DR. SHIKHA SHRIVASTAVA (Orcid ID : 0000-0001-6276-0791)

Received Date : 21-Nov-2016
Revised Date : 17-Jan-2017
Accepted Date : 12-Feb-2017
Article type : Original Paper

Manuscript Title:
Augmentation of HCV specific immunity and Sustained virological response (SVR)

Authors:
Shikha Shrivastava[1], Wilson E[1], Poonia B[1], Tang L[1], Osinusi A[2], Kohli A[3], Shyam Kottilil[1]

Affiliations:
1. Institute of Human Virology, University of Maryland School of Medicine, Baltimore MD,
2. Gilead Sciences Inc., Foster City, CA.
3. Creighton University School of Medicine, St Joseph's Hospital, Phoenix, Arizona

Shikha Shrivastava: sshrivastava@ihv.umaryland.edu
Institute of Human Virology, University of Maryland School of Medicine, Baltimore MD,

Eleanor Wilson: EWilson@ihv.umaryland.edu
Institute of Human Virology, University of Maryland School of Medicine, Baltimore MD,

Bhawna Poonia: bpoonia@ihv.umaryland.edu
Institute of Human Virology, University of Maryland School of Medicine, Baltimore MD
Lydia Tang: LydiaTang@ihv.umaryland.edu
Institute of Human Virology, University of Maryland School of Medicine, Baltimore MD,

Anu Osinusi: anu.osinusi@gilead.com
Gilead Sciences Inc., Foster City, CA.

Anita Kohli: Anita.Kohli@DignityHealth.org
Creighton University School of Medicine, St Joseph's Hospital, Phoenix, Arizona

Corresponding author: Dr. Shyam Kottilil (SKottilil@ihv.umaryland.edu)
Institute of Human Virology
725 West Lombard Street
Baltimore, MD 21201
Phone: (410) 706 8927
Fax: (410) 706 3243
Email: SKottilil@ihv.umaryland.edu

Short Title: HCV specific immunity and SVR

Abstract

Background: Treatment for chronic hepatitis C virus (HCV) infection has rapidly evolved into interferon-free directly acting antiviral regimens (DAA) that result in high sustained-virologic-response. DAAs primarily work by suppressing HCV replication and rely less on the immune system than interferon-based therapies. However it is unclear whether the immune system recovers with suppression of HCV replication and contributes to HCV clearance with DAA therapy. We previously demonstrated HCV clearance is associated with increased HCV-specific-immunity in CHCV-GT-1 infected patients during treatment with sofosbuvir (SOF)+ribavirin (RBV). Here, we aimed to analyze changes in HCV-specific immunologic responses associated with viral clearance with combination DAA therapy of SOF+ledipasvir (LDV) for 12 weeks in CHCV-GT1 (N=14) patients who relapsed without augmentation of HCV-specific-immunity during treatment with SOF+RBV. **Methods:** Phenotypic and functional changes within the T-cell compartment of PBMCs pre- and post-treatment were analyzed. **Results:** Retreatment of relapsers with LDV/SOF resulted in all patients attaining SVR_{12}. Suppression of HCV was associated with a decline in T-cell exhaustion markers (CD57; Tim3; PD1) along with augmented of HCV-specific T-cell IFN-gamma responses post-treatment. **Conclusions:** Addition of LDV to SOF was associated with augmentation of HCV-specific-immunity and SVR in patients who previously failed SOF+RBV therapy without increased immunity. These findings demonstrate a novel effect of DAA in inducing host immune responses to aid HCV clearance and achieve SVR.

Keywords: Hepatitis C, Antiviral Therapy, Cellular Immunity, Immune response

Abbreviations:
DAA: directly acting antiviral agents;, SVR: sustained virologic response, CHCV GT-1: Chronic hepatitis C virus (HCV) genotype 1, SOF: sofosbuvir (SOF) , RBV: ribavirin, LDV: ledipasvir, EOT: End of treatment, PD-1: Programmed cell death 1 (PD-1)), PE-Cy7 , CTLA-4: cytotoxic T lymphocyte antigen 4 , TIM-3: T cell immunoglobulin and mucin protein 3, IFN: Interferon, PBMCs: Peripheral blood mononuclear cells, PHA: Phytohaemagglutin , DMSO: Dimethyl sulfoxide , CFSE: Carboxyfluorescein succinimidyl ester , IL2: Interleukin-2, IFN-G: Interferon gamma, TNF-A: Tumor Necrosis Factor-alpha

Introduction

Chronic hepatitis C virus (HCV) infection is a major cause of chronic liver disease and hepatocellular carcinoma and the leading indication for liver transplantation in Western countries (1,2). Chronic HCV infection is characterized by impaired or reduced functional HCV-specific T cells, with a concomitant increase in the number of exhausted T cells, thought to be an effect of the constant presence of the viral antigens (3-7). Recent advances in the development of combination therapy of HCV-specific directly acting antivirals (DAAs) with improved efficacy, better tolerability, and shorter treatment duration have revolutionized HCV therapy (8,9). In 2013, the U.S. Food and Drug Administration approved sofosbuvir (SOF) plus ribavirin (RBV) as the first IFN-free treatment of HCV genotype 2 or 3 and for patients with genotype 1 (GT-1) who are IFN-ineligible (10). DAAs act to suppress HCV replication, by blocking different stages of the HCV life cycle (11). A previous study by Martin et al reported specific restoration of proliferative HCV-specific $CD8^+$T cells during interferon-free therapy in patients who achieved SVR compared to patients with treatment failure (12). Barrett et al also evaluated immune correlates associated with SVR using IFN-free DAA therapy with SOF and RBV and reported that HCV clearance was associated with an augmentation of HCV-specific $CD8^+$ T-cell responses and decreased immune exhaustion markers with improved HCV specific immunity in chronic HCV GT-1 infected patients who attained SVR (13). However, SOF plus RBV therapy was associated with relapse in 30% of patients and these patients lacked enhancement of HCV-specific immune responses despite suppression of HCV replication by DAA therapy. All these previous studies suggest that HCV replication specifically depresses HCV-specific immune responses and that the augmentation of HCV-specific T cell responses seen with DAA therapy aids virologic response.

