection in hepatocytes shifts the immuno-modulatory cargo of exosomes in a way that favors the expansion of Tfr cells.

Discussion

Numerous strategies for immune evasion, including increased T cell exhaustion and regulatory T cells are associated with chronic HCV infection.^(3,36,37) However, mechanisms leading to CD4⁺ T cell dysfunction are yet to be completely defined. In this report, we identified Tfr cells in the livers of chronic HCV patients, which were absent in healthy subjects and barely detectable if at all in NVH patients. There is growing interest in whether liver Tfh and the lymphoid-like structures they are associated with provide protection during infection. Given that GCs are tightly regulated by Tfr and that HCV has a propensity to facilitate Treg responses, exacerbated Tfr responses in the HCV-infected liver could suppress Tfh at the liver GCs. Accumulation of intrahepatic Tfr during infection could be due to the expansion of natural Tregs adopting a Tfr profile and/or potentially from HCV-specific differentiation and expansion. Results obtained from co-culture studies support expansion from pre-existing Tfr. Further analysis of the liver Tfr will be required to address whether they exhibit HCV specificity. Regardless, increased numbers of Tfr at the site of HCV infection will most certainly hinder Tfh function and potentially limit effective antibody responses within the liver.

We sought to understand the underlying mechanism contributing to the accumulation of Tfr. With the emergence of exosomes as central players in HCV pathogenesis and their expanding list of reported immuno-modulatory activities, we hypothesized that liver

exosomes could play a role in regulating immune cells during HCV infection. Interestingly, treatment of T cells with exosomes from HCV-infected hepatocytes resulted in an increase in the abundance of Tfr, which was dependent on exosome-associated TGF- β . This study highlights a novel pathway in which hepatocytes infected with HCV are able to release exosomes with the potential to promote the accumulation of Tfr.

Recently, several studies reported on the pathological role of Tfr cells in various disease settings such as human autoimmune disease and infection. Dysregulation of Tfr cells appears to be directly linked to the development of autoimmune diseases.⁽³⁸⁾ Interestingly, there is a high rate of HIV infection detectable in follicular lymphoid regions, resulting in dysregulated humoral immunity. As a result of HIV replication and TGF- β signaling, there was an increased frequency of Tfr cells, resulting in aberrant Tfh cell responses and B cell maturation.^(39,40)

A striking observation from our study was the significant role that hepatocyte-derived exosomes play in regulating T cell responses. Given that much of the immune modulation occurring during chronic HCV infection takes place within the liver, it underscores the importance of liver-derived exosomes and their role in intercellular communication. Exosomes have received much attention for their immuno-modulatory activities, which can include both activating and inhibitory effects on immune cells.⁽⁴¹⁾ Exosomes from HCV-infected hepatocytes could contain other suppressive factors in addition to TGF- β . The cargo of exosomes can contain viral factors as well, including HCV-core and envelope proteins.⁽²⁷⁾ Thus, these exosome-associated factors may contribute to some degree in the regulation of immune cells.

While our study demonstrates direct effects of liver exosomes on CD4⁺ T cells, it is possible that exosomes might act on other immune cells in the liver, including APC populations such as dendritic cells and macrophages, or liver sinusoidal endothelial cells.⁽⁴²⁾ Future studies will be critical to determine if hepatocyte-derived exosomes modulate these cells and what the effects are on adaptive immunity and establishment of viral persistence. A limitation of our study is the use of exosomes from hepatocytes infected *in vitro* with HCV. The drawback to this approach is that exosome content and activity may not fully recapitulate exosomes secreted by infected hepatocytes *in vivo* during infection. In future studies, exosomes from biopsied primary hepatocytes could potentially be used to evaluate these effects in a more physiological setting. Secondly, circulating exosomes can be isolated from chronically-infected patients to determine if they contain higher levels of immuno-modulatory factors. Identification of this novel exosomal pathway responsible for Tfr expansion by HCV-infected hepatocytes could have implications for developing therapeutic strategies to alleviate excessive immune suppression and/or curtail viral transmission via exosomes.

