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Recovery of Hepatitis C Specific T-cell Responses after Rituximab Therapy in Hepatitis C Mixed Cryoglobulinemic Vasculitis

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Abstract

Background & Aims—Mixed cryoglobulinemic vasculitis is associated with monoclonal B cell expansion in patients with chronic hepatitis C (HCV) infection. B cell depletion therapy using rituximab, a CD20 monoclonal antibody, has been successful in achieving remission from symptomatic disease. This study investigated whether B cell depletion therapy has an impact on activation of HCV-specific T cell phenotype and function.

Methods—Nineteen patients with Hepatitis C mixed cryoglobulinemic vasculitis were treated with 4 cycles of rituximab (375mg/m²) and variables were measured 6 months after therapy. Using flow cytometry and Enzyme-Linked Immunospot assay, the number of activated and tissue-like B cells and number of T cells expressing Programmed cell death protein 1 (PD-1), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and multiple cytokines were measured before and after rituximab therapy.

Results—B cell depletion therapy is associated with a significant ($P < 0.0001$) decline in peripheral T cells with exhaustive phenotype, from pre-therapy to post-therapy-of rituximab (mean $\pm$ standard error): CD4⁺ (16.9 $\pm$ 0.9% to 8.9 $\pm$ 1.0%) and CD8⁺ (6.8 $\pm$ 0.6% to 3.0 $\pm$ 0.5%) T cells expressing PD-1 and CD4⁺ (11.0 $\pm$ 1.0% to 6.1 $\pm$ 0.8%) and CD8⁺ (12.7 $\pm$ 0.7% to 6.4 $\pm$ 0.4%) T cells expressing TIM-3. In addition, there was a significantly higher percentage of peripheral CD8⁺ T cells responding to HCV peptide stimulation in vitro secreting IFN- γ (4.55 $\pm$ 0.3 to 9.6 $\pm$ 1.0 IFN- γ /10⁶ PBMCs, $P < 0.0001$), and more than one cytokine (1.3 $\pm$ 0.1% to 3.8 $\pm$ 0.2%, $P < 0.0001$) after therapy compared to pre-therapy.

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Conflict of interest: None of the authors have any conflicts of interest to report

Experimental Ethics: All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki. The institutional committee of the NIAID of the NIH approved the experiment. Informed consent was obtained by the human subjects for this study.

Disclaimer: This study does not represent the views of the Department of Health and Human Services.

Additional Supporting Information may be found in the online version of this article.

Conclusion—B cell depletion therapy results in recovery of T cell exhaustion and function in patients with HCV cryoglobulinemic vasculitis.

Keywords

Hepatitis C virus; Virus classification; Antibody-containing preparations; Disease control; T cell; Immune responses; Activation; Immune responses

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is one of the most prevalent viral infections in the United States (US) with an estimated 2.7 million individuals living with HCV.^{1,2} The natural history of untreated chronic HCV is characterized by a gradual progression to fibrosis and cirrhosis, hepatocellular carcinoma, liver failure, and mortality.³ HCV remains the leading cause of chronic liver disease, hepatocellular carcinoma, and liver transplantation in US and Western countries.^{4–6} Extra-hepatic manifestations (EHM) occur in approximately 40–74% of HCV-infected patients.^{7,8} EHM include mixed cryoglobulinemic (MC) vasculitis, B cell lymphomas, rheumatic disorders, autoimmune thyroiditis, hypothyroidism, papillary thyroid cancer, and type 2 diabetes mellitus.^{6,8} While most EHM are provisionally associated with chronic HCV infection,⁹ Type II MC vasculitis is perhaps the most strongly associated with HCV,^{7–11} as 70–86% of symptomatic patients have HCV viremia.^{12–14}