Combination therapy of SOF combined with ledipasvir (LDV, an NS5A inhibitor) for 12 weeks demonstrated high SVR rates (95% to 99%) in treatment-naive patients with HCV GT-1 infection (14). We have demonstrated that 14 HCV GT-1 patients who had virologic relapse after treatment with SOF plus RBV for 24 weeks in the SPARE study were able to be successfully re-treated with SOF plus LDV for 12 weeks (15). Therefore, in the present study, we hypothesize that the retreatment of these relapsers with SOF plus LDV lead to a potent suppression of HCV replication, reversal of T cell exhaustion, and augmentation of HCV-specific protective immunity with viral clearance. We aimed to evaluate the HCV-specific immune responses in these patients and specifically investigated the phenotypic and functional changes in peripheral blood T cells pre- and post-treatment to identify immune responses associated with viral clearance.

Materials and Methods

Participants

Patients were enrolled in a single center, non-randomized controlled trial conducted at the Clinical Research Center of the National Institutes of Health (NIH), Bethesda, MD, USA. Fourteen HCV GT-1 patients who had relapsed from previous treatment with SOF plus RBV for 24 weeks in the NIAID SPARE protocol (NCT# NCT01441180) were enrolled in a phase 2a open label NIAID SYNERGY study (NCT01805882) and were treated with SOF and LDV for 12 weeks. The NIAID Institutional Review Board approved this study and written informed consent was obtained from all participants. The primary study end point was the proportion of participants with undetectable HCV viral load 12 weeks after treatment completion (SVR_{12}) was reported elsewhere (11). Immunological studies were performed pre-and post-treatment at SVR.

Viral load measurement

Plasma HCV RNA levels were measured using the real-time HCV assay (Abbott Molecular), with a lower limit of quantification of 12 IU/mL and a lower limit of detection of 3 IU/ml. The Abbott assay was used to measure HCV RNA levels in all participants. Plasma HCV RNA levels were also measured using the COBAS TaqMan HCV RNA assay, version 1.0 (Roche), with a lower limit of quantification of 43 IU/mL and a lower limit of detection of 12 IU/ml.

PBMC isolation

Peripheral blood was collected in heparinized tubes, and PBMC were separated by Ficoll-Paque (GE Health Care Life Sciences) density centrifugation. Cells were counted by trypan blue exclusion and stored in liquid nitrogen until use.

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.

Immunophenotyping

Multicolor flow cytometry analyses were performed on thawed PBMCs that had been stored in liquid nitrogen. Pre- and post-treatment, T-cell phenotyping was performed to assess the expression of exhaustion markers by using the following fluorescently labeled antibodies: APC-Cy7 anti-CD3 (BD Biosciences), FITC anti-CD4 (BD Biosciences),

Horizon V500 anti-CD8 (BD Biosciences,), eFluor450 anti-CD57 (eBioscience), PE anti-programmed cell death 1 (PD-1) (Biolegend), APC anti- T cell immunoglobulin and mucin protein 3 (Tim-3) (eBioscience), PE-Cy7 anti-cytotoxic T lymphocyte antigen 4 (CTLA-4) (Biolegend). Cells were acquired on a BDFACS Canto II flow cytometer and data were analyzed by Flow Jo version 9.7.7 software (Tree Star, Inc.).

IFN-gamma ELISpot

HCV-specific T cell functions were evaluated by quantification of HCV-specific IFN-γ-production by standard ELISPOT assay (BD Biosciences). Cryopreserved PBMC were thawed and suspended in (R10) RPMI 1640 medium (Gibco, Life technologies) supplemented with FBS (10%) (Gibco, Life technologies), HEPES Buffer (Gibco) (10mM), L-glutamine (Gibco) (2mM) and penicillin-streptomycin (Gibco) (20 U/mL). Briefly, 96 well ELISPOT plates were coated with (5 μg/ml) anti-IFN-γbiotinylated capture antibody and incubated overnight at 4°C. Plates were then blocked using lymphocyte medium. PBMCs were allowed to rest for 6 hours at 37°C and then plated at $1x10^6$ cells per well with either phytohaemagglutin (PHA) as a positive control (5 μg/ml), dimethyl sulfoxide (DMSO) as a negative control (0.05%), or pooled HCV Genotype 1a or 1b peptides at the final concentration of 3 μg/ml. All cultures were performed in duplicate. After incubating for 12 hours at 37°C in 5% CO_2, cells were removed, and plates were treated 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.

12-color flow cytometry assay to assess HCV responsive T cell polyfunctionality

Here, we have used the comprehensive peptide approach and CFSE dilution to identify HCV responsive T cells. PBMC from 20 individuals (12 SVR, 8 relapsers) were assessed simultaneously for phenotype and function via a flow cytometric assay (Supplementary figure 1). Viable cells with CFSE dilution after 48-hour incubation with HCV peptides were assessed for cytokine production and exhaustion marker expression. PBMC were washed with PBS, incubated with 2.5μM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) at 37°C for 6 minutes, quenched with PBS + 5% FCS, and washed three times with lymphocyte medium. Cells were then incubated at $5x10^6$ in 1.2 mL with either pools of overlapping HCV peptides spanning the entire proteome (3μg/mL/peptide) or DMSO (1%) and NH_4OH (0.7mM) as a vehicle control along with recombinant human IL-2 (20IU/ml). Brefeldin A and monensin were added at 36 hours to block the Golgi apparatus and allow intracellular cytokine accumulation. After 48 hrs of incubation, PBMCs were harvested and stained with LIVE/DEAD Near-IR (Invitrogen) for 30 minutes on ice. Cells were then stained for surface antibodies and after a wash, permeabilized and stained with intracellular antibodies. Stained cells were acquired on a BD FACS Aria and data files were analyzed by Flow Jo version 9.7.7 software (Tree Star, Inc.). CFSE low HCV responsive cells were assessed for phenotype and function using the following antibodies Alexa Fluor 700 anti-CD3, PerCP-Cy5.5 anti-CD4, Brilliant Violet 510 anti- CD8, APC anti-Tim-3, PE-Cy5 anti-CTLA4, Brilliant Violet 785 anti- PD1, eFluor 450 anti- CD57, PE anti-IFN-γ, Brilliant Violet 605 anti-IL-2, PE-Cy7 anti- TNF-α. Gating strategy used to determine the HCV specific cytokine production is described in Supplementary figure 2.

