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Increased frequencies of circulating IFN-γ-producing Vδ1$^+$ and Vδ2$^+$ γδ T cells in patients with asymptomatic persistent hepatitis B virus infection

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Abstract

Hepatitis B virus (HBV) is a leading cause of liver cirrhosis and hepatocellular carcinoma. The outcome of HBV infection is largely determined by the host immune response with virus-specific cytotoxic T cells being able to mediate immunity against HBV as well as causing liver pathology. γδ T cells are reported to be depleted in patients with HBV-associated liver disease. However, it is not known if these cells control HBV infection in patients with asymptomatic chronic HBV infection. In this study, we have examined the frequencies, phenotypes and interferon-γ production by circulating γδ T cell subsets in a group of asymptomatic HBV carriers with low viral loads and little evidence of liver disease. We show that γδ T cells expressing Vδ1 and Vδ2 T cell receptors and effector-memory phenotypes are found at higher frequencies in these patients compared to controls. Vδ2 T cells from the patients expressed interferon-γ significantly more frequently than Vδ2 T cells from healthy donors in the absence of *ex vivo* stimulation. These data suggest that effector-memory IFN-γ-producing Vδ2 T cells may contribute to control of HBV in patients with asymptomatic infection, without mediating liver pathology.

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Introduction

Hepatitis B virus (HBV) remains a major global health problem and is attributable to 780,000 deaths each year, despite the availability of a vaccine [58]. Over 240 million people worldwide have chronic HBV infection, which can lead to cirrhosis, hepatocellular carcinoma (HCC) and liver failure [30, 47]. While the majority of adult HBV infections resolve, 80-90% of infected neonates develop chronic infection and 15–25% die from HBV-related liver disease or cancer [58]. The majority of patients with chronic HBV infection do not develop liver disease and are said to be asymptomatic carriers, however, as many as 20% of patients in the immune control phase of chronic HBV infection develop reactivation of the virus and cirrhosis within 5 years [25, 43, 46]. Therefore, although a vaccine exists with 95% efficacy, new treatments are urgently required to treat the vast numbers of HBV-infected patients worldwide who are at risk of HBV-related liver cirrhosis and cancer [58].

Resolution of HBV infection is associated with strong, polyclonal and multi-specific, $CD8^+$ cytotoxic T lymphocyte (CTL) responses directed against multiple viral epitopes, while chronic HBV infection is characterized by lower numbers and lower potency of HBV-specific CTLs [38, 43, 46, 55]. Inefficient T cell priming by dendritic cells [21, 40], immunomodulation by regulatory T cells [28, 54] and clonal exhaustion due to up-regulation of inhibitory receptors, such as PD-1 [27, 59], have been implicated as factors that contribute to the inadequate T cell responses. Moreover, $CD8^+$ CTL-mediated cytotoxicity is strongly implicated in HBV-related liver damage but does not appear to play a major role in eliminating the virus, while IFN-γ produced by virus-specific CTLs and NK cells are thought to mediate clearance of HBV by interfering with viral replication and by recruiting other antigen-non-specific effector cells [13, 31, 36, 38, 55]. Therefore, the ideal immune response against HBV must control viral replication but limit hepatocyte cytotoxicity and immune-mediated liver damage.

Roles for γδ T cells in antiviral immune responses have been reported for cytomegalovirus (17, 32), Epstein Barr virus [18], human immunodeficiency virus (HIV; [45]) and herpes simplex virus [39] infections. γδ T cells have also been implicated in immune responses to hepatitis C virus (HCV) and are thought to play a role in liver injury associated with the virus [41, 56]. Sing and co-workers [50] reported that γδ T cells were expanded in the blood of patients with HBV infection who seroconverted. Subsequently, Chen and co-workers [10, 11] reported that Vδ2 T cells, the most abundant subset of γδ T cells in human blood and liver [37], are depleted in patients with chronic HBV infection and in patients who develop HBV-associated acute-on-chronic liver failure (HBV-ACLF). In contrast, Vδ1 T cells, the most abundant γδ T cell subset in the intestine, were expanded in the blood of patients with HBV-ACLF.

γδ from HBV-ACLF patients exhibited enhanced cytotoxicity and inflammatory cytokine production, compared to their counterparts in chronic HBV patients and healthy controls, suggesting that γδ T cells play a role in liver injury in HBV-ACLF [10].