Pathogenesis of HCV-associated MC vasculitis is categorized by preferential expansion of monoclonal B cells,¹⁵ probably triggered by HCV neoantigens/epitopes, which produce soluble IgM with rheumatoid factor activity capable of forming immune complexes that fix complement, resulting in glomerulonephritis and small vessel vasculitis.^{6,9,13} Clinical signs of HCV-associated MC vasculitis include classic manifestations of systemic immune complex diseases, such as cutaneous vasculitis (non-healing ulcers), arthralgia/arthritis, peripheral neuropathy, and membranoproliferative glomerulonephritis.^{16–18}

The pathogenesis of immunological abnormalities associated with HCV-MC vasculitis, the nature of HCV infection leading to MC vasculitis, and the role of B cells are not completely understood. Recent clinical trials have shown HCV-MC vasculitis patients can be effectively treated with rituximab, an anti-CD20 monoclonal antibody, resulting in symptomatic relief.^{10,11,19,20} Rituximab is known to normalize peripheral B- and T-lymphocyte homeostasis by improving regulation, activation, Th1/Th2 imbalances,²¹ and increasing CD4+ T cells.¹¹ However, it is unclear how resetting of B cell homeostasis using rituximab therapy may have any impact on T cell phenotype and function. Chronic HCV infection is associated with increases in T cells with an exhausted phenotype (defined as cells expressing Programmed cell death protein 1 [PD-1], T-cell immunoglobulin and mucin-domain containing-3 [TIM-3]), the hallmark of which is a lack of polyfunctionality, and is more commonly observed in those cells specifically responding to HCV antigens.^{22–25} These T cells express PD-1 and TIM-3 molecules, more frequently than what is observed among normal healthy human subjects.^{23,26} Recently, we demonstrated a decline in activated and memory T cells in peripheral blood from pre-therapy to follow-up while on rituximab therapy, resolution of

active disease, and lowering of the Birmingham Vasculitis Activity Score (BVAS), among HCV-MC vasculitis patients.²⁷

In the present study, we sought to further characterize T cell phenotype and function, and reversal of T cell exhaustion, with respect to HCV specificity before and after rituximab therapy in patients with HCV-MC.

MATERIALS AND METHODS

Study Population

Patients were selected from an open-labeled, randomized controlled trial (NCT#00029107) conducted at the National Institute of Allergy and Infectious Diseases (NIAID) at the National Institutes of Health (NIH) Clinical Center, Bethesda, MD, of rituximab therapy for patients with HCV-MC.¹⁰ The clinical trial eligibility criteria were patients with HCV infection and MC vasculitis who had failed interferon alpha and ribavirin therapy or who could not tolerate this therapy and is described elsewhere.¹⁰ Patients enrolled were randomized 1:1 to receive either the therapy arm of rituximab or the control arm of best available therapy.¹⁰ Patients in the therapy arm received rituximab 375 mg/M² on days 1, 8, 15, and 22, beginning at the time of randomization as previously described (immediate therapy).¹⁰ Patients in the control arm could remain on any immunosuppressive therapies at the time of randomization. The clinical trial primary endpoint was remission 6 months from randomization. After study month 6, patients randomized to the control arm were offered therapy with rituximab if they continued to have active manifestations of vasculitis (delayed therapy). This study used 19 patients who received rituximab therapy overall (10 patients from the therapy and 9 patients from the control arm).¹⁰ All patients provided written informed consent and the clinical trial was approved by the NIAID Institutional Review Board. All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

Laboratory Measurements

Patients had comprehensive clinical and laboratory evaluations at each monthly clinical trial visit: chemistries, urinalysis, complete blood counts, cryoglobulin levels (% of cryocrit), peripheral blood flow cytometry, HCV plasma RNA levels, and remission, defined as a BVAS of 0 (indicating no new or worsened activity or no persistently active disease manifestations within the previous month).¹⁰

PBMC collection

Peripheral blood was collected by venipuncture and peripheral blood mononuclear cells (PBMCs) were isolated from white blood cells by the standard Ficoll-Hypaque Plus (Amersham Biosciences, Uppsala, Sweden) density gradient separation technique as previously described.²⁸

HCV peptide reconstitution

Genotype 1a or 1b HCV 15 to 18-mer peptides with 11 or 12 amino acid overlaps spanning the entire HCV polyprotein (BEI Resources, NIAID, NIH: Peptide Array, Hepatitis C Virus)

were reconstituted in 5% sterile dimethylsulphoxide (DMSO) and pooled consecutively into twenty-one groups. Peptides were aliquoted and stored at -80°C until use.

