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Original Research

Chronic Viral Infection Compromises the Quality of Circulating Mucosal–Associated Invariant T Cells and Follicular T Helper Cells via Expression of Inhibitory Receptors

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Abstract

Background: Chronic viral infection results in impaired immune responses rendering viral persistence. Here, we compared the quality of T-cell responses among chronic hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV)-infected individuals by examining the levels of expression of selected immune activation and exhaustion molecules on circulating MAIT cells and Tfh cells. **Methods**: Cytokines were measured using a commercial Bio-plex Pro Human Cytokine Grp I Panel 17-plex kit (BioRad, Hercules, CA, USA). Inflammation was assessed by measuring an array of plasma cytokines, and phenotypic alterations in $CD4^+$ T cells including circulating Tfh cells, $CD8^+$ T cells, and TCR $iV\alpha7.2^+$ MAIT cells in chronic HBV, HCV, and HIV-infected patients and healthy controls. The cells were characterized based on markers pertaining to immune activation (CD69, ICOS, and CD27) proliferation (Ki67), cytokine production (TNF-α, IFN-γ) and exhaustion (PD-1). The cytokine levels and T cell phenotypes together with cell markers were correlated with surrogate markers of disease progression. **Results**: The activation marker CD69 was significantly increased in $CD4^{+hi}$ T cells, while $CD8^+$ MAIT cells producing IFN-γ were significantly increased in chronic HBV, HCV and HIV infections. Six cell phenotypes, viz., TNF-α^+CD4^{+lo} T cells, $CD69^+CD8^+$ T cells, $CD69^+CD4^+$ MAIT cells, PD-1^+CD4^{+hi} T cells, PD-1^+CD8^+ T cells, and $Ki67^+CD4^+$ MAIT cells, were independently associated with decelerating the plasma viral load (PVL). TNF-α levels showed a positive correlation with increase in cytokine levels and decrease in PVL. **Conclusion**: Chronic viral infection negatively impacts the quality of peripheral MAIT cells and Tfh cells via differential expression of both activating and inhibitory receptors.

Keywords: HBV; HIV; MAIT cells; PD-1; T cell exhaustion

1. Introduction

Chronic viral infections result in immune cell dysfunctions in the host [1], but often persist without inflicting any serious cell damage [2]. Many chronic viruses, especially the hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus 1 (HIV-1) in humans are adept at circumventing the host's immune responses, primarily by imposing the expression of co-inhibitory molecules to the advantage of the pathogen [3]. Given the non-cytopathic nature of HBV and HCV, the immune system is attributed to hepatocellular damage as well as viral clearance [4]. Inability to attain viral clearance and development of chronic HBV disease is suggestive of dysfunctional immune responses [5]. The expansion of regulatory T cells (Tregs), high antigen loads, anti-inflammatory cytokines, and biosignatures of exhaustion are the likely indications of dysfunctional HBV-specific responses [6]. Evasion of the host's immune surveillance augments active replication of chronic viruses. In addition, viral persistence also stems from clonal deletion of HBV-specific T cells and/or their functional insufficiency together with increased expression of signatures associated with immune activation, senescence, and exhaustion.

Chronic HCV infection leads to persistent upregulation of genes associated with innate immune activation leading to liver inflammation and consequently cirrhosis [7]. Exhausted T cells in concert with TNF-α and IFN-γ, are capable of driving non-specific immune responses in order to prolong the infection [8]. During HIV infection, it's clear that the virus dominates with the loss of immunological control over viral replication in treatment-naïve individuals. Exhausted virus-specific $CD8^+$ T cells progressively lose their ability to clear cellular reservoirs of viruses [9], due to chronic immune activation that results in functional immune exhaustion [10].

Mucosal-associated invariant T (MAIT) cells are a unique subset of innate-like T cells that link the innate and the adaptive immune systems [11]. Although MAIT cells comprise only ~5% of the peripheral T cell pool in adults, the cell population is enriched in up to ~40% in the liver [12]. MAIT cells play a paramount role in innate host defense responses against bacterial and viral infections [13,14]. Given the primary localization of MAIT cells is confined to the peripheral mucosal tissues, their functions are weakened (characterized by heightened expression of immune exhaustion markers, reduced cytokine production and cytotoxicity) in chronic viral infections, including HIV [15], HCV [16,17] and HIV-TB co-infections [18], the current research intends to generically and descriptively determine the quality of host immune responses in chronic HBV, HIV, and HCV infections. We investigated the role of immune activation and potentially compromised T-cell responses in the three different chronic viral infections by exploring conventional $CD4^+$ and $CD8^+$ T cells along with their counterpart follicular T helper cells (Tfh) and mucosal-associated invariant T cells (MAIT).

2. Materials and Methods

2.1 Ethics Approval

The cross-sectional case-control study was carried out in accordance with the guidelines of the International Conference on Harmonization Guidelines and the Declaration of Helsinki. The study protocols were reviewed by the Institutional Ethical Committee (IEC) of the Government Medical College, Theni, for necessary approval for the conduct of the research (Ref. No. 2544/ME1/18 and Ref. No. 1515/MEIII/21). Prior approval was also obtained from the Tamil Nadu State AIDS Control Society, Chennai, India (TANSACS Approval- 00529/TANSACS/M&E/2019 NACO-T-11020/08/2020_NACO) for the conduct of work. Institutional Biosafety Committee (IBSC) approval was secured (Ref. No.: CUTN/SLS/1st IBSC/2020/04). All the human subjects were adults and written consents were duly obtained from all the participants.

2.2 Subjects and Analytical Parameters

HBV-infected individuals with plasma HBsAg and anti-HBc positivity (n = 13), HCV-infected individuals as determined by anti-HCV (n = 8), HIV-infected individuals (as per the criteria of the National AIDS Control Organization (NACO), India) (n = 7), and healthy controls (HCs) (n = 10) were recruited into the cross-sectional study. The HIV-infected patients were on ART as per NACO recommendations (at least for over five years). Peripheral blood was obtained from all the participants by a trained phlebotomist. HCs were identified as individuals free from HBV, HCV, HIV, *Mycobacterium tuberculosis* infections as well as HBV/HIV co-infections.

Plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase (GGT), and alkaline phosphatase (ALP) levels were measured on a Semi-automatic Analyzer (Rapid Diagnostics Star 20, Hyderabad, India) using commercial kits procured from TRUEchemie, Brussels, Belgium; AST assay kit (Lot. No. A1921102; TRUEchemie, Brussels, Belgium) with the cut-off values set at 33 U/L, ALT assay kit (Lot. No. A1521101; TRUEchemie, Brussels, Belgium) with the cut-off values set at 35 U/L, GGT assay kit (Lot. No. G1221122; TRUEchemie, Brussels, Belgium), with the cut-off values set at 49 U/L (male) and 32 U/L (female), ALP assay kit (Lot. No. A1321111; TRUEchemie, Brussels, Belgium) with the cut-off values set at 141 U/L, respectively.

2.3 HIV Diagnosis and Absolute $CD4^+$ T-Cell Counts

HIV infection was diagnosed using the conventional three-kit method advocated by the NACO. According to the manufacturer's instructions, the rapid immunochromatographic tests Comb Aids-RS (Arkray Healthcare, Mumbai, India), VoXpress HIV-1/2 (Voxtur Bio, Mumbai, India), and Meriscreen HIV 1-2 WB (Mumbai, Merillife, India) were employed for diagnosing HIV infection. At inclusion, all HIV-positive individuals were receiving active antiretroviral therapy (ART). According to the manufacturer's instructions, anti-CD45-PE-Cy5 (Cat. No. 05-8405-02) and anti-CD4-PE (Cat. No. 05-8405-01) (Sysmex Partec GmbH, Gorlitz, Germany) fluorochrome-tagged antibodies were used for the immunophenotyping. For absolute $CD4^+$ T-cell counts, 2 mL of whole blood in EDTA tubes was used.

2.4 Plasma Viral Load

The Pathodetect™ (Mylab Discovery, Pune, India) quantitative Real-Time PCR was used to quantify the viral loads of HBV and HCV using an *in vitro* nucleic acid amplification assay on a QuantStudio 5 real-time PCR (Applied Biosystems, ThermoFisher Scientific, MA, USA). We determined HIV-1 viral load using the Abbott Real-time HIV-1 assay (Abbott, Abbot Park, IL, USA), via an *in vitro* reverse transcription-polymerase chain reaction assay with the sensitivity 40 copies/mL volume of the sample.

Table 1. Clinico-demographic characteristics of study participants.