The identification of Tfr cells in the livers of infected patients introduces an additional component of ongoing immune regulation during HCV infection that includes Tregs, tolerogenic DCs, and immunosuppressive viral factors. While it is unclear what the extent of Tfr-mediated regulation is on protective immunity and HCV persistence, their presence suggests that they likely play an important role in limiting Tfh responses in the liver. Our

finding that Tfr were predominantly identified in livers of chronic HCV patients and barely detectable in NVH patients suggests a role for viral infection. While this phenomenon is observed in chronic HCV it may not be exclusive to HCV as expansion of Tfr have been reported in other viral infections such as HIV and SIV.⁽³⁹⁾ Although beyond the scope of our study, it would be of interest to study hepatic Tfr in HCV/HIV co-infection as well as in HBV and other hepatotropic viral infections. Additional studies are necessary to determine the impact of Tfr on disease as well as evaluating the effects of antiviral drug treatment on their presence. Studies into how intrahepatic Tfr contribute to humoral immunity may further our understanding of antibody responses during HCV infection. Thus, novel therapeutic strategies against HCV infection could include specific agent(s) targeting Tfr-mediated immune regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

Treg	regulatory T cell
Tfr	T follicular regulatory cell
Tfh	T follicular helper cell
GC	germinal center
DMEM	Dulbecco's Modified Eagle Medium
RPMI	Roswell Park Memorial Institute medium
PBMCs	peripheral blood mononuclear cells
PHH	primary human hepatocytes
CFSE	carboxyfluorescein succinimidyl ester
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
ICOS	Inducible T-cell costimulatory
NVH	non-viral hepatitis

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Fig. 1. Identification of intrahepatic T follicular regulatory cells in HCV patients

(A) Flow cytometry gating strategy for liver Tfh and Tfr cells. CD45⁺ cells from the live gate were gated to remove liver cells. Lymphocytes were gated, followed by exclusion of CD56⁺ NK cells and CD14⁺/CD11b⁺ myeloid cells. CD4⁺ cells were then gated to analyze CXCR5⁺PD-1⁺ cells. Within the CXCR5⁺PD-1⁺ population, Tfr were identified by co-expression of CD25 and Foxp3. (B) Percentage of CD4⁺ T cells within CD45⁺ leukocyte population. (C) Percentage of Tfh (CXCR5⁺PD-1⁺) within the CD4⁺ T cell population. (D) Percentage of Tfr (CD25⁺Foxp3⁺) within CXCR5⁺PD-1⁺ population. Symbols represent individual patients with a total of 7 healthy subjects, 6 non-viral hepatitis (NVH) patients, and 8 HCV patients. Horizontal bars indicate the median value in each group. Statistical analyses were performed by Mann-Whitney tests to compare unpaired, non-parametric values. **P*<0.05.

Fig. 2. HCV infection in hepatocytes promotes Tfr cell expansion

(A) PBMCs from healthy subjects were co-cultured with uninfected or HCV-infected hepatoma cells for 4 days. PBMCs were evaluated by flow cytometry to assess the frequency of Tfr within the CXCR5⁺PD-1⁺ population. A representative experiment is shown in A with Tfh and Tfr gating. (B) Symbols in graphs represent individual donors and horizontal bars indicate the median value in each group for the percentages of CD4⁺ T cells, Tfh cells, and Tfr cells following co-culture or when PBMCs were cultured alone. The bar graph for Foxp3 MFI represents the means $\pm$ SEM for each group. (C) Tonsillar MNCs were co-cultured with uninfected or HCV-infected hepatoma cells for 4 days. (D) Symbols in graphs represent individual donors and horizontal bars indicate the median value in each group for the percentages of CD4⁺ T cells, Tfh cells, Tfr cells, absolute number of Tfr. The bar graph for Foxp3 MFI represents the means $\pm$ SEM. (E) Tfr cells were depleted from healthy subject PBMCs by sorting prior to co-culture. On day 4 of co-culture, the percentage of Tfr was analyzed. Statistical analyses were performed by Wilcoxon tests to compare paired, non-parametric values or by two-tailed *t*-test for paired, parametric values. **P*<0.05, ***P*<0.01.

Fig. 3. Exposure to HCV-infected hepatocytes leads to an enhanced immune-suppressive phenotype of Tfr cells

(A) Tonsillar MNCs were co-cultured with uninfected or HCV-infected hepatoma cells for 4 days. Intracellular cytokine staining for IL-10 was performed. Shown are representative flow plots gated on Tfr cells. IL-10⁺ cells were gated according to unstimulated controls. (B) Average percentage of IL-10⁺ Tfr following co-culture or when tonsillar MNCs were cultured alone. (C) Average percentage of IL-10⁺ Tfh following co-culture. (D) Representative histogram of CTLA-4 (surface and intracellular) expression on Tfr following culture with uninfected hepatocytes (gray line), infected hepatocytes (black line), or on CD25⁻Foxp3⁻ CD4⁺ T cells (gray-filled). (E) Average CTLA-4 MFI on Tfr. Data are averages of 3–6 individuals $\pm$ SEM. * P <0.05 by two-tailed paired t -test or by Wilcoxon test.