IFN- γ ELISPOT to characterize HCV-specific T cell responses

To characterize HCV responsive T cell function, PBMCs were incubated with either genotype 1a or 1b HCV 15 to 18-mer peptides with 11 or 12 amino acid overlaps that spanned the entire HCV polyprotein (BEI Resources, NIAID, NIH: Peptide Array, Hepatitis C Virus). The number of HCV-responsive IFN- γ -producing PBMCs was quantified by standard ELISPOT assay (BD Biosciences). In summary, 96 well ELISPOT plates were coated with anti-IFN- γ biotinylated capture antibody and incubated overnight at 4°C . Plates were then blocked using lymphocyte medium, and PBMCs were allowed to rest for 6 hours at 37°C . PBMCs were plated between 250,000–400,000 cells per well with either phytohemagglutinin as a positive control (5 $\mu\text{g}/\text{ml}$), dimethyl sulfoxide as a negative control (0.05%), or pooled HCV peptides (3 $\mu\text{g}/\text{ml}$). All cultures were performed in duplicate. After incubating for 12 hours at 37°C , cells were removed, and plated with streptavidin detection antibody, enzyme conjugate, and substrate. The plates were air dried in the dark overnight, and developed spots were enumerated using an ELISPOT plate reader. The frequency of IFN- γ -producing cells was calculated as described previously.²⁸

Multi-color flow cytometry assay to assess HCV responsive T cell polyfunctionality and phenotype

We performed multicolor flow cytometry to enumerate CD4⁺ and CD8⁺ T cells expressing exhaustive phenotype before and after B cell depletion therapy. To identify HCV responsive cell populations, CFSE dilution based flow cytometry was utilized. PBMCs were washed with PBS, incubated with 2.5 μM CFSE (Invitrogen) at 37°C for 6 minutes, quenched with PBS + 5% FCS, and washed three times with lymphocyte medium. PBMC were incubated at 5×10^6 in 1.2 mL with either pools of overlapping HCV peptides spanning the entire proteome (3 $\mu\text{g}/\text{mL}/\text{peptide}$) or DMSO (1%) and NH_4OH (0.7mM) as a vehicle control for a total of 48 hours. Brefeldin A and monensin were added at 36 hours to block the Golgi apparatus and allow intracellular cytokine accumulation. Following incubation, PBMCs were harvested and stained with LIVE/DEAD Near-IR (Invitrogen) for 30 minutes on ice. Cells were harvested and live, CFSE cells were assessed for phenotype and function using the antibodies described previously. The gating strategy is shown in supplemental Figure 1.

To assess secreted cytokine production, 100 μL of supernatant from cultured PBMC was collected at 36 hours and frozen at -80°C until used. Samples were thawed and analyzed for cytokine production using Mesoscale Discovery Human TH1/TH2 10-plex ultra-sensitive kit (catalog K#15010C-1), according to the manufacturer's protocol. All samples were tested in duplicate.

Statistical Analysis

The primary analysis assessed the individual differences between CD4⁺ and CD8⁺ T cell expressing exhaustive phenotype and secreting one or more cytokines after stimulation with HCV peptides from pre-therapy to post-therapy (6 months after starting rituximab therapy) using a paired *t*-test. We also explored if there was a difference in the change of these

parameters between patients who received immediate vs. delayed therapy. All analysis was conducted in GraphPad Prism 5 with a p -value < 0.05 (two-sided) indicating statistical significance.