Characteristics	Total	HBV	HCV	HIV	HC	*p* value
	(n = 38)	(n = 13)	(n = 8)	(n = 7)	(n = 10)	
Age, years	43 (32.5–58.0)	55 (33.3–67)	53.5 (45–65.5)	34 (32–42)	34 (28–42)	0.103
Sex, male (%)	24 (63.2%)	10 (76.9%)	6 (75%)	3 (42.9%)	5 (50%)	0.274
SGOT, U/L†	37 (27.1–66)	33.3 (21.4–75.2)	59.8 (40.7–68.2)	36.7 (30.7–48.2)	…	0.126
SGPT, U/L†	15.2 (10.5–26.2)	12.3 (5.4–13.4)	23.7 (17.8–47.8)	16.9 (12–21.6)	…	0.010*
GGT, U/L†	41.5 (18.4–93.9)	18.6 (13.4–71.2)	68.8 (37.6–117)	55.5 (33.1–93.4)	…	0.113
ALP, U/L†	58.4 (45.3–66.5)	52.7 (32.5–66.1)	63.7 (55.7–86.7)	59.5 (47.9–64)	…	0.188
PVL, copy/μL†	4.03 (1.63–4.64)	2.1 (1.46–2.5)	4.67 (1.9–4.78)	4.63 (4.6–6.66)	…	0.086
$CD4^+$ T cell count, cells/μL	…	…	…	333 (241–265)	…	…

All data reported as median and interquartile range (IQR) unless specified. n, numbers; %, percentages; IQR, interquartile range; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HC, healthy control; PVL, plasma viral load; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic-pyruvic transaminase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase.

* represents $p < 0.05$.

†The median (IQR) was calculated in HBV, HCV, and HIV-infected individuals only.

2.5 Luminex Bio-Plex Cytokine Array

Cytokines were measured using the Bio-plex Pro Human Cytokine Grp I Panel 17-plex kit (BioRad, Hercules, CA, USA) that quantifies the levels of MCP-1, G-CSF, GM-CSF, IL-7, IL-12 (p70), IL-1β, MIP-1β, TNF-α, CXCL8 (IL-8), IFN-γ, IL-6, IL-2, IL-4, IL-5, IL-13, IL-17 and IL-10 following the manufacturer's instructions.

2.6 Peripheral Blood Mononuclear Cells

Ten milliliters of peripheral blood were collected by venipuncture, and stored in lithium heparin BD Vacutainer (BD Biosciences, Franklin Lakes, NJ, USA) tubes at room temperature. PBMCs were extracted using a commercial Sepmate™ (Stemcell Technologies, Vancouver, Canada) by density gradient centrifugation. Cell viability was determined by 0.4% Trypan blue vital staining. Purified PBMCs were suspended in a Bambanker™ serum-free cell freezing medium (Nippon Genetics Europe GmbH, Duren, Germany) for storage at –80 °C, not more than 3 months before use in the experiments. PBMCs were thawed in a water bath at 37 °C before use in the experiments.

2.7 Flow Cytometry

Multi-Parametric Immunophenotyping

All antibodies were purchased from BD Pharmingen™ (BD Biosciences, San Jose, CA, USA) unless otherwise specified. Immunostaining was performed with one panel each for MAIT cells, Tfh cells, and along with various markers. The MAIT cell panel included allophycocyanin H7 (APC-H7)–conjugated anti–CD3, brilliant violet 510 (BV510) anti–CD4, fluorescein isothiocyanate (FITC)–conjugated anti–CD8, phycoerythrin (PE)–conjugated TCR iVα7.2, brilliant violet 421 (BV421) conjugated anti–CD56, peridinin chlorophyll protein (PerCP)-Cy5.5–conjugated anti–ICOS, Alexa 647 anti–PD-1, PE-Cy7 anti–CD69. The TFH cell panel was performed with APC H-7 conjugated anti–CD3, BV421-conjugated anti–CXCR5, Alexa647-conjugated anti–PD-1, BB515 conjugated anti–ICOS, PE–Cy7-conjugated anti–CD27.

2.8 Intracellular Cytokine Staining

Mononuclear cells were incubated with PMA (50 ng/mL) and ionomycin (500 ng/mL) or cultured in RPMI containing 10% FBS (R10) alone. Samples were incubated at a concentration of 10 μg/mL, and Golgi Plug (brefeldin A) and Golgi Stop (monensin) were included at 10 μg/mL. Samples were incubated for overnight at 37 °C in 5% CO_2 and then permeabilized using Fix & Perm reagents (BD Bioscience) and stained intracellularly with BV421–conjugated anti–IFN-γ and APC–conjugated anti–TNF-α. At the end of stimulation, cells were washed once with FACS wash (PBS containing 2% [vol/vol] FBS and 0.25% of sodium azide) and surface stained with anti-CD3, anti-TCR7.2 (3C10), anti-Ki67 (B56) anti-CD8 (SK1), anti-CD4 (OKT4). Cell stain at room temperature for 30 minutes. Cells were then fixed with Cytofix/Cytoperm (BD Pharmingen) for 20 minutes at 4 °C and washed with Perm wash (BD Pharmingen). Cells were then incubated for 30 minutes at 4 °C with antibodies specific to IFN-γ and TNF-α, washed once with Perm wash and once with FACS wash, and resuspended in PBS containing 1% formalin. Cells were acquired on a BD FACS Canto II Immunocytometric system. FlowJo for Windows, Ver.10.0.8 (FlowJo LLC, Ashland, OR, USA) was used to perform the analysis. At least 100,000 events were acquired for each sample.

2.9 Statistical Analyses

We examined the percentages and expression of biomarkers on distinct subsets of T cells, MAIT and Tfh cells between the four study groups. For multiple group comparisons, categorical variables were examined using

the Chi-square test of Fisher's Exact Test, while continuous variables were tested using non–parametric Kruskal–Wallis Test. If the p values were <0.05, four–way comparisons were made between the four patient groups using the Mann–Whitney Test. The Spearman's Rank correlation was used to compare the correlation between two continuous variables. The association between the surface markers, and functional markers and plasma viral load (PVL) were assessed using the linear regression model. $^{*}p < 0.05$, $^{**}<0.01$, $^{***}<0.001$, and $^{****}<0.0001$ were used to determine significance. GraphPad Prism Ver.6.0 software (GraphPad, La Jolla, CA, USA) was used to perform all the analyses.

3. Results

3.1 Clinico-Demographic Characteristics of Participants

The four groups, non-randomized study design consisted of 38 individuals. Thirteen subjects with chronic HBV infection who tested positive for HBsAg, anti-HBc as well as HBV DNA: Group 1 (G1), eight subjects with HCV RNA positive and anti-HCV positive; G2, seven subjects with HIV RNA positive, and 10 healthy controls (HC) (G4). The samples were collected between September and October of 2021. As per the analytical parameters, 54% HBV-infected individuals, 87% HCV-infected individuals, and 47.5% HIV-infected individuals were diagnosed with signs of liver injury, while 46% of the HBV-infected, 13% of the HCV-infected, and 52.5% of the HIV-infected participants were chronically infected without any underlying clinical or biochemical signs of liver injury (Table 1).

3.2 Activated T Cells with High PD-1 Expression was Observed in Chronic HBV, HCV and HIV-Infected Subjects

The flow cytometry gating strategy to delineate the $CD4^{+hi}$, $CD4^{+lo}$ and $CD8^{+}$ T cells is depicted in Fig. 1A. The $CD8^{+}$ T cell levels were significantly higher for HIV (Fig. 1B) as expected. There was negligible alteration in CD69, ICOS, or PD-1 (CD279)-expressing T cells between the different groups (Fig. 1C,D). CD69 was significantly increased on $CD4^{+hi}$ T cells in chronic HBV, HCV and HIV in comparison with HCs ($p < 0.05$, $p < 0.01$, and $p < 0.05$, respectively). The expression of co-stimulatory (ICOS) and co-inhibitory (PD-1) markers were significantly higher in $CD4^{+hi}$ T cells in HBV ($p < 0.05$), HCV-infected ($p < 0.01$) and HIV-infected ($p < 0.01$) individuals. Likewise, $CD69^{+}CD4^{+lo}$ T cell levels ($p < 0.01$) $PD\text{-}1^{+}CD4^{+lo}$ T cell levels and ($p < 0.001$) were significantly higher in all infected groups whereas the levels of $ICOS^{+}CD4^{+lo}$ T cells were only significantly higher in chronic HBV ($p < 0.01$) infection. $CD69^{+}CD8^{+}$ T cell levels were significantly increased in HBV ($p < 0.05$) and HCV-infected individuals ($p < 0.01$). Furthermore, all groups had significantly higher levels of $PD\text{-}1^{+}CD8^{+}$ T cells (Fig. 1C,D). Together, these data highlight that markers of T-cell activation coupled with exhaustion were increasingly expressed on T cells in chronic HBV, HCV and HIV infections.