In the present study, we investigated the potential role of γδ T cell subsets in immunity against HBV in the absence of liver injury, by studying a cohort of patients with persistent HBV infection (HBsAg-positive) but low viral burden (<20,000 copies/ml) and no evidence of liver disease (ALT below 70 IU/ml). This patient cohort can be considered as having an efficient immune response against HBV, which is under sufficient regulatory control and does not cause significant pathology but fails to completely eliminate the virus [34]. We examined the frequencies, differentiation status and IFN-γ production of the $V\delta1^+$ and $V\delta2^+$ subsets of γδ T cells to assess, for the first time, their potential roles in controlled asymptomatic HBV infection.

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Materials and Methods

Subjects

62 consecutive patients persistently infected with HBV, attending the Hepatology Outpatient clinic at St. James Hospital, Dublin were studied. The patient cohort included 32 men and 30 women with ages ranging from 18 to 60 years. The patients were ethnically diverse and comprised 27 Africans, 25 Caucasians and 10 Asians. Alanine aminotransferase (ALT) levels ranged from 8 to 143 IU/ml with 50 patients having ALT below 40 IU/ml and 12 patients having higher ALT levels above 40 IU/ml. Liver biopsy was performed for those patients with high ALT (>70 IU/ml) relating to HBV. Blood samples for our study were obtained at one time-point for each patient and no patients in our cohort were receiving treatment at the time of sample collection. The viral load was measured at the time of blood collection and varied from 7 copies/ml to 4.5×10^8 copies/ml, but was less than 20,000 copies/ml in the majority of patients. For our analysis, 100,000 copies/ml was chosen as the cut-off to distinguish between higher and lower viral load, with only 5 patients in our cohort having such a high viral burden. All patients were HBV surface antigen (HBsAg) positive and HBeAg negative. There was no clinical or sonographic evidence of cirrhosis or portal hypertension for patients in the study cohort and all patients were negative for HIV and HCV antibody.

Our control population consisted of 66 healthy donors obtained as buffy coat packs from the Irish Blood Transfusion Service. The demographics of our control subjects were mostly unknown; therefore, we also studied a cohort consisting of 13 African, 14 Caucasian and 9 Asian healthy uninfected control subjects attending the Genitourinary and Infectious Diseases Clinic, St. James's Hospital, Dublin. Ethical approval for the study was obtained from the Research Ethics Committees of St. James Hospital and Adelaide and Meath Hospital incorporating the National Children's Hospital, Dublin and informed consent was obtained from all donors.

Antibodies and flow cytometry

Peripheral blood mononuclear cells (PBMC) were prepared from blood samples by density gradient centrifugation over Lymphoprep (Nycomed, Oslo, Norway). Freshly-isolated PBMC were stained with monoclonal antibodies (mAbs) specific for human CD3, Vδ1, Vδ2, CD45RA and CD27, obtained from BD Biosciences (Oxford, UK), Immunotools (Friesoythe, Germany), eBioscience (Hatfield, UK) and R&D Systems (Abingdon, UK). Cells were analysed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

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Investigation of IFN-γ-production by Vδ2 T cells in HBV

Freshly isolated PBMC were incubated for 4 hours in medium alone or with 10 ng/ml phorbol myristate acetate and 1 μg/ml ionomycin (PMA/I), in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich, Dublin, Ireland). Cells were then stained for surface expression of CD3 and Vδ2 and intracellular expression of IFN-γ. The frequencies of IFN-γ^+ Vδ2 T cells were then detected by flow cytometry.

Statistical analyses

Statistical analysis was carried out using Prism GraphPad Version 5.0. Differences between groups were assessed using the Mann-Whitney U test or unpaired t test, where appropriate. P values of <0.05 were considered significant. P values were corrected for multiple testing (p_c) by the Bonferroni method.

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Results

Circulating Vδ1 and Vδ2 T cell frequencies are increased in asymptomatic patients with chronic HBV infection.

PBMC were prepared from 20 HBV-infected subjects and from the buffy coat packs of 23 control subjects. Surface staining with PE-labelled anti-Vδ2 mAb and PE-Cy5-labelled anti-CD3 mAb was performed to identify Vδ2 T cells in the peripheral blood. The mean frequencies of circulating Vδ2 T cells were 3.6±0.4% in controls and 7.8±0.9% in HBV (p=0.0001, Figure 1). PBMC from 23 HBV-infected subjects and from the buffy coat packs of 21 control subjects were indirectly surface stained with unconjugated anti-Vδ1 mAb (murine IgG), followed by PE-labelled anti-mouse IgG, in combination with direct surface staining with PE-Cy5-labelled anti-CD3 mAb. Mean frequencies of circulating Vδ1 T cells were 0.8±0.2% in controls and 1.2±0.2% in HBV patients (p=0.02, Figure 1). These data show significant expansions in both the $V\delta1^+$ and $V\delta2^+$ proportions of circulating γδ T cells in asymptomatic patients with HBV infection compared to uninfected control subjects.