Fig. 4. Reduced Tfh cell functionality following co-culture with HCV-infected hepatocytes (A) PBMCs were co-cultured with uninfected or HCV-infected hepatoma cells for 4 days. Intracellular cytokine staining for IL-21, IFN- γ , and IL-17 was performed. Shown are representative flow plots gated on Tfh. Numbers in quadrants represent the average number of cytokine⁺ cells from a representative experiment. Cytokine⁺ cells were gated according to unstimulated controls. (B) Percentage (top) of IL-21⁺ Tfh and the MFI of IL-21 (bottom) following co-culture. (C) Percentage (top) of IFN- γ ⁺ Tfh and the MFI of IFN- γ (bottom) following co-culture. (D) Percentage (top) of IL-17⁺ Tfh and the MFI of IL-17 (bottom) following co-culture. Data points in B–D represent values from individual PBMC donors. (E) Representative histogram of ICOS expression on Tfh following PBMC co-culture with uninfected hepatocytes (gray line), infected hepatocytes (black line), or in PBMC only controls (gray-filled). (F) Average ICOS MFI on Tfh. Symbols in graphs represent individual donors. Data are averages of 10–12 individuals $\pm$ SEM. NS=Not significant. * P <0.05 by two-tailed paired t -test.

Fig. 5. Tfr cells impair Tfh cell cytokine production and proliferation during co-culture with HCV-infected hepatocytes

(A) Tfr cells were depleted by cell sorting prior to co-culture. PBMCs were stimulated followed by intracellular cytokine staining. Shown are representative flow plots gated on Tfh. Numbers in quadrants represent the number of cytokine⁺ cells from a representative experiment. (B) Bar graphs represent the percentages of IL-21⁺ Tfh (left) and IFN-γ⁺ Tfh (right) in each condition following co-culture ±SEM. (C) Tonsillar MNCs were co-cultured with HCV-infected hepatocytes for 4 days. Tfr were sorted and co-cultured with autologous Tfh to assess their ability to suppress proliferation. Representative flow plots for CFSE dilution are shown on day 2 (top) and day 5 (bottom). (D) The graph represents the percent of proliferating Tfh (CFSE low) on day 5. **P*<0.05 by two-tailed paired *t*-test.

Fig. 6. Characterization of hepatocyte exosomes and their effects on Tfr cell expansion and Tfh function

Isolated liver exosomes were characterized by electron microscopy (A), western blot, and flow cytometry (B). (C) On day 4, PBMCs cultured in the presence or absence of exosomes were evaluated by flow cytometry to assess the frequency of Tfr within the CXCR5⁺PD-1⁺ population. (D) Bar graphs represent the average percentages of Tfh and Tfr following culture with exosomes. (E) The percentage of Tfr on day 4 following co-culture of PBMCs with uninfected or HCV-infected hepatoma cells in the presence of the exosome release inhibitor spiroepoxide (5μM) or DMSO. Data represents the means of 5 individuals ±SEM. (F) On day 4, intracellular cytokine staining for IL-21, IFN-γ, and IL-17 was performed. Average percentage of IL-21⁺, IFN-γ⁺, or IL-17⁺ Tfh is shown in the graphs. Symbols in graphs represent individual donors. NS=Not significant. **P*<0.05 by two-tailed paired *t*-test.

Fig. 7. Exosomes from HCV-infected hepatocytes act directly on CD4⁺ T cells to promote Tfr cell development in a TGF-β-dependent manner

(A) Isolated exosomes were labeled with CFSE and cultured with PBMCs for 24 hours. PBMCs were analyzed for CSFE to evaluate interaction with exosomes. (B) *Left*: CD4⁺ T cells were cultured alone, with exosomes from uninfected hepatocytes, or with exosomes from infected hepatocytes for 4 days. *Right*: CD4⁺ T cells were cultured alone, with exosomes isolated from uninfected PHHs, or exosomes isolated on day 12 post-infection from HCV sera-infected PHHs for 4 days. Tfr frequency was determined by flow cytometry. (C) Exosomes were incubated with beads coated with anti-CD63 antibody and subsequently stained with anti-CD63 and anti-TGF-β fluorescent antibodies. Bead-bound exosomes were then analyzed on a flow cytometer. (D) Average percentage of bead/exosomes positive for TGF-β. (E) Exosomes were incubated with isotype or anti-TGF-β blocking antibody, re-isolated, and cultured with CD4⁺ T cells for 4 days. CD4⁺ T cells were evaluated by flow cytometry to assess the frequency of Tfr. Shown are representative flow plots gated on CXCR5⁺PD-1⁺ cells. (F) Percentage of Tfr within CXCR5⁺PD-1⁺ population. Symbols in graphs represent individual donors of PBMCs or CD4 T cells. Error bars represent ±SEM. **P*<0.05 by two-tailed paired *t*-test or Friedman nonparametric test followed by Dunn's multiple comparison.