RESULTS

Study Population

The clinical and demographic characteristics of patients from the clinical trial, and that rituximab therapy was successful with a clinical response, have been previously described.¹⁰ The rituximab and control group (immediate and delayed therapy) were balanced in demographics, clinical manifestations, disease activity, laboratory values, and exposure to glucocorticoids and other immunosuppressive therapies.¹⁰

Decline in CD4 and CD8+ T cells with Exhaustive Phenotype after B cell depletion therapy

Collectively, CD4+ and CD8+ T cells from all patients experienced a decline in PD-1 and TIM-3 expression with rituximab therapy. Overall, there was a significant decline in change from pre-therapy to post-therapy in CD4+ T cells expressing both PD-1 (pre-therapy $16.9 \pm 0.9\%$ and post-therapy $8.9 \pm 1.0\%$, $P < 0.0001$) and TIM-3 (pre-therapy $11.0 \pm 1.0\%$ and post-therapy $6.1 \pm 0.8\%$, $P < 0.0001$) (Figures 1 and 2). There was no difference in the change between patients who received immediate rituximab compared to those who received delayed therapy.

Overall, there was a significant decline in change from pre-therapy to post-therapy in CD8+ T cells expressing both PD-1 (pre-therapy $6.8 \pm 0.6\%$ and post-therapy $3.0 \pm 0.5\%$, $P < 0.0001$) and TIM-3 (pre-therapy $12.7 \pm 0.7\%$ and post-therapy $6.4 \pm 0.4\%$, $P < 0.0001$) (Figures 1 and 2). There was no difference in the change between patients who received immediate rituximab compared to those who received delayed therapy.

Enhancement of CD8+ T cells secreting multiple cytokines in response to HCV peptide stimulation with B cell depletion therapy

There was a significant increase in polyfunctionality (more than one cytokine) from pre-therapy ($1.3 \pm 0.1\%$) to post-therapy ($3.8 \pm 0.2\%$), $P < 0.0001$ (Figure 3). Second, there was a significant decrease in IFN- γ from pre-therapy (229.6 ± 20.1 pg/mL) to post-therapy (165.1 ± 22.5 pg/mL), $P = 0.0031$ (Figure 4). Finally, there was a significant decrease in TNF- α from pre-therapy (128.8 ± 13.5 pg/mL) to post-therapy (88.8 ± 11.4 pg/mL), $P = 0.0003$ (Figure 4). There was no difference in the change between patients who got immediate rituximab compared to those who received delayed therapy for polyfunctionality, IFN- γ , and TNF- α .

Augmentation of CD8+ T cells secreting IFN- γ in response to HCV peptide stimulation with B cell depletion therapy

There was an increase in IFN- γ spot forming cells with rituximab therapy from pre-therapy (4.55 ± 0.3 IFN- γ /10⁶ PBMCs) to post-therapy (9.6 ± 1.0 IFN- γ /10⁶ PBMCs), $P < 0.0001$ (Figure 3). When we measured the levels of IFN- γ and TNF- α from the cell culture supernatants, there was a significant increase in IFN- γ from pre-therapy ($0.09 \pm 0.02\%$) to

post-therapy ($1.0 \pm 0.1\%$, $P < 0.0001$) (Figure 3). Patients who received delayed therapy had a larger change than patients who received immediate therapy, $P = 0.027$.

DISCUSSION

We demonstrate an association between rituximab therapy and improved HCV-specific T cell function in patients with HCV-MC vasculitis. The data supports the functional recovery of HCV-responsive polyfunctional CD8⁺ T cells, as there was a significant increase in the HCV-specific T cells secreting either IFN- γ or secreting more than one cytokine. In addition, both CD4⁺ and CD8⁺ T cells in the periphery expressed lower levels of PD-1 and TIM-3, both associated with immunologic exhaustion. Consistent with the lowered expression of exhaustive markers, T cells exhibited increased secretory function when stimulated with HCV peptides, suggesting reversal of exhaustion as the mechanism of this phenomenon. These results provide novel insights into the interactions between B and T cells in the pathogenesis of HCV-MC vasculitis. The monoclonal expansion of B cells that leads to immune complex mediated small vessel vasculitis also leads to T cell exhaustion. Rituximab therapy results in rapid depletion of circulating tissue-like memory B cells, and our study showed an increase in HCV-specific T cell function following rituximab therapy. Previously, we described B cell depletion in the same patients result in T cell activation²⁷. These results further extend these observations suggesting depletion of B cells and reversal of inflammatory response lead to recovery of HCV-specific T cells. The exact mechanism involved is not well understood, but an indirect effect of B cells that reduce inflammatory response and drive immune exhaustion may be the major driver of the aberrant antiviral response.^{6,29} Our data demonstrates a significant reversal of exhaustive phenotype of T cells with depletion of B cells, suggesting a mechanism that involves B and T cell interaction or indirect effect of B cell recovery on non-B-cells.