Frequencies of circulating Vδ2 and Vδ1 T cells are similar in African, Caucasian and Asian healthy control subjects.

Our patient cohort consisted of individuals of African, Caucasian and Asian origin. Therefore, blood samples were obtained from healthy controls that were demographically-matched to our patient cohort in an effort to identify differences in Vδ1 and Vδ2 T cell frequencies that relate to race and not HBV infection. Blood samples were taken from 13 African, 14 Caucasian and 9 Asian healthy donors. PBMC preparation and surface staining were performed as above and the frequencies of Vδ1 and Vδ2 T cells were quantified. The mean frequencies of circulating Vδ1 and Vδ2 T cells were similar in African, Caucasian and Asian control subjects (Figure 2). Although Vδ1 T cells were found at slightly higher frequencies in Asians, these data suggest that the frequencies of circulating Vδ1 and Vδ2 T cells do not significantly differ between persons of the 3 ethnicities investigated in this study and, therefore, that the higher frequencies of $V\delta1^+$ and $V\delta2^+$ T cells in HBV patients (Figure 1) are not biased by the demographics of the patient group.

The frequencies of circulating IFN-γ-producing Vδ2 T cells are higher in HBV patients than in healthy control subjects.

Freshly-isolated PBMC from 10 HBV-infected subjects and 18 control subjects were incubated for 4 hours in medium alone or with PMA/I, in the presence of brefeldin A. Cells were then stained for surface expression of Vδ2 and CD3 and intracellular expression of IFN-γ to identify IFN-γ-producing Vδ2 T cells. The frequencies of IFN-γ-producing Vδ2 T cells were calculated as percentages of the Vδ2 T cells and as percentages of the total T cells. In the absence of stimulation, the mean frequencies of IFN-γ-producing Vδ2 T cells, as a percentage of total Vδ2 T cells, were 2.7±0.7% in controls and 6.7±1.4% in HBV patients (Figure 3, p=0.01). After PMA/I stimulation, the mean frequencies of IFN-γ-producing Vδ2 T cells were similar in HBV patients and uninfected controls (Figure 3, p=0.6). In the absence of stimulation, the mean frequencies of IFN-γ-producing Vδ2 T cells, as a percentage of total T cells, were 0.06±0.02% in controls and 0.4±0.2% in HBV-infected subjects (Figure 5, p=0.005) and these differences were not observed following PMA/I stimulation (Figure 5, p=0.2). Thus, in the absence of *ex vivo* stimulation, Vδ2 T cells from the HBV-infected individuals more likely produce IFN-γ than Vδ2 T cells from uninfected controls.

Vδ1 and Vδ2 T cells from patients with HBV infection predominantly display memory phenotypes. PBMC from 17 HBV-infected subjects and from the buffy coat packs of 21 control subjects were stained with mAbs specific for Vδ1, CD3, CD45RA and CD27. The scheme described by Dieli [19] was then used to identify the naïve, central memory (T_{CM}), effector memory (T_{EM}) and terminally differentiated (T_{EMRA}) Vδ1 T cells (Figure 4A). The frequencies of naive Vδ1 T cells, as a proportion of the total Vδ1 T cell population, were significantly lower in HBV patients compared with controls (Figure 4B, p=0.0002), while the frequencies of $T_{CM,}$ T_{EM} and T_{EMRA} Vδ1 T cells were significantly higher (Figure 3B, p=0.02, 0.004 and 0.02). These data show that all memory subsets of Vδ1 T cells are expanded in HBV infection while, naïve Vδ1 T cells are significantly lower, suggesting that Vδ1 T cells are actively involved in the immune control of HBV.