3.3 Circulating MAIT and Tfh Cells Represented Activated Phenotypes Coupled with Higher PD-1 Expression in Chronic HBV, HCV, and HIV-Infected Subjects

Next, we set out to study the MAIT and Tfh cells across the different study groups with a pre-determined gating strategy (Fig. 2A). Total TCR $iV\alpha7.2^{+}$ MAIT cells were significantly lower in all the infected groups ($p < 0.01$) compared with HCs. The $CD4^{+}$MAIT cell levels were significantly increased in HCV and HBV groups ($p < 0.01$) but not in the HIV-infected group compared to HCs. The level of $CD8^{+}$MAIT cells ($p < 0.01$) as compared to HCs were higher in all the groups (Fig. 2B). The circulating Tfh cell levels in HIV-infected individuals were significantly higher ($p < 0.001$) in HIV infected group, but comparable in the HBV and HCV-infected groups (Fig. 2C). The $CD69^{+}CD4^{+}$MAIT cell levels were significantly increased in the HIV and HBV groups (<0.05) but not in the HCV-infected group. $PD\text{-}1^{+}CD4^{+}$MAIT cell levels were significantly elevated in all the infected groups (Fig. 2D). The $ICOS^{+}CD8^{+}$ MAIT cells were significantly decreased for HCV and HBV groups, and the $PD\text{-}1^{+}CD8^{+}$ T cell levels were highly significant in HIV-infected individuals with $p < 0.001$ (Fig. 2D). Spearman correlation of $CD4^{+}$ TCR $iV\alpha7.2$, $CD8^{+}$ TCR $iV\alpha7.2$ with CD69, ICOS, and PD-1 is presented in Fig. 2E. The functional markers for Tfh viz., CD27, ICOS and PD-1 were comparable between HBV, HCV and HCs. Nonetheless, PD-1 was highly elevated in the HIV-infected group suggesting that Tfh cells become activated (see Fig. 2F).

3.4 Significant Elevation in Cytokine-Producing T Cells during Chronic Viral Infection

Next, we compared the cytokine-producing ability of T cells with respect to $CD4^{+hi}$, $CD4^{+lo}$ and $CD8^{+}$ T cells. Our results indicates that there was a significant increase in the cytokine^{+} $CD4^{+}$ T cells and $CD8^{+}$ T cells during chronic viral infection when compared to HCs. The comparison analysis of $CD4^{+hi}$ IFN-γ was significant in HBV and HIV-infected individuals ($p < 0.0001$). $Ki67^{+}CD4^{+hi}$ was significantly higher in HIV-infected individuals ($p < 0.0001$). $CD4^{+lo}$TNF-α was significant in HCV-infected individuals ($p < 0.001$). $CD4^{+lo}$IFN-γ and Ki67 was highly significant in HIV ($p < 0.0001$) infection. $CD8^{+}$ IFN-γ and Ki67 had significance ($p < 0.0001$) in HBV and HCV-infected subjects, respectively (Fig. 3A,B).

Next, we also looked at the proliferating potential of T cells, which indicated that $Ki67^{+}$ T cells were higher in chronic viral infections as compared to HCs. Then, we examined the production of cytokines by the $CD4^{+}$ and $CD8^{+}$ MAIT cells. Both $CD4^{+}$ and $CD8^{+}$ MAIT cells expressed higher levels of TNF-α, and IFN-γ, together with higher proliferating ability as compared to HCs. The proliferating MAIT cells, $Ki67^{+}CD4^{+}$MAIT and $Ki67^{+}CD8^{+}$ MAIT cells, had higher significance in HIV and HBV-infected individuals (Fig. 3C) compared to the HCV-infected group.

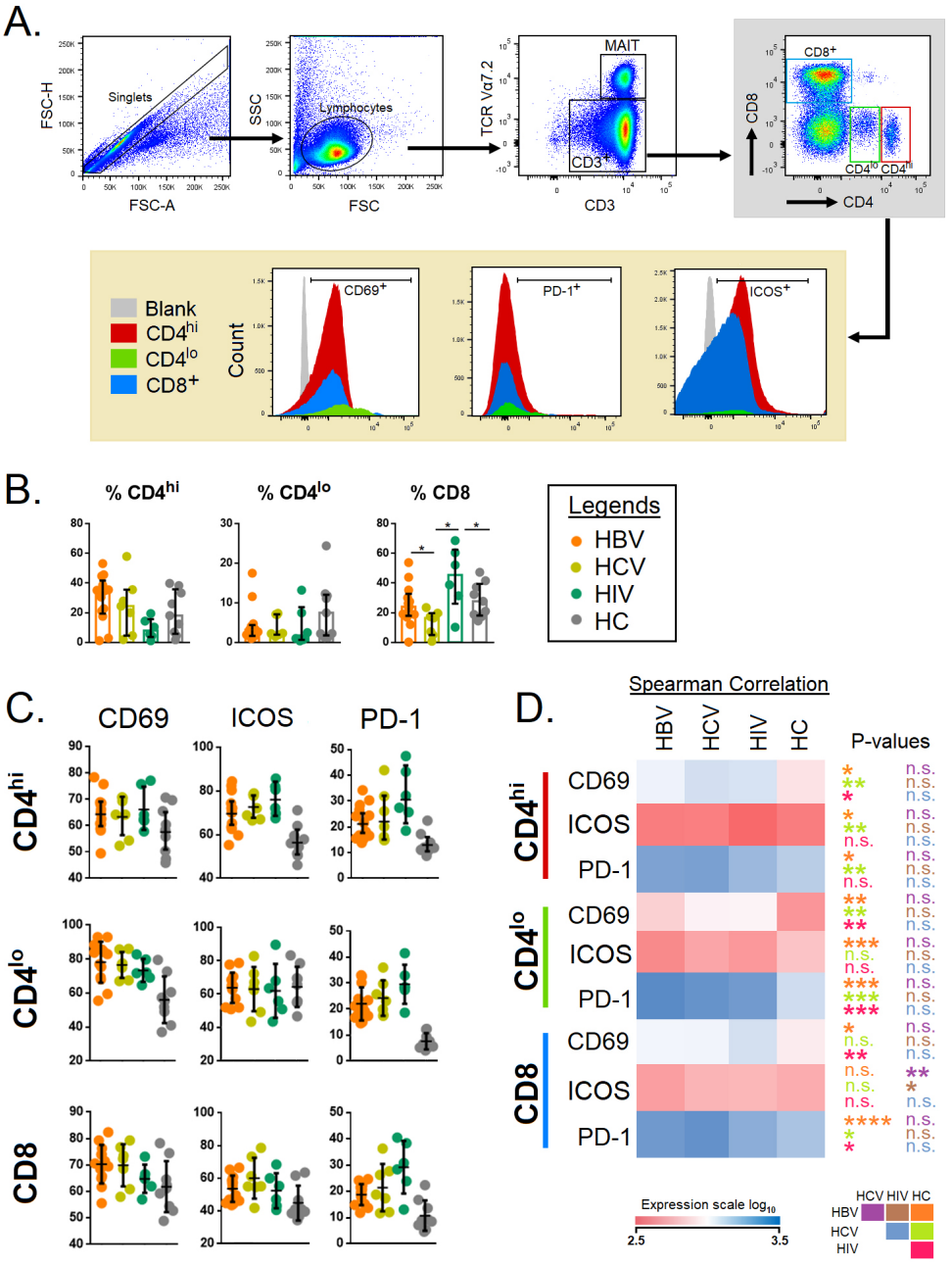

Fig. 1. Immune activation and exhaustion markers associated with CD4$^+$ T cells and CD8$^+$ T cells. (A) Gating strategy for CD69, ICOS and PD-1 expression on peripheral CD4^{+hi}, CD4^{lo}, and CD8$^+$ T cell populations. Lymphocytes were gated from whole human PBMCs using height and area of forward scatter, then singlet gates were utilized to remove doublet populations. This was followed by lymphocyte gating using forward and side scatters areas. This was followed by a total CD3$^+$ cell gate against TCR iVα7.2. From CD3$^+$ cells, total CD8$^+$ cells CD4^{+hi}, and CD4^{+lo} were gated out. From this CD8$^+$, CD4^{+hi}, and CD4^{+lo} we determined CD69$^+$, PD-1 and ICOS. (B) The results of these gates are three T cell populations: CD8$^+$, CD4^{+hi}, and, CD4^{+lo}. Comparison of the levels of, CD4^{+lo}, CD4^{+hi} and CD8$^+$ among patients chronically infected with HBV, HCV, HIV, and HCs. (C) CD69, PD-1 and ICOS expression was determined by using a CD69, PD-1, and ICOS mean fluorescence intensity (MFI), respectively, which was used as a negative control for CD69, PD-1, and ICOS staining and allowed for accurate gating on the positive populations only. (D) Expression (MFI) of CD69, ICOS, and PD-1 in CD4^{+hi}, CD4^{lo} and CD8$^+$ T cells among patients chronically-infected with HBV, HCV, HIV and HCs. The level of expression of each marker was reflected by the color scale of the heatmap. The cells were compared across the four groups by the Kruskal–Wallis test. *Post-hoc* Mann–Whitney U tests were subsequently performed only for those biomarkers with a Kruskal–Wallis test p value of <0.05. $p < 0.05$ are considered significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. n.s. represents 'not significant'.