When we measured cytokine production by PBMCs in general, there was no significant change in IFN- γ production, while there was an increase in IFN- γ production by HCV-specific cells, which represents only a small fraction of all cells secreting IFN- γ . These results suggest a specific effect of B cell depletion therapy and remission on HCV specific immunity, which is a unique finding. Recently, all oral directly-acting antiviral (DAA) drugs have been shown to result in a complete cure of HCV infection in patients by achieving sustained virologic response (SVR), defined as the absence of plasma HCV RNA levels 12 weeks after completing therapy.³⁰ DAA therapy has revolutionized the HCV therapy paradigm and is likely to reduce the prevalence of HCV-MC vasculitis by eliminating chronic HCV infection. Recent studies have also demonstrated that HCV-specific immunity plays a role in the elimination of HCV and achieving SVR when treated with DAA therapy.²² In this regard, a recent study of immunologic recovery with DAA therapy suggested a dichotomous response of T and B cells with SVR.²² Barrett *et al* demonstrated normalization of T cell activation and exhaustion while persistence of B cell abnormalities in the peripheral blood of patients who achieved SVR with sofosbuvir and ribavirin.²² Our findings align with this concept that patients with HCV-MC are unable to mount an effective antiviral immune response and hence fail to achieve SVR with interferon based therapy. It should be noted that most patients with HCV-MC are previously therapy-experienced and non-responders to HCV therapy.^{10,17} Future studies will demonstrate the response rate of

patients with HCV-MC when treated with DAAs. We anticipate a decline and possible elimination of HCV-MC vasculitis as use of DAA regimens becomes more prevalent.

Our study had limitations. The sample size was small and the follow-up time was limited; long-term follow-up would have allowed us to study T cell functionality with B cell recovery and, sustenance of response, and disease relapse. Second, it is difficult to quantify immunological response, which makes it challenging to evaluate the clinical significance of enhanced antiviral immunity. In our study, the augmentation of cellular immunity against HCV was not associated with a significant decline in HCV VL. Since we did not have long-term follow-up, we were not able to evaluate substance of this response. Other factors that could mediate inflammatory response in these patients such as B lymphocyte stimulator (BLyS-1), interleukin-1 (IL-1), and interleukin 1 receptor antagonist (IL-IRA), could also play a role in explaining these findings. Last, there is inconsistency between the ELISPOT data and in vitro culture data with regards to the IFN- γ detection. In this regard, we have 3 possible explanations. First, the variability in the T cell frequency in input cells is a confounding factor that could impact the levels of cytokine. Second, IFN- γ secretion by bystander T cells in vitro culture may relate to this variability. Third, the difference in the detection platforms, particularly variability in anti-IFN- γ antibodies used in EIA vs. ESISPOT, may also contribute to this discrepancy.

B cell depletion therapy of HCV-MC vasculitis patients offered us a unique perspective into B cell interface with HCV specific T cells. Future studies should focus on evaluating B and T cell phenotype and specificity in HCV-MC vasculitis patients treated with DAAs, in order to understand whether recovery of antiviral immunity is associated with enhanced clearance of virus and achievement of SVR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Mohd Hanafiah K, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology*. Apr; 2013 57(4):1333–1342. [PubMed: 23172780]
2. Denniston MM, Jiles RB, Drobeniuc J, et al. Chronic hepatitis C virus infection in the United States, National Health and Nutrition Examination Survey 2003 to 2010. *Annals of internal medicine*. Mar 04; 2014 160(5):293–300. [PubMed: 24737271]
3. Chen SL, Morgan TR. The natural history of hepatitis C virus (HCV) infection. *International journal of medical sciences*. 2006; 3(2):47–52. [PubMed: 16614742]
4. Davis GL, Alter MJ, El-Serag H, Poynard T, Jennings LW. Aging of hepatitis C virus (HCV)-infected persons in the United States: a multiple cohort model of HCV prevalence and disease progression. *Gastroenterology*. Feb; 2010 138(2):513–521. 521 e511–516. [PubMed: 19861128]