Further phenotypic studies were performed to ascertain whether the frequencies of naïve, T_{CM}, T_{EM} and T_{EMRA} Vδ2 T cells were altered in HBV infection. PBMC from 27 HBV-infected subjects and from 40 control subjects were surface stained for Vδ2, CD3, CD45RA and CD27 expression. The frequencies of naïve and T_{CM} Vδ2 T cells, as a proportion of the total Vδ2 T cell population, were significantly lower in HBV patients, compared with controls (Figure 3B, p=0.006, p=0.03), while the frequencies of T_{EM} Vδ2 T cells were similar (Figure 3B, p=0.1). The frequencies of T_{EMRA} Vδ2 T cells were significantly higher in HBV patients (Figure 3B, p=0.02). These data suggest that effector Vδ2 T cells are also expanded in order to facilitate the control of HBV infection.

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The frequencies of circulating Vδ2 T cells do not correlate with viral load, serum ALT and age in HBV but are slightly higher in female subjects.

Circulating Vδ1 and Vδ2 T cell frequencies in groups of HBV patients within the study cohort were compared based on clinical parameters. There were no significant differences in the frequencies of Vδ1 and Vδ2 T cells between patients with low and high viral loads, low and high ALT, or in patients aged 19-35 years compared to patients aged 35-55 years (Figure 5). There were significantly higher frequencies of Vδ2 T cells in HBV-infected females compared with HBV-infected males, while, the frequencies of Vδ1 T cells were similar (Figure 5, $p=0.02$). Spearman correlation tests confirmed that there were no linear correlations between the frequencies of Vδ1 and Vδ2 T cells and viral load, ALT or age. These data suggest that while, the frequencies of circulating Vδ2 T cells are slightly higher in female HBV-infected subjects than their male counterparts, the frequencies of Vδ1 and Vδ2 T cells do not significantly correlate with the clinical parameters investigated here.

Discussion

Vδ2 T cells are the predominant γδ T cell subset in human blood and are capable of producing IFN-γ and TNF-α [23, 24], killing target cells [3, 22, 23], inducing activation and maturation of neutrophils [15, 16], monocytes [24], B cells [7, 9] and dendritic cells [14, 23, 35] and presenting antigen to conventional $CD4^+$ and $CD8^+$ T cells [8]. Their multi-functional capacity makes them ideal candidates for immunotherapy and they are already the focus of several clinical trials [5, 12, 20, 42, 51]. Vδ1 T cells are mainly found at mucosal surfaces and can exhibit immunostimulatory [26, 29] and immunoregulatory [6] functions and are also under consideration as therapeutic targets [49].

Previous studies have revealed that Vδ2 T cell frequencies are decreased in the peripheral blood of chronic HBV patients with liver disease [11] and with HBV-ACLF [10], whereas Vδ1 T cells are expanded in the blood of HBV-ACLF patients and exhibit enhanced cytotoxicity and cytokine production compared to Vδ1 T cells from healthy controls. We studied a cohort of asymptomatic, HBeAg-negative patients with persistent HBV infection with little evidence of liver disease, to provide an insight into the possible roles of γδ T cells in immune control of HBV infection without significant liver injury. We report that IFN-γ-producing Vδ1 and Vδ2 T cells with effector memory phenotypes are found at higher frequencies in the peripheral blood of these patients compared to controls. This suggests that these γδ T cell subsets are expanded in the circulation of patients, although we cannot exclude the possibility that their increased frequencies could be the result of a contraction of other T cell subsets. Future studies are required to determine if Vδ1 and Vδ2 T cells are also expanded in the livers of our patients, however, one study [44] has demonstrated that the numbers of peripheral and intrahepatic lymphocyte subtypes correlate closely with each other. Therefore it is likely that effector memory Vδ1 and Vδ2 T cells may contribute to the immune control of HBV infection without causing liver pathology. Our finding contrasts with the depletions of these cells in patients with HBV-associated liver disease and HBV-ACLF [10, 11], which may facilitate liver damage. A role for Vδ2 T cells in immunity against HCV was reported by Agrati and co-workers [1], while the same group has provided evidence that Vδ1 T cells contribute to liver damage in patients with HCV infection [2].

As well as being expanded, our study has revealed that greater proportions of Vδ1 and Vδ2 T cells from asymptomatic HBV patients displayed effector memory phenotypes compared to Vδ1 and Vδ2 T cells from uninfected control subjects, while the proportions of naïve Vδ1 and Vδ2 T cells were substantially lower. This suggests that Vδ1 and Vδ2 T cells are actively involved in immunity against HBV.

