

# Hbc Alone Profile: Susceptibility versus Protection against *Hepatitis B Virus* could be associated with TNF Production

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## Abstract

**Background:** Donors with occult HBV (*Hepatitis B virus*) infection, defined as those who lacked detectable HBsAg but whose exposure to HBV infection was indicated by a positive anti-HBc (HBc alone) profile and the presence of HBV DNA, are a potential source of HBV infection. The aim of this study was to evaluate HBcAg-specific T cell responses, NK cell activity and cytokine levels in blood donors with HBc alone profiles with and without detectable viral DNA.

**Methods:** From January 2010 to December 2012, a total of 4,252 HBc alone donations were obtained. Of the 4,252 donors, 681 donors had spontaneous HBV clearance (Co/s >10,0 by chemiluminescent assay, undetectable HBV DNA and reactivity to anti-HBc in subsequent donations), 3,097 were classified as false-positive for anti-HBc (Co/s <6,0 in chemiluminescent assay, undetectable HBV DNA and no reactivity to anti-HBc in subsequent donations), 438 were classified as having chronic HBV infection (Co/s >10,0 in chemiluminescent assay, detectable HBV DNA and reactivity to anti-HBc in subsequent donations) and 36 were OBI (anti-HBc positive and detectable HBV DNA). There were 500 healthy blood donors and 434 HBV carriers (HBsAg, anti-HBc positive and detectable HBV DNA). NK cells were tested for cytotoxicity against K562 cells, serum levels of specific cytokines (IL-8, IL-1, IL-10, IL-12, IL-6 and TNF) were assayed by flow cytometry and HBcAg-specific T cell responses were assessed by lymphoproliferation reported in stimulation index (SI) units.

**Results:** The IL-8 and IL-12 serum levels increased significantly ( $p < 0.001$ ) in HBV carriers and OBI, whereas the TNF- $\alpha$  serum levels increased significantly ( $p < 0.001$ ) in spontaneous HBV resolvers compared to HBV carriers and OBI. The serum levels of IL-1 and IL-10 were similar in HBV carrier, OBI and spontaneous HBV resolvers. Higher NK cytotoxic activity was observed in spontaneous HBV resolvers compared to HBV carriers, healthy donors and OBI. TNF- $\alpha$  correlated with NK cell activity in spontaneous HBV resolvers. A low intensity of HBcAg-specific T cell responses was observed in healthy donors (SI < 3.0) and OBI (SI = 8.0 to 10.0) compared to spontaneous HBV resolvers (SI > 22.0). TNF- $\alpha$  was correlated with HBcAg-specific T cell responses in spontaneous HBV resolvers.

**Conclusion:** Our results suggest that TNF- $\alpha$  production may be associated with protection against *hepatitis B virus* through increased NK cell activity and HBcAg-specific T cell responses in HBc alone blood donors.

## Introduction

*Hepatitis B virus* (HBV), as a member of the *hepadnavirus* family, is a small, enveloped, partially double-stranded circular DNA virus that primarily infects hepatocytes. HBV causes acute and persistent liver diseases, which are among the most critical human health problems in high-prevalence regions, such as Brazil [1-7].

Consequently, the pathogenesis of HBV has been the focus of HBV research for years. However, the precise pathogenic mechanism responsible for the various forms of associated liver diseases are poorly defined, especially in regards to occult HBV infection due to its low frequency of occurrence [8-10].

Most studies indicate that the host immune response to the virus has a critical role in determining pathogenesis, as HBV itself is not cytopathic to hepatocytes [11]. The T cell response to viral antigens is essential for both clearance and pathogenesis in HBV infection [11-17]; the specific adaptive cellular immune response to HBV-encoded proteins, principally HBc (core of *hepatitis B virus*), is decisive in determining the out-come of the infection [18]. In a recent study by our group, we observed that spontaneous HBV resolvers showed a strong peripheral blood mononuclear cell (PBMC) response to HBcAg when compared with HBV carriers and occult HBV infection (OBI) corroborating data in the literature [19].

Another important defense against viruses, including HBV, is the natural killer (NK) cell response. Similar to T cells, NK cells can produce high levels of cytokines when these cells are activated and thereby kill infected cells directly or indirectly through cell-cell contacts. NK cells not only have antiviral effects, but they also have regulatory effects on

other lymphocytes such as T cells via cytokine production [20,21]. For example, INF- $\gamma$  can induce MHC class I expression and promote Th1-type T cell responses, and TNF- $\alpha$  can promote the differentiation and maturation of monocytes/macrophages and dendritic cells [22].

In an animal model, it has been shown that NK cell activity, T cells and cytokines (e.g., interleukin-12) can inhibit HBV replication in transgenic mice, thus implying a role for innate immunity in controlling HBV infection in this model [23].