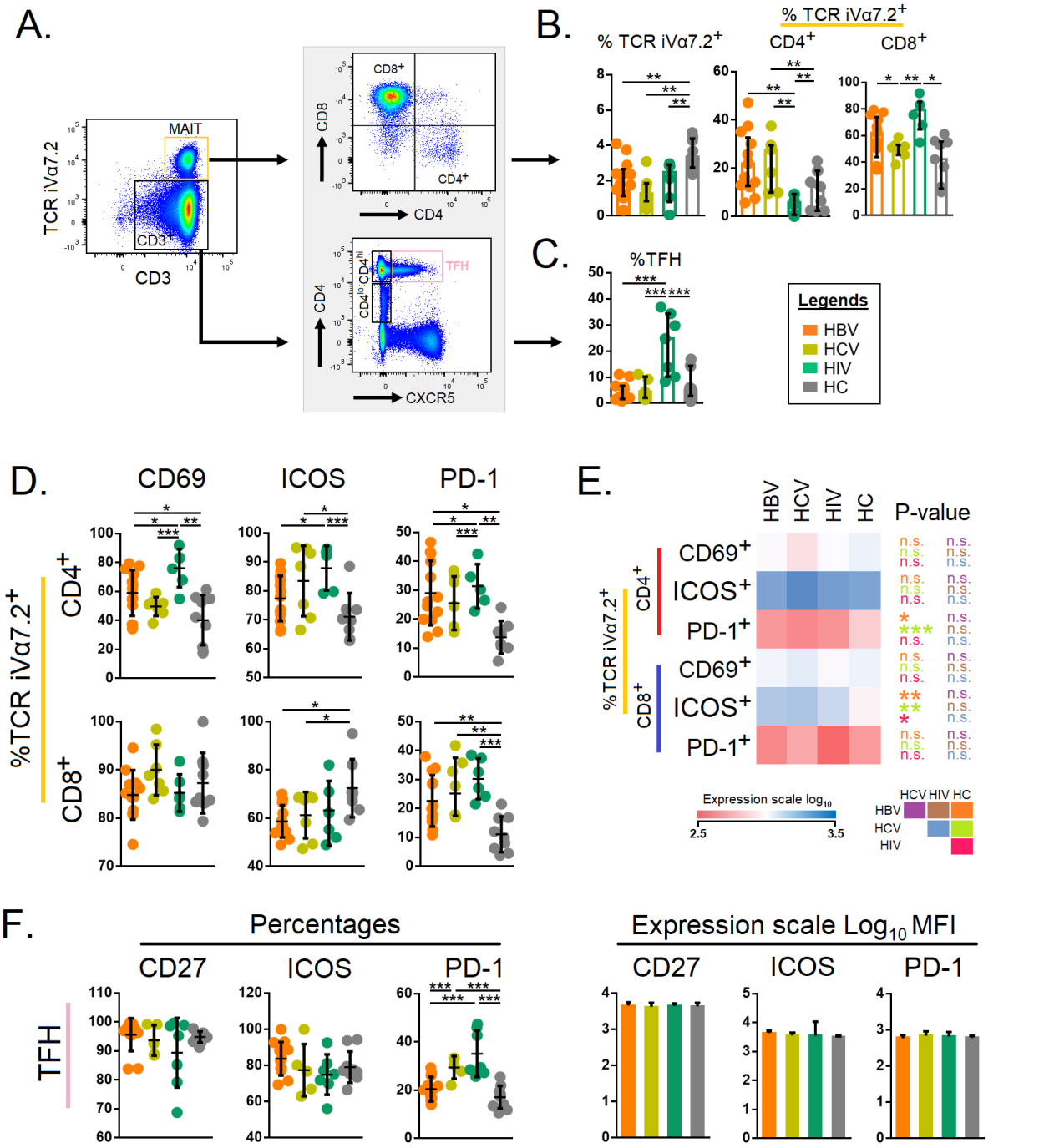

Fig. 2. Expression of immune activation and exhaustion markers on mucosal-associated invariant T cells and follicular T helper cells. (A) Gating strategies for mucosal-associated invariant T cells and follicular T helper cells. Total $CD3^+$ cells were gated against TCR iVα7.2 (MAIT cells). From MAIT cells $CD8^+$ and $CD4^+$ T cell populations were gated. From $CD3^+$ cells, $CD4^+$ cells were gated against CXCR5. The different gates were determined: TCR iVα7.2, $CD4^+$ TCR iVα7.2, $CD8^+$ TCR iVα7.2, $CD4^+$ CXCR5. (B) Comparison of the levels of TCR iVα7.2, $CD4^+$ TCR iVα7.2, $CD8^+$ TCR iVα7.2 among individuals chronically infected with HBV, HCV, HIV, and HCs. (C) Comparison of the levels of TFH among patients chronically infected with HBV, HCV, HIV, and HCs. (D) Percentage level of $CD4^+$ TCR iVα7.2, $CD8^+$ TCR iVα7.2 were compared with CD69, ICOS expression and PD-1. (E) Expression (mean fluorescence intensity, MFI) of CD69, ICOS, and PD-1 in $CD4^+$ TCR iVα7.2, $CD8^+$ TCR iVα7.2 with among individual chronically infected with HBV, HCV, HIV, and HCs. (F) Percentage levels of TFH were compared with CD27, PD-1, and ICOS expression. The level of expression of each marker was reflected by the color scale of the heatmap. The cells were compared across the four groups by the Kruskal–Wallis test. *Post-hoc* Mann–Whitney U tests were subsequently performed only for those biomarkers with a Kruskal–Wallis test p value < 0.05. $p < 0.05$ are considered significant; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. n.s. represents 'not significant'.