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Previous studies have shown that circulating HBV-specific $CD8^+$ T cells from patients with acute HBV infection predominantly express memory T cell phenotypes suggesting that they are also actively involved in immunity against HBV [52, 57]. We also found that, in the absence of *ex vivo* stimulation, the frequencies of IFN-γ-expressing Vδ2 T cells were higher in the HBV-infected patients compared to healthy controls. This suggests that Vδ2 T cells may control HBV infection and prevent immune-mediated damage by a mechanism that involves IFN-γ-mediated viral clearance. A similar role for subsets of NK cells in IFN-γ-mediated control of HBV without liver-damaging cytotoxicity has been proposed [13, 48, 60]. The elevated frequencies of circulating Vδ1 T cells with effector memory phenoptypes, although not as significant as those observed for Vδ2 T cells, suggest that these cells also play a role in the antiviral immune response against HBV, possibly via the production of IFN-γ and TNF-α [52]. Since Vδ1 T cells have previously been implicated in the pathogenesis of HCV infection and arthritis, they might require strict regulation in asymptomatic HBV infection [2, 4]. However, they have also previously been shown to regulate inflammatory responses of $CD8^+$ T cells in the small intestine via the suppression of IFN-γ, granzyme-B and NKG2D expression [6]. Further work is required to determine whether this γδ T cell subset plays a predominant antiviral or regulatory role in the control of HBV infection.

In summary, a previous study has provided evidence that failure to clear HBV infection is associated with depletions of Vδ2 T cells and reduced IFN-γ expression, with the greatest depletions observed in patients with the highest viral loads [11]. Our data support this hypothesis, having found higher frequencies of IFN-γ-producing, effector memory Vδ2 T cells in a cohort of asymptomatic HBV patients with low viral loads. These findings implicate Vδ2 T cells as key players in the control of HBV replication, most likely via the production of IFN-γ. Since, Vδ2 T cells have already been identified as targets for immunotherapy and they are already the focus of several clinical trials [5, 12, 20, 42, 51], we propose that they might be used as the basis for future immunotherapies to treat HBV patients with persistent symptomatic infection.

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Author disclosure statement

No competing financial interests exist for all authors of this manuscript.

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Legends to Figures

Figure 1. γδ T cell subset frequencies are higher in the peripheral blood of patients with chronic HBV infection compared to controls. **A**, Representative flow cytometry dot plots showing circulating total Vδ1 T cells (left) and Vδ2 T cells. (right) **B**, Scatterplots showing the percentages of T cells that express the Vδ1 (left) or the Vδ2 (right) T cell receptor in 21 uninfected control subjects (filled circles) and 23 HBV-infected patients (unfilled circles). Horizontal bars show means.

Figure 2. Frequencies of circulating Vδ1 and Vδ2 T cells are similar in African, Caucasian and Asian healthy control subjects. Frequencies of circulating Vδ1 (left) and Vδ2 T cells (right), as percentages of total T cells, in 13 African, 14 Caucasian and 9 Asian healthy control subjects. Horizontal bars show means.

Figure 3. Frequencies of circulating IFN-γ-producing Vδ2 T cells are higher in HBV-infected patients than in control subjects. Scatterplots showing the frequencies of IFN-γ-producing Vδ2 T cells as a percentage of Vδ2 T cells (top) and as a percentage of total T cells (bottom), following incubation in medium alone (left) and following incubation in medium conditioned with PMA/I (right) in 18 uninfected control subjects (filled circles) and 10 HBV-infected patients (unfilled circles). Horizontal bars show means.

Figure 4. Circulating effector-memory Vδ1 and Vδ2 T cells are expanded in HBV infection while naive Vδ1 and Vδ2 T cells are reduced. **A,** Representative flow cytometry dot plot showing CD27 and CD45RA expression by gated Vδ2 T cells for the enumeration of naïve, central memory, effector memory and terminally-differentiated Vδ2 T cells. **B,** Scatterplots showing the frequencies of circulating naïve, central memory, effector memory and terminally-differentiated Vδ1 (top) and Vδ2 (bottom) T cells in 40 (21 for Vδ1 T cells) uninfected control subjects (filled circles) and 27 (17 for Vδ1 T cells) HBV-infected patients (unfilled circles). Horizontal bars show means.

Figure 5. Frequencies of circulating Vδ1 and Vδ2 T cells in HBV patient subsets based on viral load, ALT, gender and age. Scatterplots showing frequencies of Vδ1 (top) and Vδ2 (bottom) T cells in male and female HBV-infected patients with viral load between both 10 and 100,000 copies/ml (Low) or between

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100,000 and $5x10^8$ copies/ml (High), with ALT below 40 IU/ml (<40) or above 40 IU/ml (>40) and aged between 19 and 35 years or 35 and 55 years. Horizontal bars show means.