Cytokine assay

To estimate secreted cytokine production, 100 μl of supernatant was collected at 36 hours and frozen at -80C until evaluated for cytokine secretion by Meso Scale Discovery technology, using a Human TH1/TH2 10-plex ultra-sensitive kit (Cat No. K15010C-1). Kits were used according to the manufacturer's instructions. Samples were tested in duplicate.

Statistical analysis

Changes in the percentage of T cell subsets expressing exhaustion markers pre- and post- treatment was analyzed using paired Wilcoxon signed-rank test. Changes in the number of IFN-γ spots in ELISPOT assay was assessed by using nonparametric Wilcoxon matched pairs signed-rank test. Changes in polyfunctional T cells pre- and post-treatment was calculated using Wilcoxon matched pairs signed-rank test. All the statistical analyses were done with Graph pad prism version 6. P values less than 0.05 were considered as significant.

Results:

Patient characteristics:

Fourteen patients who had relapsed after 24 weeks of treatment with SOF plus RBV in the NIAID SPARE study were retreated with SOF plus LDV for 12 weeks in the phase 2a, open-label NIAID SYNERGY study. All fourteen patients were included in the immunological studies. Most of the patients were male (M: F; 13:1), over 50 years old and African American with an unfavorable interleukin-28B non-CC genotype (Table 1). The majority of the individuals were infected with HCV genotype 1a, had high baseline HCV viral loads (>800 000 IU/mL), increased body mass index (>30 kg/m2), and

Histology Activity Index fibrosis score of 3 or 4. All 14 patients retreated with SOF plus LDV achieved SVR 12 weeks after the completion of treatment as previously reported (14).

Decline in Exhausted T cells with SVR at post treatment

We examined the frequency of peripheral T cells with an exhausted phenotype in patients undergoing retreatment. Both the $CD4^+$ and $CD8^+$ T cell compartments showed similar trends. The frequency of PD-1^+ (18.99±1.4 vs. 14.2±1.8; p=0.001), Tim-3^+ (68.5±11vs. 51±12; p=0.04), $CD57^+$ (9.3±1.7 vs. 6.3±1.6; p=0.04) on $CD4^+$ T cells, PD-1^+ (17.24±1.8 vs. 12.67±1.5; p=0.003), Tim-3^+ (72.3±11 vs. 55±12; p=0.04), $CD57^+$(40.7±3.6 vs. 33.7±3.1; p=0.002) on $CD8^+$ T cells decreased rapidly with treatment (Figure 1). Thus SVR following retreatment of relapsers with the combination therapy of SOF plus LDV was associated with a decline in peripheral exhausted T cells post-treatment.

Augmentation of HCV specific T cell responses with increased polyfunctionality post-treatment

In order to investigate whether this improved T-cell phenotype was associated with enhanced function, we evaluated the polyfunctionality of HCV-specific T cells. To further ascertain the correlates of HCV viral suppression, HCV-specific immunity was analyzed by stimulating the PBMC *in vitro* with a panel of HCV peptides spanning the entire HCV genome via intracellular cytokine staining, ELISPOT and ELISA (IL2, IFN-γ and TNF-α) pre- and post-treatment. We observed improved HCV-specific responses post-treatment compared to baseline [Intracellular cytokine assay. Figure 2 (A-B): CFSE low

HCV-responsive cells: (0.97± 0.29 vs. 5.5±1.5 p=0.02); HCV-peptide-specific IFN-γ secreting cells: (26.6± 9.6 vs. 34.15±10.5 p=0.06); HCV peptide specific TNF-α secreting cells: (7.05± 1.3 vs. 10.9±2.1 p=0.03); HCV peptide specific IL-2 secreting cells: (21.6± 5.3 vs. 29.5±4.9 p=0.03)]; (E) IFN-γ ELISPOT: Number of IFN-γ spots/million cells: (18± 4 vs. 39 ±8 p=0.04) (F) ELISA: HCV specific IFN–γ: 45± 5 vs. 140±20 p=0.01).

To analyze the HCV-specific T cell polyfunctional responses, we assessed the changes in the ability of T cells stimulated with HCV peptides to secrete more than one cytokine pre- and post-treatment with LDV/SOF. Figure 3 illustrates the increased number of HCV responsive cells producing cytokines pre- vs. post-treatment. All the three cytokines: (50 ± 9% vs. 67± 10% p=0.02) post-treatment compared to pre-treatment.

Declined expression of exhaustion markers PD-1, Tim-3, and CD57 on HCV-specific T cells

We further evaluated whether improved polyfunctionality of HCV-specific T cells was due to a decline in the exhausted phenotype by correlating changes in exhaustion markers and functionality. The distribution of exhaustion markers HCV-responsive cells was assessed pre- and post-treatment. Post treatment, the detection of HCV-specific $CD57^+$ (9.3 ± 1.4 % vs. 6.3± 1.8% p=0.02), $PD1^+$ (18.9±1.8 % VS. 14.2±1.5% p=0.03), and $Tim\text{-}3^+$ (68.5±10.2 % VS. 51±9.3 %p=0.03) cells declined (Fig.4a). The majority of the elicited cytokine responses were found to be produced by cells negative for the exhaustion markers CD57, PD-1, or Tim-3 or, proving the fact that exhausted T cells have lost their functionality and could not produce any cytokines (Fig. 4 b).