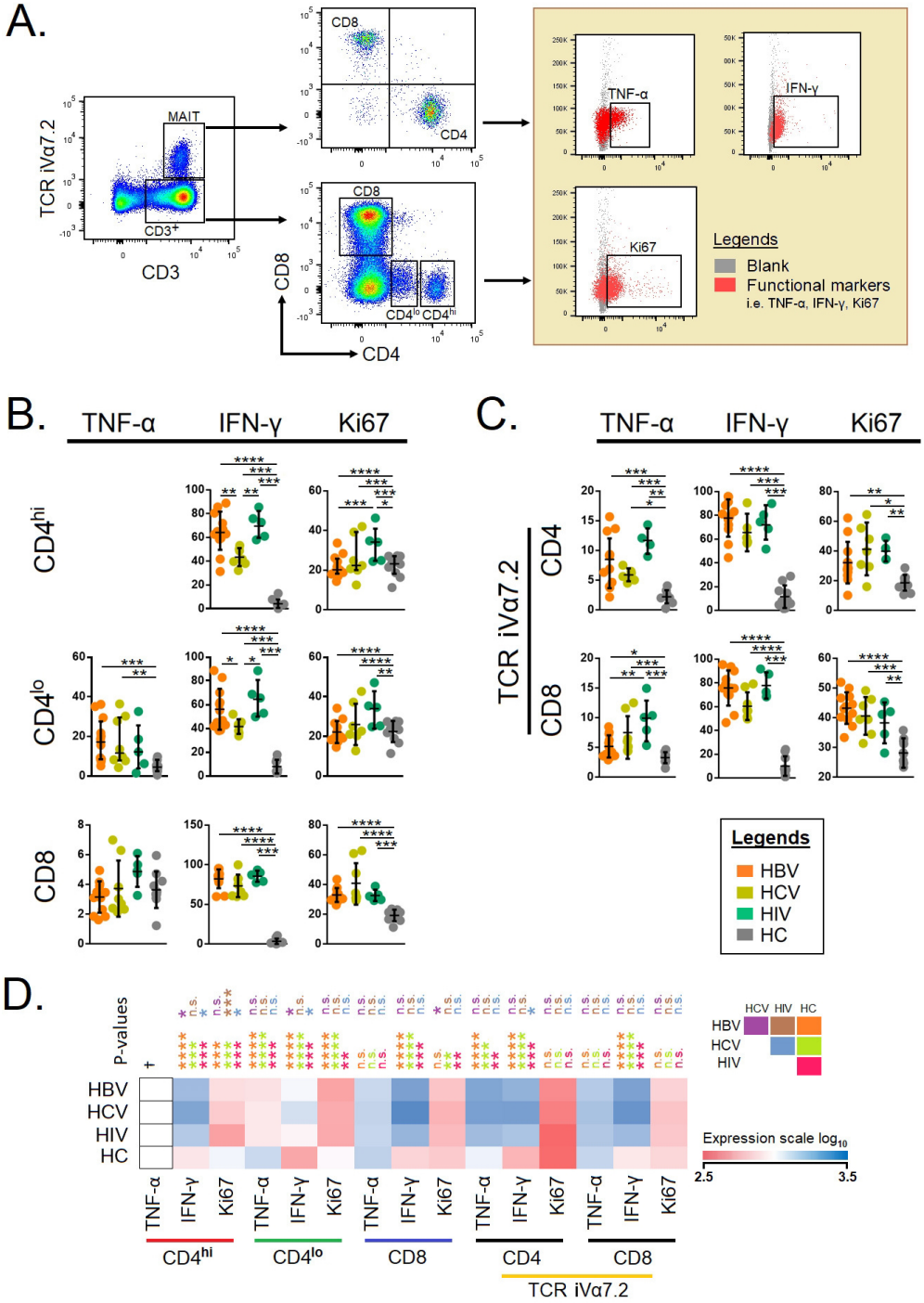

Fig. 3. Functional markers on conventional T cells and mucosal-associated invariant T cells (A) Gating strategies of intercellular cytokines in conventional and unconventional T cells. Total $CD3^+$ cells were gated against TCR iVα7.2 (MAIT cells). From MAIT cells $CD8^+$ and $CD4^+$ T cell populations were gated whereas $CD3^+$ was gated as $CD4^{+hi}$, $CD4^{+lo}$, and $CD8^+$ T cells. The functional markers of different gates were determined: $CD4^+$ TCR iVα7.2, $CD8^+$ TCR iVα7.2, $CD4^+$, and $CD8^+$ cells. (B) Comparison of the levels of TNF-α, IFN-γ, and Ki67 in $CD4^{+hi}$, $CD4^{+lo}$ and $CD8^+$ T cells among individuals chronically-infected with HBV, HCV, HIV, and HCs. (C) Comparison of the levels of $CD4^+$ MAIT cells, and $CD8^+$ MAIT cells among patients chronically-infected with HBV, HCV, HIV, and HCs. (D) The level of expression of TNF-α, IFN-γ, Ki67 with $CD4^{+hi}$, $CD4^{+lo}$, $CD8^+$, $CD4^+$ TCR iVα7.2, $CD8^+$ TCR iVα7.2. The level of expression for each marker was reflected by the color scale of the heatmap. The cells were compared across the four groups by the Kruskal–Wallis test. *Post-hoc* Mann–Whitney U tests were subsequently performed only for those with a Kruskal–Wallis test p value of <0.05. $p < 0.05$ are considered significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. †, TNF-α did not express in the $CD4^{hi}$ population. n.s. represent not significant.

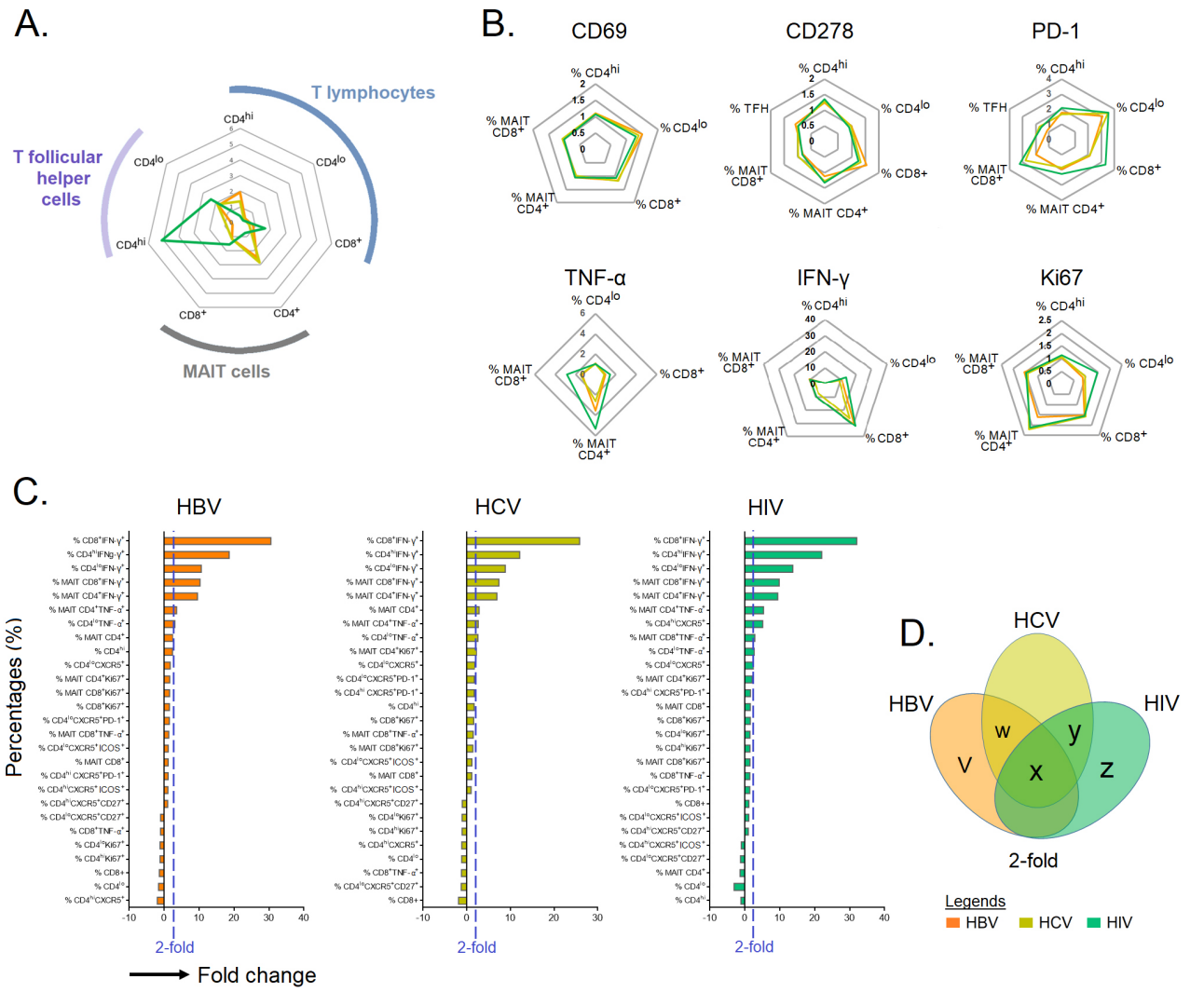

Fig. 4. Immune cell profiling in chronic HBV-, HCV-, and HIV-infected individuals. (A,B) The fold change in immune cells with identified intracellular and extracellular markers in individuals chronically infected with HBV, HCV and HIV normalized against HCs. (C) Bar plot depicting mean 2-fold change among identified markers in specific immune cell type. (D) Venn diagram showing immune cells that are upregulated >2-fold. The Venn diagram identified a profile of seven immune cell populations that are commonly expressed among chronically HBV-, HCV-, and HIV-infected individuals. (v) %CD4^{hi}; (w) %MAIT CD4^{+}; (x) %CD4^{lo} TNF-α^{+}, %MAIT CD4^{+} TNF-α^{+}, %MAIT CD4^{+} IFN-γ^{+}, %MAIT CD8^{+} IFN-γ^{+}, %CD4^{hi} IFN-γ^{+}, %CD4^{lo} IFN-γ^{+}, CD8^{+} IFN-γ^{+}; (y) %MAIT CD4^{+} Ki67^{+}, %CD4^{lo} CXCR5^{+}; (z) %CD4^{hi} CXCR5^{+}, %MAIT CD8^{+} TNF-α^{+}.