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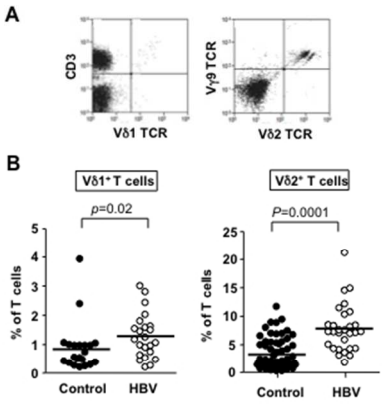

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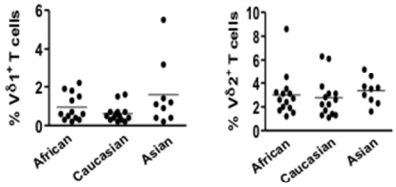
Conroy *et al*. Figure 2



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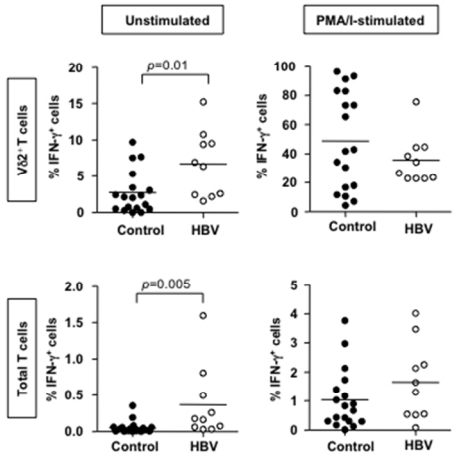

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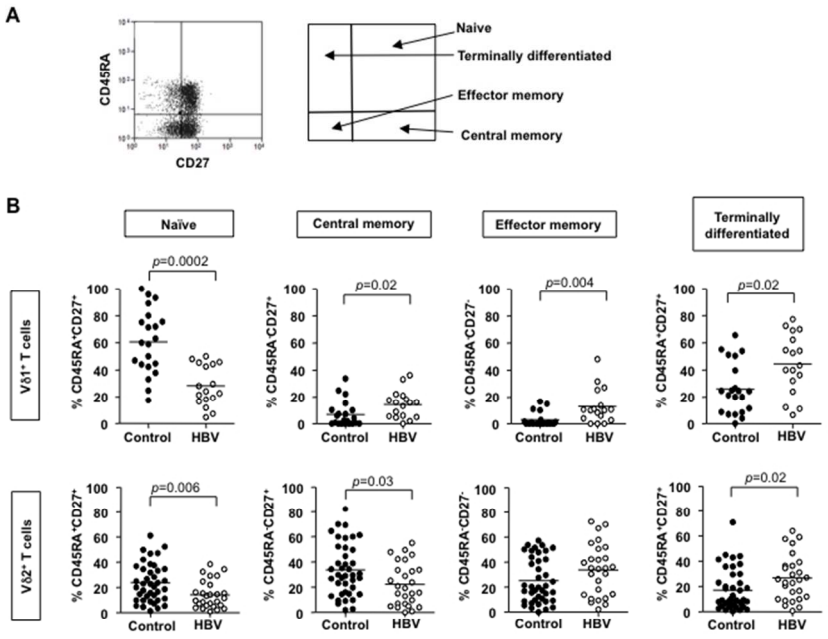

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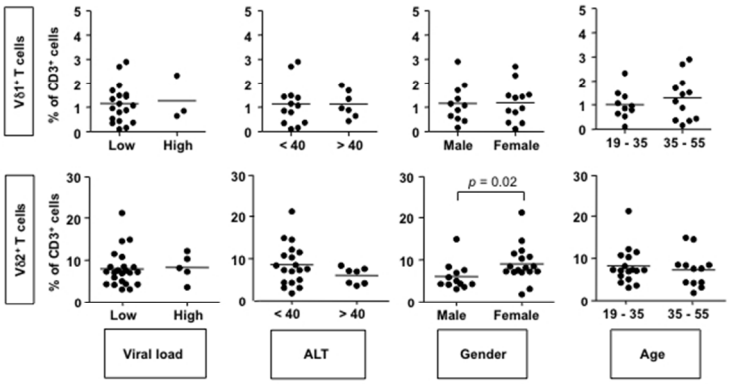

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