5. Kabiri M, Jazwinski AB, Roberts MS, Schaefer AJ, Chhatwal J. The changing burden of hepatitis C virus infection in the United States: model-based predictions. *Annals of internal medicine*. Aug 05; 2014 161(3):170–180. [PubMed: 25089861]
6. Charles ED, Brunetti C, Marukian S, et al. Clonal B cells in patients with hepatitis C virus-associated mixed cryoglobulinemia contain an expanded anergic CD21low B-cell subset. *Blood*. May 19; 2011 117(20):5425–5437. [PubMed: 21421840]
7. Galossi A, Guarisco R, Bellis L, Puoti C. Extrahepatic manifestations of chronic HCV infection. *Journal of gastrointestinal and liver diseases: JGLD*. Mar; 2007 16(1):65–73. [PubMed: 17410291]
8. Antonelli A, Pistello M. New Therapies, Markers and Therapeutic Targets in HCV Chronic Infection, and HCV Extrahepatic Manifestations. *Current drug targets*. Nov 01. 2015
9. Dore MP, Fattovich G, Sepulveda AR, Real di G. Cryoglobulinemia related to hepatitis C virus infection. *Digestive diseases and sciences*. Apr; 2007 52(4):897–907. [PubMed: 17380399]
10. Sneller MC, Hu Z, Langford CA. A randomized controlled trial of rituximab following failure of antiviral therapy for hepatitis C virus-associated cryoglobulinemic vasculitis. *Arthritis and rheumatism*. Mar; 2012 64(3):835–842. [PubMed: 22147444]
11. Zaja F, De Vita S, Mazzaro C, et al. Efficacy and safety of rituximab in type II mixed cryoglobulinemia. *Blood*. May 15; 2003 101(10):3827–3834. [PubMed: 12560225]
12. Ferri C, La Civita L, Longombardo G, Greco F, Bombardieri S. Hepatitis C virus and mixed cryoglobulinaemia. *European journal of clinical investigation*. Jul; 1993 23(7):399–405. [PubMed: 8397090]
13. Ferri C, Mascia MT. Cryoglobulinemic vasculitis. *Current opinion in rheumatology*. Jan; 2006 18(1):54–63. [PubMed: 16344620]
14. Schamberg NJ, Lake-Bakaar GV. Hepatitis C Virus-related Mixed Cryoglobulinemia: Pathogenesis, Clinical Manifestations, and New Therapies. *Gastroenterology & hepatology*. Sep; 2007 3(9):695–703. [PubMed: 21960882]
15. Sansonno D, Dammaco F. Hepatitis C virus, cryoglobulinaemia, and vasculitis: immune complex relations. *The Lancet. Infectious diseases*. Apr; 2005 5(4):227–236. [PubMed: 15792740]
16. Lamprecht P, Gause A, Gross WL. Cryoglobulinemic vasculitis. *Arthritis and rheumatism*. Dec; 1999 42(12):2507–2516. [PubMed: 10615995]
17. Cacoub P, Comarmond C, Domont F, Savey L, Saadoun D. Cryoglobulinemia Vasculitis. *The American journal of medicine*. Sep; 2015 128(9):950–955. [PubMed: 25837517]
18. Jacobson IM, Cacoub P, Dal Maso L, Harrison SA, Younossi ZM. Manifestations of chronic hepatitis C virus infection beyond the liver. *Clinical gastroenterology and hepatology: the official clinical practice journal of the American Gastroenterological Association*. Dec; 2010 8(12):1017–1029. [PubMed: 20870037]
19. Saadoun D, Delluc A, Piette JC, Cacoub P. Treatment Treatment of hepatitis C-associated mixed cryoglobulinemia vasculitis. *Current opinion in rheumatology*. Jan; 2008 20(1):23–28. [PubMed: 18281853]
20. Dammaco F, Sansonno D. Therapy for hepatitis C virus-related cryoglobulinemic vasculitis. *The New England journal of medicine*. Sep 12; 2013 369(11):1035–1045. [PubMed: 24024840]
21. Saadoun D, Rosenzweig M, Landau D, Piette JC, Klatzmann D, Cacoub P. Restoration of peripheral immune homeostasis after rituximab in mixed cryoglobulinemia vasculitis. *Blood*. Jun 01; 2008 111(11):5334–5341. [PubMed: 18292291]
22. Barrett L, Shivasabesan G, Wang C, Osinusi A, Kohli A, Meissner EG, Heytens L, Nelson A, Polis MA, Masur H, Symonds W, McHutchinson J, Kottitil S. Altered HCV specific T cell immunity very early in interferon free HCV DAA therapy. *Hepatology*. 2013; 58(S1)
23. Barrett L, Trehanpati N, Poonia S, et al. Hepatic compartmentalization of exhausted and regulatory cells in HIV/HCV-coinfected patients. *Journal of viral hepatitis*. Mar; 2015 22(3):281–288. [PubMed: 25174689]
24. Abdel-Hakeem MS, Boisvert M, Bruneau J, Soudeyhs H, Shoukry NH. Selective expansion of high functional avidity memory CD8 T cell clonotypes during hepatitis C virus reinfection and clearance. *PLoS pathogens*. Feb. 2017 13(2):e1006191. [PubMed: 28146579]