Discussion:

In this study we demonstrated that patients who previously relapsed after SOF and RBV without augmentation of $CD8^+$ T cell responses have a significant increase in HCV-specific T cell response and are able to attain SVR when retreated with SOF and LDV for 12 weeks. This is possibly due to the addition of LDV to SOF resulting in potentiation of HCV-specific immune responses in $CD8^+$ T cells aiding achievement of SVR. It is also plausible that more potent DAA therapy, leading to complete suppression of HCV replication, may reverse immune exhaustion, resulting in enhancement of HCV-specific immune responses. Our study suggests that host immunity does play an important role in aiding HCV clearance during treatment with combination DAA-based therapy.

Previously, several studies have demonstrated that host immunity plays a major role in resolution of acute HCV infection. In this regard, studies have established that during acute HCV infection, viral clearance is correlated with vigorous, sustained and multi-epitope-specific $CD8^+$ T-cell responses (16, 17,18). Chronic HCV infection is associated with a lower frequency of HCV-specific $CD8^+$ T cells characterized by so-called stunned phenotype with impaired proliferation, IFN-γ production, and cytotoxicity (19, 20,21). It has also been demonstrated that HCV infection is associated with an exhausted phenotype, which may impair the ability of the host to mount an effective immune response against HCV (22). Numerous studies have evaluated the effect of HCV therapy in the reconstitution of HCV-specific immunity. Studies have shown that SVR is associated with increased HCV-specific responses, but this was after IFN-based combination therapy for HCV (23,24,25). Since IFN-α itself leads to host immune enhancement, it is difficult to distinguish the overall effect of IFN-α from SVR. The use of DAAs, without IFN, offers us a unique opportunity to examine the effects of HCV therapy

on the immune system and identify components of host immunity that aid in HCV clearance to attain SVR. In this regard, Barrett et al also demonstrated that during treatment with IFN-free DAA therapy using SOF and RBV, HCV clearance was associated with increased HCV-specific immunity in chronic HCV GT-1 infected patients (13). However, treatment with SOF plus RBV therapy was associated with relapse in about 30% of patients. In this study, we asked if addition of LDV would further enhance HCV-specific immunity of CD8+ T cells in patients who had relapsed after single DAA therapy, SOF and RBV, therapy and is this augmentation of HCV-specific immunity associated with SVR.

Our results showed that retreatment with combination DAA therapy with agents active against NS5A and NS5B is highly effective in inducing SVR with a concomitant increase in HCV-specific protective T-cell immunity in patients who had relapsed on SOF plus RBV. DAA therapy was associated with a profound decline in the proportion of circulating T cells expressing the exhaustion phenotype. Previous studies also suggested that, in patients with chronically evolving hepatitis, antigen-driven exhaustion may contribute to the failure to generate successful HCV-specific $CD8^+$ T cell responses. Exhaustion is mediated in part by the expression of receptors like PD-1, TIM-3, 2B4, and CTLA- 4 that deliver inhibitory signals to $CD8^+$ T cells upon engagement of their respective ligands (26,27). In vitro blockade of Tim-3, PD-1, and CTLA-4 is necessary to increase the frequency HCV-specific T cells, (28,29,30) and to rescue their in vitro function (31). Barrett et al also reported that HCV clearance was associated with decreased immune exhaustion markers in HCV GT-1-infected patients who attained SVR compared to those who relapsed during the treatment with SOF and RBV in NIAID SPARE trial. We therefore assessed the frequency of various exhaustion markers such

as Tim-3, PD-1, CD57 and CTLA-4 on peripheral blood T cells in patients with virologic relapse who were retreated with SOF plus LDV. Our results demonstrate that HCV suppression with the combination of SOF and LDV is associated with a significant decline or reversal of T cell exhaustion, illustrated by down regulation of inhibitory molecules such as PD-1, TIM-3, and CD57 in those patients.

When we further enumerated HCV-specific T cells that secrete IFN-γ in response to stimulation with HCV peptides using ELISPOT, we found that there was an enhancement in all treated patients. This is in contrast to what was observed with the same patients when they were treated with SOF and RBV and failed to achieve SVR. When we examined the polyfunctionality of T cells specific to HCV, we observed that HCV suppression by LDV/SOF enhanced the ability of T cells to secrete more than one cytokine when stimulated with HCV peptides. The majority of HCV-responsive T cells at baseline did not express all three cytokines such as IFN-γ, TNF-α, or IL-2. The number of cells expressing one, two or all three of these cytokines at the end of treatment was significantly higher. Increased levels of IFN-γ, IL-2 and TNF-α cytokines at the end of treatment compared to baseline suggest augmentation of the HCV-specific immune responses with viral suppression. Thus, after viral suppression HCV-specific T cell polyfunctional responses were increased and may have contributed to HCV clearance, in contrast to the lack of a polyfunctional response to HCV antigens in the same patients when initially treated with SOF and RBV without achieving SVR.

Finally, we demonstrated that the enhancement of HCV-specific responses in $CD8^+$ T cell compartment is a direct result of reduced expression of exhaustive markers on these cells. T cells post-treatment producing one or more cytokines expressed low levels of

exhaustion markers. This correlation succinctly connects the intricate interplay between HCV and the host system as unraveled by DAA therapy. Thus, the addition of LDV, an NS5A inhibitor to SOF is associated with augmentation of HCV-specific immunity and SVR in patients who previously failed to produce HCV-specific immune responses with SOF and RBV therapy and relapsed. These findings demonstrate a novel effect of DAAs in inducing a host response aiding HCV clearance and achieving SVR.

There are two major changes in DAA therapy between the SPARE and the SYNERGY studies. One was the addition of a second potent DAA to SOF instead of RBV. It is likely that potent suppression of HCV with two DAAs were required to achieve host immune recovery and SVR in these patients. Second, the specific role of LDV on HCV replication and host immunity, which were not completely evaluated *in vivo* prior to this study. These findings suggest a novel effect of DAAs in inducing a host response that aids HCV clearance and SVR achievement.

In conclusion, DAA therapy is effective in suppressing HCV replication and may allow for HCV clearance and SVR. However, augmentation of HCV-specific immunity is also associated with successful clearance of HCV. Monitoring for reconstitution of HCV-specific responses may allow for more accurate prediction of those who will go on to successfully clear HCV and inform future strategies to treat HCV, including shorter duration of DAA therapy or concomitant vaccination to aid in the development of protective immunity.