Spearman correlation analysis and the significance of various cytokines in chronic HBV, HCV and HIV-infected individuals are shown in Fig. 3D.

3.5 Similar Expression Profile of Markers Across Individuals with Chronic HBV, HCV and HIV Infections

In this study, we observed that the infected groups shared a similar activation and cytokine profiles with total CD4^{+}, CD8^{+} T cells along with Tfh, and MAIT cells displaying elevated activation and cytokine levels (Fig. 4A,B). The cells that showed >2-fold for each chronic infection were identified and displayed in Venn diagrams after the cytokine fold change was graded by descending order (Fig. 4C,D). The analysis revealed that among all the immune cells, seven altered phenotypic profiles viz., CD4^{+lo}TNF-α, MAIT CD4^{+}TNF-α, MAIT CD4^{+}IFN-γ, MAIT CD8^{+}IFN-γ, CD4^{+lo}IFN-γ, CD4^{+hi} IFN-γ, CD8^{+} IFN-γ was common among patients chronically-infected with HBV, HCV, and HIV. Ki67^{+}MAIT CD4^{+} and CXCR5 CD4^{+lo} was common among those chronically infected with HCV and HIV. CD4^{+}MAIT was common among chronic HBV and HCV-infected individuals. (Fig. 4C,D and **Supplementary Fig. 1**). The activation, exhaustion and functional markers in association with viral load of T-cell subsets are presented in Table 2.

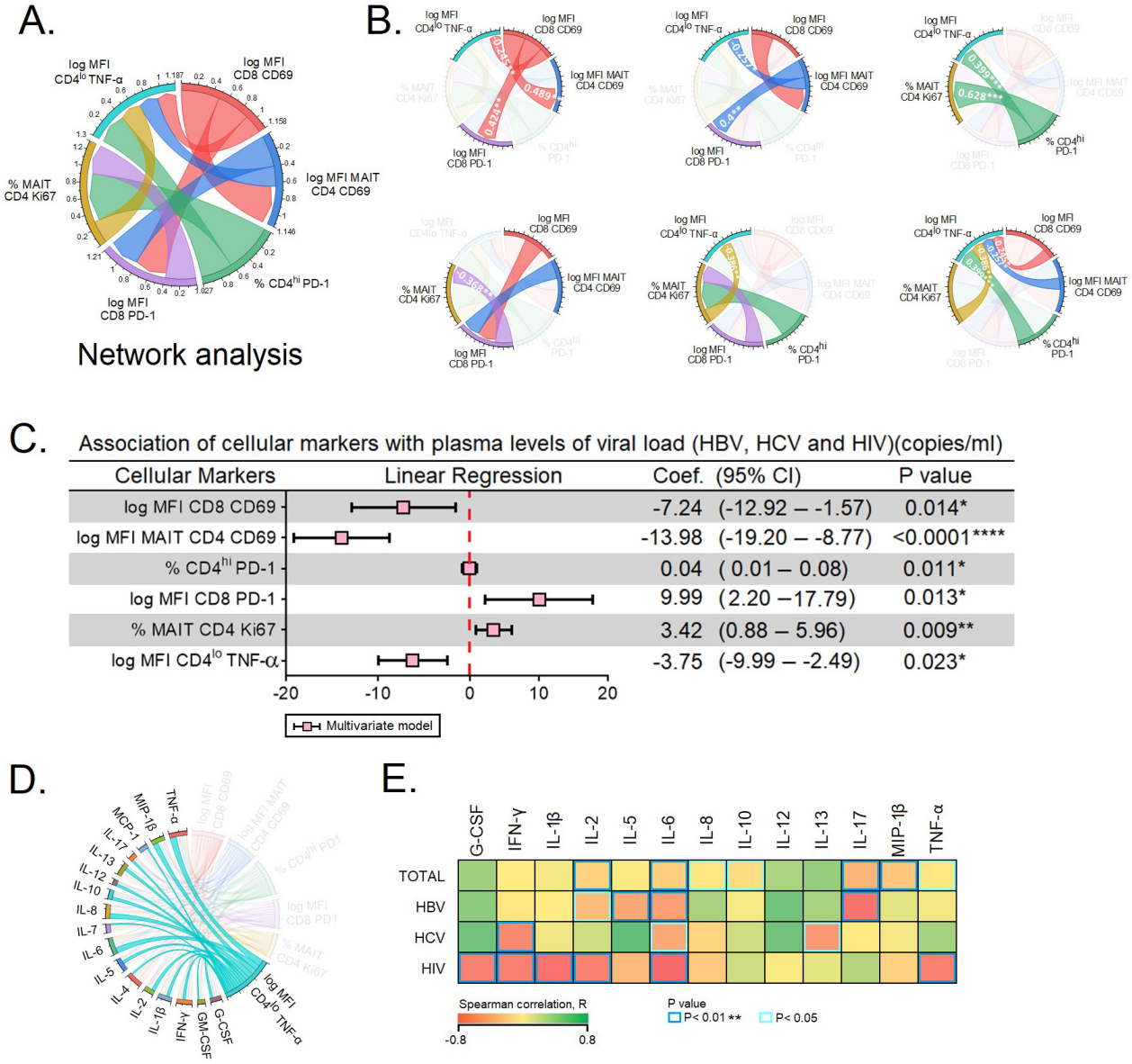

Fig. 5. Predictors of plasma viral load. (A,B) Network analysis of the six predictors of plasma viral load. (A) depicts the complexity of the interactions between the six predictors. (B) Spearman correlation between the six predictors of plasma viral load. (C) The six markers were subjected to multivariate linear regression analysis to determine the markers that independently predict the plasma viral load. (D) Expression of TNF-α and their association with plasma cytokines. (E) Plasma level of cytokines associated with plasma viral load. Variables with p values < 0.05 were considered independent predictors in their respective models. *, **, **** represent $p < 0.05$, <0.01, and <0.0001, respectively.

3.6 Increased CD69 Expression on MAIT Cells Correlates with Low Plasma Viral Loads in HBV, HCV and HIV Infections

We performed a network analysis for the six predictors of PVL, viz., $CD4^{+lo}$TNF-α, $CD69^{+}CD8^{+}$, $CD69^{+}CD4^{+}$MAIT, $PD\text{-}1^{+}CD4^{+hi}$, $PD\text{-}1^{+}CD8^{+}$, $Ki67^{+}CD4^{+}$MAIT cells (Fig. 5A). $CD69^{+}CD8^{+}$ showed a positive correlation with $CD69^{+}$ $CD4^{+}$MAIT, $PD\text{-}1^{+}CD8^{+}$, and a negative correlation with $CD4^{+lo}$TNF-α. Further, $CD69^{+}CD4^{+}$MAIT had a positive correlation with $PD\text{-}1^{+}CD8^{+}$ and a negative correlation with $CD4^{+lo}$TNF-α. Moreover, $PD\text{-}1^{+}CD4^{+hi}$ correlated positively with $Ki67^{+}CD4^{+}$ and $CD4^{+lo}$TNF-α. We also found that $PD\text{-}1^{+}CD8^{+}$ were positively correlated with $Ki67^{+}CD4^{+}$. $Ki67^{+}CD4^{+}$MAIT were negatively correlated with $CD4^{+lo}$TNF-α. In short, $CD4^{+lo}$TNF-α had a positive correlation with $PD\text{-}1^{+}CD4^{+hi}$ and a negative correlation with $CD69^{+}CD8^{+}$, $CD69^{+}$ $CD4^{+}$MAIT, and $Ki67^{+}CD4^{+}$MAIT (Fig. 5B). Together, we found that the markers associated with immune activation, proliferation and exhaustion were differentially expressed and correlated with the different T cell phenotypes.

Table 2. Activation, exhaustion and functional markers associated with plasma viral load.