One of the challenges in understanding HBV pathogenesis is elucidating the full repertoire of immune responses that controls viral replication, especially in spontaneous HBV clearance. Additionally, insight into the control of the HBV infection in these individuals could clarify the mechanism of HBV persistence. The goal of this study was

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to evaluate HBcAg-specific T cell responses, NK cell cytotoxicity and cytokine production in blood donors with isolated serology reactivity for anti-HBc (HBc alone) with HBV DNA (occult HBV infection) or without HBV DNA (spontaneous HBV resolvers).

## Material and Methods

### Blood donors included for cellular immunity assessment

The blood donor population from COLSAN-Associação Beneficente de Coleta de Sangue (São Paulo, Brazil) was tested between January 2010 and October 2012. We selected 4,252 donors found positive only for anti-HBc from a total of 250,136 donations. As control subjects, we enrolled 500 healthy blood donors who had no reactivity for HBV, including HBV PCR, and who were nonreactive for *hepatitis C virus* (HCV), *human immunodeficiency virus* (HIV), *human T-lymphotropic virus* (HTLV), *syphilis* and Chagas disease and 438 HBV carriers (HBsAg, anti-HBc and detectable HBV DNA). All subjects gave their informed consent, and the study was approved by the UNIFESP Ethical Committee.

### Detection of serological HBV markers and HBV DNA detection

Blood units collected at COLSAN-Associação Beneficente de Coleta de Sangue (São Paulo, Brazil) were screened for the presence of serum anti-HBc and HBsAg using a commercial chemiluminescent microparticle immunoassay (CMIA) (Abbott, Germany). HBe, anti-HBe, anti-HBs and Core IgM testing was performed using the ELFA system (BioMérieux, France). HBsAg was confirmed by HBsAg Qualitative Confirmatory-CMIA (Abbott, Germany). All of the samples were also screened by CMIA for infection with HCV, HTLV, HIV, syphilis or Chagas disease (Abbott, Germany) and were excluded from this study if found positive. For the detection of HBV DNA, DNA was extracted and subsequently subjected to a commercial test for HBV DNA (HBV Monitor; Roche) with a higher detection limit of 10 UI/mL.

### HBcAg specific T cell-response

Peripheral blood mononuclear cells preparation was performed as previously described by Araujo [23]. T cells were cultured at 106 cells per mL in RPMI-1640 (Gibco, Invitrogen, Beijing, P.R. China) supplemented with 10% heat-inactivated human AB serum, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin and incubated overnight at 37°C and 5% CO<sub>2</sub>. Seventeen overlapping 20-mer peptides covering the entire HBV core sequence (ayw subtype) (amino acids 1 to 183) were synthesized using a multiple-peptide synthesizer with standard 9-fluorenylmethoxycarbonyl chemistry (Syro, MultiSynTech, Bochum, Germany). Then, 10 µg/mL of HBV peptide was added, and the cells were further incubated for 5 days. Control cultures included 2 µg/mL phytohemagglutinin (PHA), medium alone and recombinant HCV NS5 protein, (American Research Products, Belmont, MA, USA). [3H]-Thymidine (specific activity 5 mCi/mmol) was added for the last 20 hours of culture. The results were expressed as counts per minute (cpm) for PHA-stimulated cultures in stimulation index (SI) units, defined as the ratio between median cpm in the HBcAg-containing cultures and median cpm of control HCV NS5-containing cultures. The 100th percentile of HBcAg-specific SI in 500 healthy blood donors was 2.01. For practical purposes, HBcAg-specific T cell responses were scored positive when SI>3.

### NK cell cytotoxicity assay

Ex vivo NK cytotoxicity (NK-CTL) was performed with K562 cells as

targets. K562 cells were resuspended in RPMI medium and dispensed in 96-well U-bottomed plates at 5 × 10<sup>3</sup> cells per well. PBMCs were then added to the K562 cells at an effector-to-target ratio of 40:1, 20:1 and 10:1. Cultures were incubated for 4 h before 25 µl culture supernatant was collected and mixed with 125 µl of scintillation fluid (Optiphase Supermix; Wallac). The amount of 51Cr released in culture supernatants was measured in a gamma counter (1450 Microbeta Trilux; Wallac). The percentage of cytotoxicity was determined using the formula  $(E - M/D - M) \times 100$ , where E is the experimental 51Cr release, M is the 51Cr released by targets in the presence of culture medium (which was 15 to 25% of the total release) and D is the total release of 51Cr in the presence of 5% Triton X-100 detergent.