Acknowledgements: We would like to thank all the patients who participated in this study. Data from this study was partly presented as a poster in 66th Annual Meeting of the American Association for the Study of Liver Diseases: The Liver Meeting. San Francisco, CA, 13–17 November 2015. SS got the travel and best paper award in the Asian Pacific

Association for the Study of Liver Diseases, Single Topic conference on HCV, 18th-20th December, New Delhi, India.

Financial Support:
Division of Intramural Research, National Institute of Allergy and Infectious Diseases supported this study.
***Ethical approval*:** The NIAID Institutional Review Board approved this study and is in accordance with Declaration of Helsinki 1975.
Conflict of Interest: All the authors declare that they have no conflicts of interest to disclose
Informed consent: All patients provided written informed consent to participate in the study

Author Contributions: **SK** and **SS** had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. **SS** Performed the experiments; data analysis and interpretation; preparation of the manuscript draft and figures **EW and BP:** Revision of the manuscript for important intellectual content. **LD AO and AK**: Revision of the manuscript. **SK:** Study concept and design, infrastructure support, critical revision of the manuscript for important intellectual content.

References

1. National Institutes of Health Consensus Development Conference Statement: Management of hepatitis C 2002 (June 10-12, 2002). *Gastroenterology.* 2002; 123(6): 2082-99
2. Brown RS. Hepatitis C and liver transplantation. *Nature* 2005; 436:973–978.
3. Raghuraman S, Park H, Osburn WO et.al. Spontaneous clearance of chronic hepatitis C virus infection is associated with appearance of neutralizing antibodies and reversal of T-cell exhaustion. *The Journal of infectious diseases* 2012; 205(5): 763-771.
4. Bowen DG, Walker CM. Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature* 2005; 436(7053): 946-952.
5. Wedemeyer H, He XS, Nascimbeni M *et al*. Impaired effector function of hepatitis C virus-specific $CD8^+$ T cells in chronic hepatitis C virus infection. *J Immunol* 2002; 169: 3447–3458.
6. Semmo N, Day CL, Ward SM *et al*. Preferential loss of IL-2-secreting $CD4^+$ T helper cells in chronic HCV infection. *Hepatology* 2005; 41: 1019–1028.
7. Maini MK, Boni C, Lee CK et al. The role of virus-specific CD8+ cells in liver damage and viral control during persistent hepatitis B virus infection. *J Exp Med* 2000; 191: 1269–1280
8. Afdhal N, Reddy KR, Nelson DR et al. Ledipasvir and sofosbuvir for previously treated HCV genotype 1 infection. *N Engl J Med* 2014; 370:1483–1493.
9. Lawitz E, Poordad FF, Pang PS et al. Sofosbuvir and ledipasvir fixed-dose combination with and without ribavirin in treatment-naive and previously treated patients with genotype 1 hepatitis C virus infection (LONESTAR): an open-label, randomized, phase 2 trial. *Lancet.* 2014; 383:515-23.
10. FDA. Soldavi US prescribing information. 2013.

http://www.accessdata.fda.gov/drugsatfda docs/label/2013/204671s000lbl.pdf.
11. Poordad F, Dieterich D. Treating hepatitis C: current standard of care and emerging direct-acting antiviral agents. *J Viral Hepatitis* 2012; 19:449–464.
12. Martin B, Hennecke N, Lohmann V, Kayser A, Neumann-Haefelin C, Kukolj G, et al. Restoration of HCV-specific CD8+ T-cell function by interferon-free therapy. J Hepatol 2014; 61:538–543.
13. Barrett L., Shivasabesan G., Wang C. et.al. Altered HCV Specific T cell immunity very early in interferon free HCV DAA therapy. [Abstract] *Journal of Hepatology,* Volume 58, Supplement1:(S1)
14. Afdhal N, Zeuzem S, Chojkier M et al. Ledipasvir and sofosbuvir for untreated HCV genotype 1 infection. *N Engl J Med* 2014; 370:1889–1898
15. Osinusi A, Kohli A, Marti MM et.al. Re-treatment of Chronic Hepatitis C Virus Genotype 1 Infection After Relapse: An Open-Label Pilot Study. *Ann Intern Med.* 2014; 161(9): 634-638.
16. Schulze ZurWiesch J, Ciuffreda D, Lewis-Ximenez L et al. Broadly directed virus-specific CD4+ T cell responses are primed during acute hepatitis C infection, but rapidly disappear from human blood with viral persistence. *J Exp Med*. 2012; 209(1): 61-75
17. Lauer GM, Ouchi K, Chung RT et al. Comprehensive analysis of CD8 (+)-T-cell responses against hepatitis C virus reveals multiple unpredicted specificities. *Journal of virology* 2002, 76(12): 6104-6113.
18. Gruener NH, Lechner F, Jung MC et al. Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *Journal of virology* 2001, 75(12): 5550-5558.
19. Lechner F, Wong DK, Dunbar PR et al. Analysis of successful immune responses in persons infected with hepatitis C virus. *The Journal of experimental medicine* 2000; 191(9): 1499-1512.
20. Thimme R, Oldach D, Chang KM et.al. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med* 2001; 194: 1395–1406.
21. Urbani S, Amadei B, Fisicaro et al. Outcome of acute hepatitis C is related to virus-specific CD4 function and maturation of antiviral memory CD8 responses. *Hepatology* 2006; 44:126–139.
22. Cooper S, Erickson AL, Adams EJ et al. Analysis of a successful immune response against hepatitis C virus. *Immunity* 1999; 10:439-449.
23. Larrubia JR, Lokhande MU, Moreno-Cubero E et.al . HCV-specific CD8+ cell detection at week 12 of chronic hepatitis C treatment with PEG-interferon-α2b/ribavirin correlates with infection resolution. *Cell Immunol*. 2013; 286(1-2): 31-38.
24. Missale G, Pilli M, Zerbini A et.al Lack of full CD8 functional restoration after antiviral treatment for acute and chronic hepatitis C virus infection. *Gut.* 2012; 61(7): 1076-1084.
25. Larrubia JR, Moreno-Cubero E, Miquel J et.al. Hepatitis C virus-specific cytotoxic T cell response restoration after treatment-induced hepatitis C virus control. *World J Gastroenterol*. 2015; 21(12): 3480-3491.
26. Kasprowicz V, Schulze ZurWiesch J, Kuntzen T et al. High level of PD-1 expression on hepatitis C virus (HCV)-specific CD8+ and CD4+ T cells during