Marker	Univariate		Multivariate	
	Coef. (95% CI)	*p* value	Coef. (95% CI)	*p* value
Demographic				
Age, years	–0.002 (–0.022 to 0.018)	0.849	–	–
Gender, male	0.244 (–0.729 to 1.216)	0.617	–	–
Activation markers				
% $CD4^{hi}$ CD69	0.006 (–0.060 to 0.071)	0.863	–	–
% $CD4^{hi}$ ICOS	–0.021 (–0.075 to 0.034)	0.450	–	–
% $CD4^{lo}$ CD69	–0.054 (–0.102 to 0.007)	0.025*	–	–
% $CD4^{lo}$ ICOS	–0.043 (–0.075 to –0.010)	0.012*	–	–
% CD8 CD69	–0.043 (–0.110 to 0.024)	0.199	–	–
% CD8 ICOS	–0.036 (–0.077 to –0.005)	0.041	–	–
% MAIT CD4 CD69	–0.011 (–0.070 to 0.048)	0.706	–	–
% MAIT CD4 ICOS	0.029 (–0.022 to 0.079)	0.262	–	–
% MAIT CD8 CD69	–0.010 (–0.070 to 0.091)	0.799	–	–
% MAIT CD8 ICOS	–0.023 (–0.050 to 0.003)	0.040*	–	–
% TFH CD27	–0.042 (–0.104 to 0.019)	0.173	–	–
% TFH ICOS	–0.015 (–0.060 to 0.030)	0.499	–	–
log MFI $CD4^{hi}$ CD69	–1.942 (–8.766 to 4.882)	0.570	–	–
log MFI $CD4^{hi}$ ICOS	5.457 (0.463 to 11.377)	0.040*	–	–
log MFI $CD4^{lo}$ CD69	–5.135 (–9.189 to –1.081)	0.014*	–	–
log MFI $CD4^{lo}$ ICOS	–3.891 (–9.766 to 1.984)	0.189	–	–
log MFI CD8 CD69	–5.759 (–11.744 to –0.226)	0.039*	–10.066 (–15.148 to –4.984)	<0.0001****
log MFI CD8 ICOS	–1.759 (–11.4 to 7.883)	0.716	–	–
log MFI MAIT CD4 CD69	5.602 (1.359 to 9.844)	0.011*	–8.725 (–12.431 to –5.019)	<0.0001****
log MFI MAIT CD4 ICOS	0.709 (–1.992 to 3.411)	0.600	–	–
log MFI MAIT CD8 CD69	–3.214 (–7.058 to –0.631)	0.037*	–	–
log MFI MAIT CD8 ICOS	–2.251 (–4.868 to –0.365)	0.040*	–	–
log MFI MAIT TFH CD27	0.255 (–5.456 to 5.967)	0.929	–	–
log MFI MAIT TFH ICOS	–1.564 (–4.093 to 0.966)	0.220	–	–
Exhaustion markers				
% $CD4^{hi}$ PD-1	0.059 (0.015 to 0.103)	0.009**	0.055 (0.034 to 0.077)	<0.0001****
% $CD4^{lo}$ PD-1	0.003 (–0.055 to 0.060)	0.930	–	–
% CD8 PD-1	0.036 (–0.023 to 0.096)	0.224	–	–
% MAIT CD4 PD-1	0.037 (0.003 to 0.077)	0.041*	–	–
% MAIT CD8 PD-1	0.025 (–0.015 to 0.065)	0.209	–	–
% TFH PD-1	0.053 (0.006 to 0.100)	0.027	–	–
log MFI $CD4^{hi}$ PD-1	2.363 (–2.885 to 7.611)	0.370	–	–
log MFI $CD4^{lo}$ PD-1	5.860 (0.538 to 12.258)	0.042*	–	–
log MFI CD8 PD-1	–3.964 (–11.878 to 3.949)	0.319	–	–
log MFI CD4 PD-1	0.014 (–5.058 to 5.087)	0.996	–	–
log MFI CD8 PD-1	–2.618 (–4.480 to –0.755)	0.007**	5.468 (1.552 to 9.384)	0.007**
log MFI TFH PD-1	5.223 (0.303 to 10.142)	0.038*	–	–
Functional markers				
% $CD4^{hi}$ TNF-α	0.103 (–0.124 to 0.330)	0.365	–	–
% $CD4^{hi}$ IFN-γ	0.001 (–0.024 to 0.026)	0.949	–	–
% $CD4^{hi}$ Ki67	0.059 (0.026 to 0.092)	0.001***	†	–
% $CD4^{lo}$ TNF-α	0.017 (–0.024 to 0.057)	0.414	–	–
% $CD4^{lo}$ IFN-γ	0.016 (–0.023 to 0.055)	0.410	–	–
% $CD4^{lo}$ Ki67	0.013 (–0.022 to 0.048)	0.447	–	–
% CD8 TNF-α	–0.021 (–0.053 to 0.011)	0.191	–	–
% CD8 IFN-γ	–0.041 (–0.081 to –0.002)	0.042*	–	–
% CD8 Ki67	0.031 (–0.013 to 0.076)	0.164	–	–

Table 2. Continued.

Marker	Univariate		Multivariate	
	Coef. (95% CI)	*p* value	Coef. (95% CI)	*p* value
% MAIT CD4 TNF-α	0.009 (–0.080 to 0.098)	0.840	–	–
% MAIT CD4 IFN-γ	0.008 (–0.015 to 0.031)	0.467	–	–
% MAIT CD4 Ki67	0.053 (0.026 to 0.079)	<0.0001****	0.034 (0.015 to 0.053)	<0.001***
% MAIT CD8 TNF-α	0.003 (–0.025 to 0.032)	0.806	–	–
% MAIT CD8 Ki67	0.001 (–0.055 to 0.056)	0.985	–	–
% MAIT CD8 IFN-γ	0.046 (–0.009 to 0.101)	0.101	–	–
log MFI $CD4^{hi}$ TNF-α	–0.019 (–0.857 to 0.820)	0.964	–	–
log MFI $CD4^{hi}$ IFN-γ	1.188 (–5.475 to 7.851)	0.720	–	–
log MFI $CD4^{hi}$ Ki67	4.314 (1.968 to 6.659)	0.001***	†	–
log MFI $CD4^{lo}$ TNF-α	–5.354 (–11.066 to –0.357)	0.045*	–7.794 (–13.400 to –2.188)	0.007**
log MFI $CD4^{lo}$ IFN-γ	–0.148 (–3.729 to 3.434)	0.934	–	–
log MFI $CD4^{lo}$ Ki67	–1.815 (–7.845 to 4.215)	0.546	–	–
log MFI CD8 TNF-α	–1.367 (–3.472 to 0.738)	0.197	–	–
log MFI CD8 IFN-γ	1.747 (–3.022 to 6.515)	0.463	–	–
log MFI CD8 Ki67	2.541 (–0.552 to 5.633)	0.104	–	–
log MFI MAIT CD4 TNF-α	4.39 (–3.233 to 12.030)	0.249	–	–
log MFI MAIT CD4 IFN-γ	1.817 (–2.528 to 6.162)	0.402	–	–
log MFI MAIT CD4 Ki67	4.632 (1.989 to 7.275)	0.001***	†	–
log MFI MAIT CD8 TNF-α	1.935 (–1.795 to 5.666)	0.300	–	–
log MFI MAIT CD8 Ki67	3.763 (0.177 to 7.703)	0.041*	–	–
log MFI MAIT CD8 IFN-γ	–1.671 (–4.186 to 0.844)	0.187	–	–

Univariate analyses by linear regression were performed to identify potential predictors of PVL. Given the small sample size, the linear regression modeling for T-cells subsets, activation, exhaustion and functional markers were performed separately. Variables with $p < 0.05$ were considered as candidates. The candidate predictors were then included in a multivariate model, and variables with *p* values were <0.05 were considered as independent predictors in their respective models. *, **, ***, **** represent $p <$ 0.05, <0.01, <0.001 and <0.0001, respectively. †, the Ki67 expression of these subpopulations were in co-linearity with % MAIT CD4 Ki67. For all the Ki67+ subpopulation, only % MAIT CD4 Ki67 was included in multivariate analysis. Coef, coefficient; CI, Confidence interval.

3.7 Inverse Association of Cellular Markers with Plasma Levels of Viral Load in Chronic HBV, HCV and HIV

Next, we performed multivariate linear regression analysis among the six subsets of T cells to determine independent prediction over the PVLs of HBV, HCV and HIV-infected individuals. The multivariate linear regression analysis of cellular markers were as follows: $CD69^+CD8^+$, –7.24 ($p = 0.014$), $CD69^+CD4^+$MAIT, –13.98 ($p = 0.0001$), PD-1^+CD4^{+hi}, 0.04 ($p = 0.011$), PD-1^+CD8^+, 9.99 ($p = 0.013$), $Ki67^+CD4^+$MAIT, 3.42 ($p = 0.009$) and $CD4^{+lo}$TNF-α, –3.75 ($p = 0.023$) (Fig. 5C). We also determined the expression of $CD4^{+lo}$TNF-α and its association with plasma cytokines. We observed that $CD4^{+lo}$TNF-α was significant among almost all the cytokines (Fig. 5D). The PVLs across all the three viral infections were associated with various cytokines of which 13 revealed positive correlations (Fig. 5E). These 13 cytokines, in turn, had an inverse correlation with the PVL. In HBV PVL IL-5, IL-6, and IL-17 were significant ($p <$ 0.01), and IL-2 ($p < 0.05$) with a negative correlation. In comparison with HCV PVL, IFN-γ was significant ($p <$ 0.01); IL-6 and IL-13 also revealed significance ($p < 0.05$) with a negative correlation. In the case of HIV PVL, G-CSF, IL-1β, IL-2, IFN-γ, IL-6, and TNF-α were negatively significant ($p < 0.01$). Among all the infected individuals IL-17, and MIP-1β were inversely significant ($p < 0.01$): IL-8, IL-10, TNF-α ($p < 0.05$). Together, our data suggest that the inverse correlation of cellular markers may independently play a vital role in determining the plasma viral load of chronic HBV, HCV and HIV-infected patients.