25. Rodrigue-Gervais IG, Rigsby H, Jouan L, et al. Dendritic cell inhibition is connected to exhaustion of CD8+ T cell polyfunctionality during chronic hepatitis C virus infection. *Journal of immunology*. Mar 15; 2010 184(6):3134–3144.
26. Nakamoto N, Cho H, Shaked A, et al. Synergistic reversal of intrahepatic HCV-specific CD8 T cell exhaustion by combined PD-1/CTLA-4 blockade. *PLoS pathogens*. Feb.2009 5(2):e1000313. [PubMed: 19247441]
27. Emmanuel B, Sidique N, Zhang X, Poonia B, Sneller MC, Kottlil S. Decline of cellular activation in non-B cells after rituximab treatment in hepatitis C-associated mixed cryoglobulinemia vasculitis. *Journal of viral hepatitis*. Feb; 2017 24(2):128–131. [PubMed: 27666584]
28. Shrivastava S, Wilson E, Poonia B, et al. Augmentation of hepatitis C virus-specific immunity and sustained virologic response. *Journal of viral hepatitis*. Mar 07.2017
29. Spangenberg HC, Viazov S, Kersting N, et al. Intrahepatic CD8+ T-cell failure during chronic hepatitis C virus infection. *Hepatology*. Oct; 2005 42(4):828–837. [PubMed: 16175596]
30. Kohli A, Shaffer A, Sherman A, Kottlil S. Treatment Treatment of hepatitis C: a systematic review. *JAMA*. Aug 13; 2014 312(6):631–640. [PubMed: 25117132]

Figure 1. Percent of PD-1+ CD4+ and CD8+ T-cells pre-and post-rituximab therapy (mean $\pm$ standard error).

Figure 2.
Percent of TIM-3 + CD4+ and CD8+ T-cells pre-and post-rituximab therapy(mean $\pm$ standard error).

Figure 3. Percent of HCV-specific CD8+ T-cells secreting IFN- γ and more than one cytokine pre-and post-rituximab therapy (mean $\pm$ standard error).

Figure 4.
Levels of IFN- γ and TNF- α pre and post rituximab therapy (mean $\pm$ standard error).