acute HCV infection, irrespective of clinical outcome. *Journal of virology* 2008; 82(6): 3154-3160.
27. McMahan RH, Golden-Mason L, Nishimura MI et al. Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity. *The Journal of clinical investigation* 2010; 120(12): 4546-4557.
28. Golden-Mason L, Palmer B, Klarquist J et.al. Upregulation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8+ T cells associated with reversible immune dysfunction. *Journal of virology* 2007; 81(17): 9249-9258.
29. Fuller MJ, Callendret B, Zhu B et al. Immunotherapy of chronic hepatitis C virus infection with antibodies against programmed cell death-1 (PD-1). *Proc Natl Acad Sci* U S A. 2013; 110(37): 15001-15006.
30. Radziewicz H, Ibegbu CC, Fernandez ML et al. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol.* 2007; 81:2545–2553.
31. 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 Pathog*. 2009; 5(2): e1000313.

Patient Characteristics	Sofosbuvir+Ledipasvir (n=14)
Median Age (IQR)	59(48-70)
Male Sex, n (%)	13(93%)
Median BMI (range), Kg/m^2	28.5(19.8-38.4)
Black race, n (%)	13 (93)
Interleukin- 28B genotype, n (%)	
CC	2(14)
TT	7(50)
CT	5(36)
Histological Activity index score fibrosis score, n (%)	
0-1	7(50)
3-4	7(50)
HCV Genotype 1 subtype, n (%)	
1a	8(57)
1b	6(43)
Median baseline HCV RNA level (range) log_{10} IU/ml, n (%)	6.31(5.50-6.76)

Table 1. **Baseline Demographics and Clinical Characteristics of Study Participants**

Abbreviations: BMI =body mass index; GT-1 = Genotype 1; HCV = Hepatitis C virus; IQR =Interquartile range

Figure legends

Figure 1(A-H)**:** ***Changes in T cell exhaustion markers with SVR.*** T-cell phenotyping was performed in 14 patients who had relapsed and were retreated with sofosbuvir plus ledipasvir at baseline and end of treatment, to assess the expression of exhaustion markers such as CD57, TIM3, PD1, CTLA-4 on $CD4^+$ and $CD8^+$ T cells. Data are expressed as (Mean ± SEM) of the percentage expression of these markers on $CD4^+$ and $CD8^+$ T cells. Changes in percentage of T cell subsets expressing exhaustion markers pre and post treatment was analyzed using paired Wilcoxon signed-rank test in Graph Pad Prism version 6. P value less than 0.05 was considered as significant.

Figure 2: ***Augmentation of HCV specific immunity post-treatment***

Cryopreserved PBMCs were thawed and stimulated with a panel of HCV peptides spanning the entire HCV genome and HCV-specific immunity was analyzed via intracellular cytokine staining (IL2, IFN-γ and TNF-α) (A-D) and IFN-γ ELISPOT. Changes in the number of IFN-γ spots in ELISPOT assay pre- and post-treatment were analyzed by using nonparametric Wilcoxon matched pairs signed-rank test. (E) ELISA: Changes in IFN-γ production at baseline and pre- and post-treatment.

Figure 3: ***Changes in HCV specific T cell polyfunctional responses pre- and post-treatment with viral suppression*** To analyze HCV specific T cell responses, 12 color flow cytometric assay was done along with CFSE assay. Cell population that express lower CFSE were defined as HCV responsive cells and intracellular cytokine production was assessed on those HCV responsive cells. To analyze the HCV-specific T cell polyfunctional responses, we assessed the changes in the ability of T cells stimulated with HCV peptides to secrete more than one cytokine pre- and post-treatment. Figure 3(a) illustrates the changes in the percentages of HCV responsive cells that are cytokine negative, producing IFN-γ, IL-2 and TNF-α respectively. Bars represent the mean ±

standard error. P<0.05 in paired Wilcoxon signed rank test was considered significant. 3(b) displays the changes in the percentages of HCV responsive cells producing all the three cytokines pre- vs. post-treatment or co-expressing IFN-γ, TNF-α and IL-2. Figure 3(c) illustrates the proportion of HCV responsive cells that are cytokine negative, producing IFN-γ, IL-2 and TNF-α pre- and post-treatment.

Figure 4: ***Exhaustion markers PD-1, Tim-3, CD57 expression on HCV specific T cells.*** Exhaustion marker distribution on HCV responsive cells was also assessed pre- and post- treatment. Figure 4(a) describes the changes in the expression of CD57, PD1 and Tim-3 on HCV responsive cells pre- and post-treatment. Figure 4(b) displays the exhaustion marker expression on HCV peptide responsive cells and describe that T cells expressing exhaustion markers were mostly negative for HCV specific cytokine production, as most of the cytokines are produced by $CD57^-$, $PD\text{-}1^-$ or $Tim\text{-}3^-$ non-exhausted T cells. Bars represent the mean ± standard error. P<0.05 in paired Wilcoxon signed rank test was considered significant.