4. Discussion

Virus-specific T cells express multiple inhibitory receptors during chronic infections eventually impairing T cell functions. Evidence suggests that exhausted virus-specific $CD8^+$ T cells eventually lose their ability to clear intracellular viral reserves [9] associated with the onset of chronic immune activation [10]. Studies in murine [19] simian [20] and humans have shown that blockade of the inhibitory molecules restores immune functions *in vitro* as well as *in vivo* [9]. $CD4^+$ T cells provide help to other effector cells, especially $CD8^+$ T cells to aid in their activation and cytokine production (TNF-α and IFN-γ), and contact-dependent cytotoxicity via perforin/granzyme syn-

thesis and/or Fas-FasL interactions to render viral elimination [21]. Chronic viral infections frequently result in decreased $CD8^+$ T cell functions as compared to the potent effector T cells activated during acute infections [22]. Exhausted T cells in chronic infections can be classified into terminally-exhausted $CD8^+$ T cells, and cells with a preserved ability to proliferate [23]. Exhausted T cells along with TNF-α, and IFN-γ induce non-specific immune responses aiding in controlling the infection [9].

Recent evidence suggest that MAIT cells are key to immune surveillance, especially in chronic viral infections. MAIT cells aid in host defense in an antigen-independent manner, as they respond to a variety of cytokines such as IL-12 and IL-18 [24] during viral infections. In chronic HBV and HIV infections plasma IL-5 and IL-7, respectively may play a significant role in viral suppression [25]. MAIT cells have been shown to act against several viral agents, particularly HBV, HCV, HIV, dengue, and influenza viruses [26,27]. Although available findings portray their significance in chronic HBV, HCV and HIV infections, very few compared their role in cross-sectional studies conducted across the infections from same region. In addition, we have also explored into the activation and functional status of circulating Tfh cells across the different study groups.

In the current study, we observed the activation and proliferation of MAIT cells with higher expression of CD69 and Ki67, respectively. Similar findings have also been observed during chronic HCV infection, where they exhibit an activated phenotype with higher levels of the activation markers CD69, HLA-DR, and CD38 [28]. Importantly, here we found that the co-stimulatory potential of MAIT cells were elevated in the three chronic viral infections, as these cells expressed higher levels of ICOS as compared to HCs. The correlation between CD69 levels and polyfunctional TCR $iV\alpha7.2^+CD4^+$ T cells points to the critical role of CD69 against chronic HBV infection [29]. Similarly, here we observed that the levels of CD69-expressing $CD8^+$ T cells and MAIT $CD4^+$ cells were associated with a decrease in PVL indicating the likely importance of CD69 in viral control. Further, chronically-infected individuals were in a hyper-activated state; a stage that precedes immune exhaustion because the later refers to a phase that occurs sequentially only next to immune activation (i.e., sequentially, immune activation will be followed by immune exhaustion, culminating in immune senescence) [30]. The major limitation of our study is that HBV and HCV are hepatotropic viruses, whereas HIV is lymphotropic macrophage-tropic, and these distinct virus tropisms should have had an impact on the phenotypes of various T cell subsets during the chronic phase, which have not been contemplated in the current work.

Experiments conducted thus far on $CD4^+$ T cells during chronic infections have focused on Th1, and only recently investigators have started looking at Tfh cells, in particular the circulating Tfh cells. Tfh, a diverse group of $CD4^+$ T cell subsets are essential for complete B cell responses that include germinal center (GC) reactions, isotype-switching, and affinity maturation [31]. Data available from all the chronic infections that we have studied herein have shown evidence on the induction of Tfh cells. However, the circulating Tfh activation, proliferation and their cytokine profiles have seldom been studied in head-to-head comparisons. Similar to the MAIT cells reported herein, we also observed increased activation of Tfh cells along with higher proliferation (Ki67), and co-stimulation with higher levels of ICOS expression. In all the chronic infections studied herein, we have shown evidence for higher levels of Tfh cells compared to HCs.

5. Conclusion

In summary, our findings suggest a likely association of compromised immune responses in chronic HBV, HCV, and HIV infections although not conclusive of the role of the markers studied herein. The upregulation of CD69 may aid in regulating immune response by determining the patterns of cytokine and chemokine release as well as the activation of lymphocytes during chronic infections. Enhanced TNF-α levels during chronic infection likely imply their protective role in viral elimination possibly via recruiting T cells.

Abbreviations

AIDS, Acquired immunodeficiency syndrome; ALP, Alkaline phosphate triphosphate; ALT, Alanine aminotransferase; APC, Antigen presenting cells; ART, Antiretroviral therapy; AST, Aspartate aminotransferase; BB, Brilliant blue; BV, Brilliant violet; CD, Cluster of differentiation; CXC, Chemokine ligand; CXCL, C-X-C chemokine ligand; CXCR, C-X-C chemokine receptor; EDTA, Ethylene diamine tetra acetic acid; FACS, Fluorescence-activated cell sorting; FBS, Fetal bovine serum; FITC, Fluorescein isothiocyanate; GC, Germinal center; G-CSF, Granulocyte colony-stimulating factor; GGT, Gamma-glutamyl transferase; GM-CSF, Granulocyte-macrophage colony-stimulating factor; HBV, Hepatitis B virus; HC, Healthy control; HCV, Hepatitis C virus; HIV, Human immunodeficiency virus; HLA-DR, Human leukocyte antigen D-related; IBSC, Institutional Biosafety Committee; IC, Internal control; ICOS, Inducible co-stimulator; IEC, Institutional ethical committee; IFN, Interferon; IL, Interleukin; MAIT, Mucosal-associated invariant T cell; MCP-1, Monocyte chemoattractant protein-1; MFI, Mean fluorescence index; MIP-1β, Macrophage inflammatory protein-1 beta; NACO, National AIDS Control Organization; PBS, Phosphate buffered saline; PCR, Polymerase chain reaction; PD, Programmed cell death; PE, Phycoerythrin; PerCP, Peridinin chlorophyll protein; PMA, Phorbol-12-myristate-13-acetate; PVL, Plasma viral load; RNA, Ribonucleic acid; RPMI, Roswell Park Memorial Institute; SGOT, Serum glutamate oxaloacetate transaminase;

SGPT, Serum glutamate pyruvate transaminase; TCR, T cell receptor; Th, T helper; Tfh, Follicular T helper cell; TNF-α, Tumor necrosis factor-alpha; Treg, Regulatory T cell.

Availability of Data and Materials

The original contributions presented in this study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author Contributions

EMS, VV, AM, PB and ML designed the study. AM coordinated patient recruitment and sample collections. JV, RA, SR and AM developed the laboratory works and coordinated the development of the study. YKY and EMS per-formed data analysis. YKY, HYT, and YZ did the biostatistical analysis. JV, EMS, YKY and VV co-wrote the manuscript. All authors critically reviewed the article, have accepted responsibility for its entire contents and approved its submission.

Ethics Approval and Consent to Participate

The studies involving human participants were reviewed and approved by Institutional Ethical Committee (IEC) of the Government Medical College, Theni, India (Ref. Nos. 2544/ME1/18 and 1515/MEIII/21). Prior approval was also obtained from the Tamil Nadu State AIDS Control Society, Chennai, India (TANSACS Approval- 00529/TANSACS/M&E/2019 NACO-T-11020/08/2020_NACO) for the conduct of work. Institutional Biosafety Committee (IBSC) approval was secured from the IBSC of the Central University of Tamil Nadu, Thiruvarur (Ref. No.: CUTN/SLS/1st IBSC/2020/04). The patients/participants provided their written informed consent to participate in this study.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2903128.

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