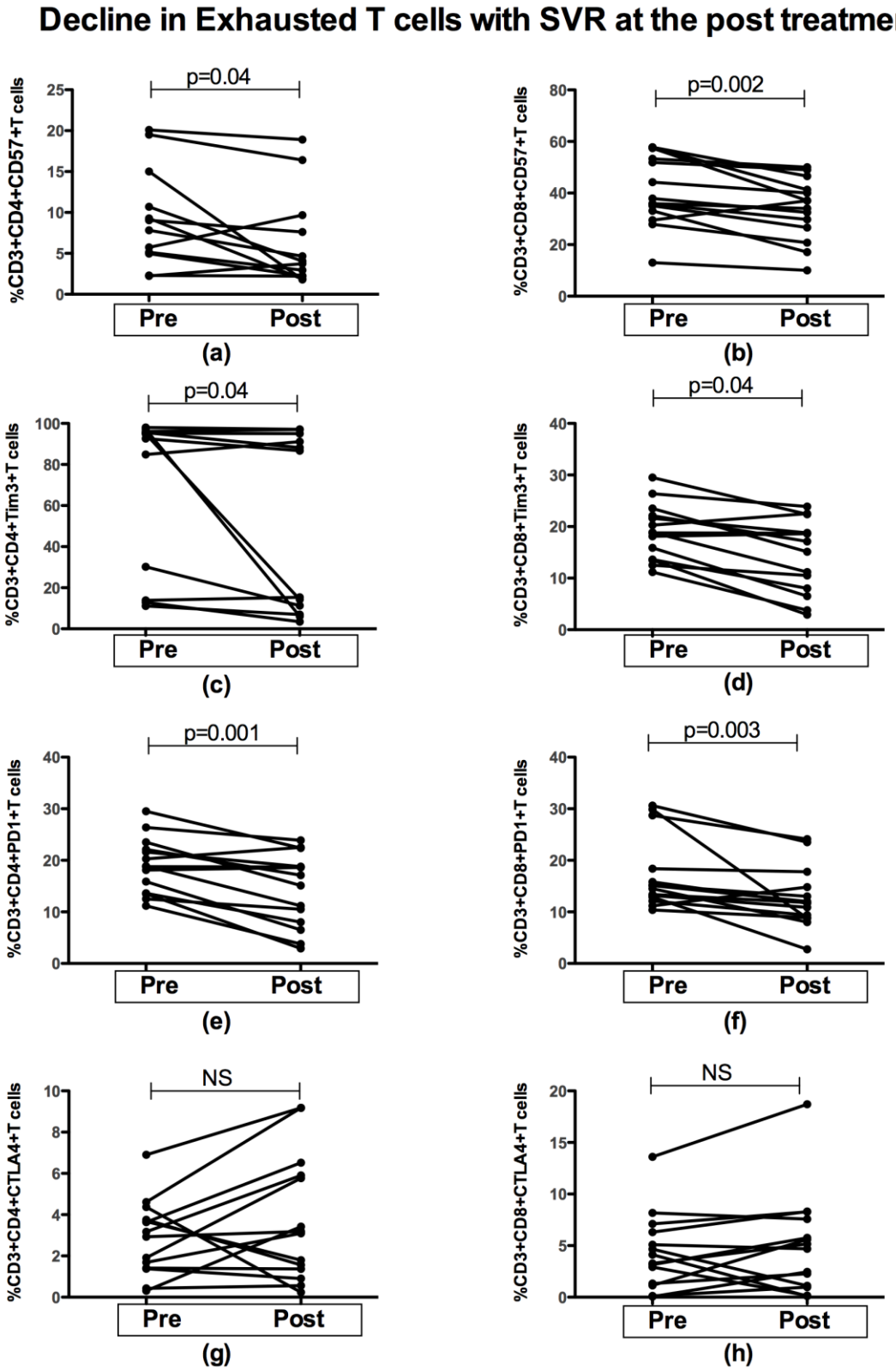


Figure.1

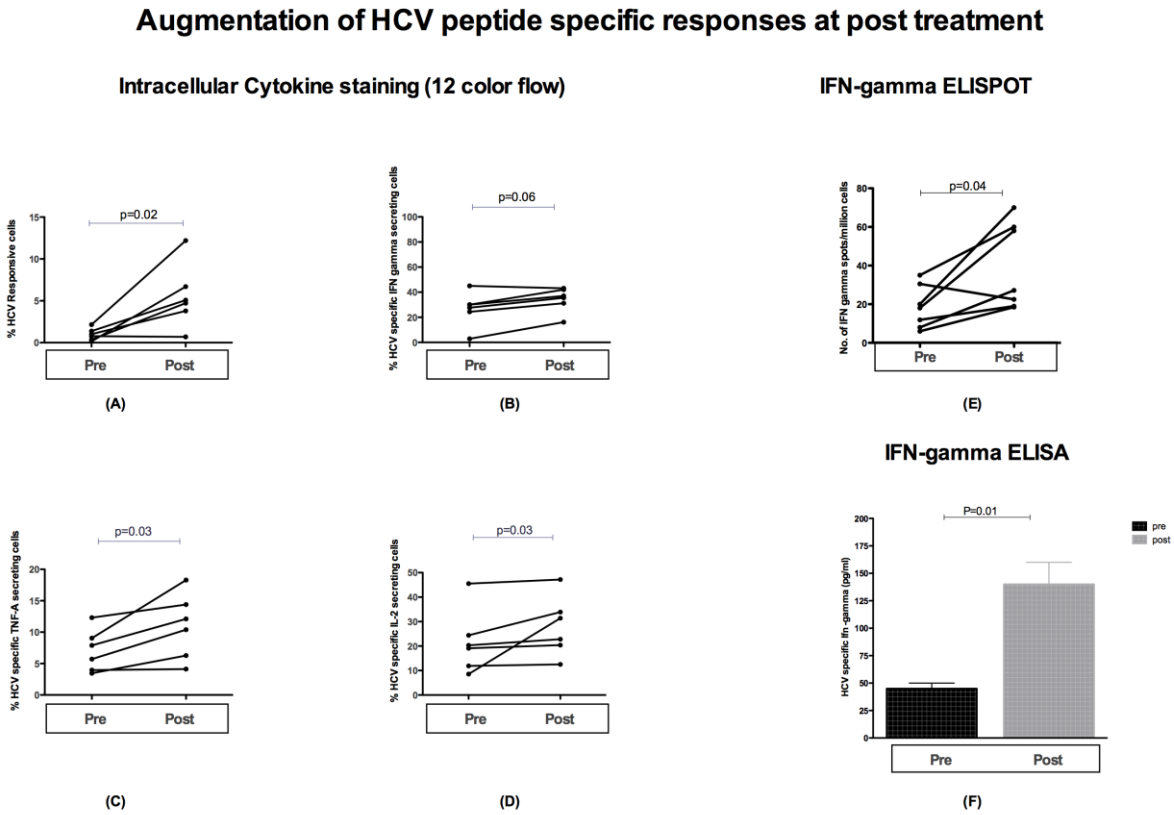
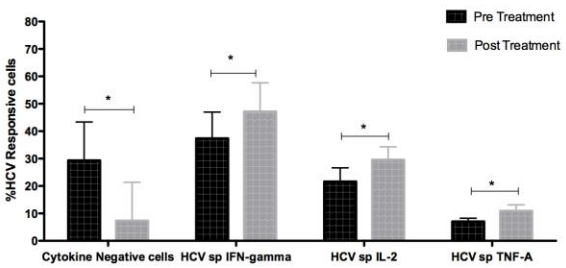
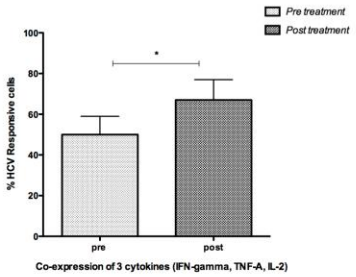


Figure 2

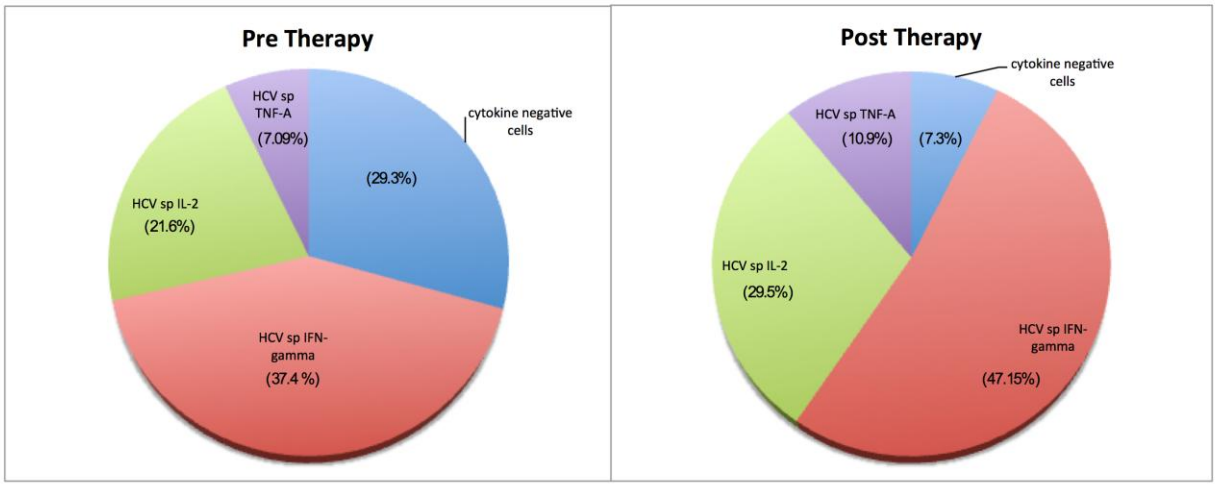
Increased polyfunctionality of HCV responsive cells at post treatment



(a)



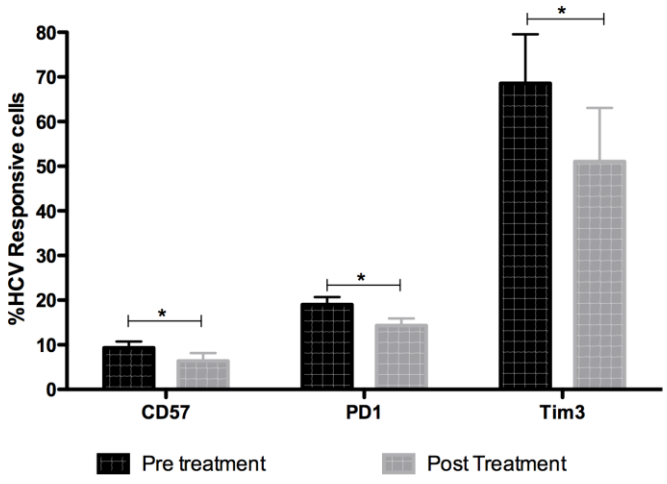
(b)



(c)

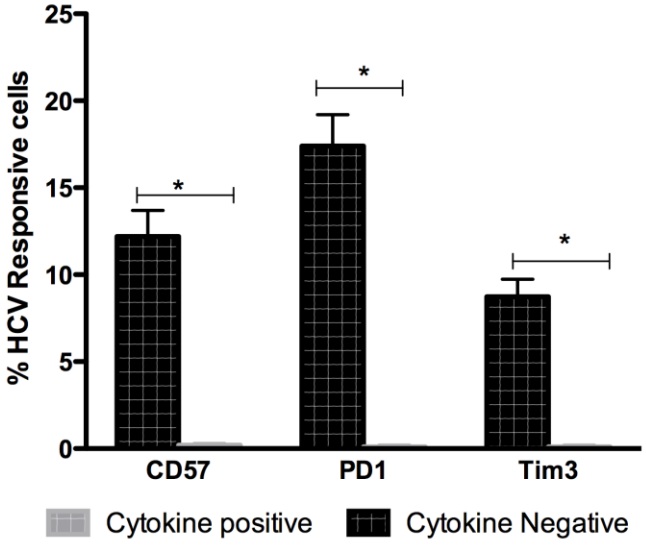
Figure 3

T cell exhaustion markers significanlty declined at post treatment



(a)

Exhaustion marker expression on HCV peptide responsive cells : Cytokine secreting cells less frequently express any exhaustion marker compared to non secreting cells



(b)

Figure 4

.