### Enzyme-linked immunospot assay (ELISpot) for interferon-gamma (IFN-γ)

Ninety-six-well milliliters plates (Millipore, Bedford, MA, USA) were coated overnight at 4°C with a primary antibody to human IFN-γ (1-DIK; Mabtech, Nacka, Sweden) at a concentration of 10 µg/mL. In parallel, PBMCs at 2 × 10<sup>5</sup> per well were cultured in triplicate in RPMI/10% human AB serum with HBcAg peptide (final concentration, 10 µg/mL), and phytohemagglutinin (2 µg/mL) or medium only. After 28 hours, the cells were transferred to the coated plates and cultured under the same conditions for 20 hours. The plates were washed, and 100 µL biotin-conjugated anti-IFN-γ antibody (Mabtech) was added to each well for 2 hours. Next, the plates were washed and incubated for an additional 2 hours with 100 µL streptavidin/alkaline phosphatase (Mabtech). The enzyme reaction was developed with freshly prepared nitroblue tetrazolium chloride/bromo-chloro-indolyl-phosphate toluidine salt (NBT/BCIP; Roche Diagnostics Ltd., Lewes, England). The reaction was stopped with distilled water, and the spots were counted by 2 observers (E.I.R. and I.M.) under a dissection microscope (Nikon SMZ800; Nikon Ltd., Kingston upon Thames, United Kingdom) equipped with a graticule eyepiece. The number of specific spot-forming cells (SFCs) was determined as the mean number of spots in the presence of an antigen minus the mean number of spots in the wells with medium only and expressed per 1 × 10<sup>6</sup> PBMCs. The positive control consisted of PBMCs stimulated with 10 µg/mL of PHA, and the criteria for a positive response for the ex vivo ELISpot assays were more than 5 SFCs per well and more than twice the number of SFCs than the unstimulated control well. Therefore, in this study, more than 20 SFCs per 10<sup>6</sup> PBMCs comprised a positive response.

### Cytokine patterns

Plasma cytokine levels were measured in duplicate in all patients. The cytokines TNF-α, IL-1, IL-6, IL-8, IL-10, and IL-12 were evaluated by cytometric bead array (CBA) assays (human Th1/Th2 cytokine kit, BD Biosciences, San Diego, CA, USA). For this assay, soluble cytokines are captured on microparticle beads and measured using a fluorescence-based detection system and flow cytometric analysis, as previously described [32]. A series of 10 dilutions of cytokine standards was run in each assay for the generation of standard curves. Samples were analyzed on a FACSCalibur flow cytometer using the BD CBA analysis software.

### Statistical analysis

Paired data from patients at different times of follow up were compared using the Wilcoxon signed-rank test. Data from groups of unrelated subjects were compared using the Mann-Whitney U-test. Correlation between HBV DNA and HBc-specific SI was assessed using Spearman's ρ test.

## Results

### Signal-to-cut-off ratios to predict true positive in a chemiluminescent assay for anti-HBc and classified as OBI carriers or spontaneous HBV resolvers

Among the group of 4,252 samples with anti-HBc only, test reactivity (represented as Co/s) ranged between 1.1 to 20.6. Of the 4,252 samples collected, 36 samples had detectable HBV DNA and presented  $Co/s \geq 10.0$  in the CMIA assay and were thus classified as “true positive to anti-HBc” (i.e., OBI); HBV DNA was not detected in the remaining 4,216 anti-HBc only samples. Of the 4,216 anti-HBc only samples, 3,907 samples presented  $Co/s \leq 6.0$  in the CMIA assay, undetectable HBV DNA and no reactivity to anti-HBc in the subsequent donations. Thus, we classified these samples (n=3,907) as “false-positive” to anti-HBc in the CMIA assay, and these samples were excluded from this study. The remaining 309 samples presented  $Co/s > 8.0$  in the CMIA assay, reactivity to anti-HBc in the subsequent donations and undetectable HBV DNA and were classified as “true positive” – spontaneous HBV resolvers reactive to anti-HBc in the CMIA assay (Figure 1).

### Serological HBV screening and HBV DNA detection

Of the 250,136 blood donations, HBsAg reactivity was found in only 250 (0.1%) donations, and HBsAg plus anti-HBc was observed in 1125 (0.45%) donations. Both HBsAg and anti-HBc were absent in 244,507 (97.75%). Of these blood donations, 4,252 (1.7%) were positive for anti-HBc, and none of them had detectable HBsAg in the serum. Of these 4,252 (HBsAg-/anti-HBc+), only 36 samples had detectable HBV DNA (OBI). Among the 250 samples with primary infection (HBsAg+/anti-HBc-), 54 samples had detectable HBV DNA. Among the 1125 samples with chronic infection (HBsAg+/anti-HBc+), 438 had detectable HBV DNA (Figure 1). Serological markers of HBV exposure or vaccination (anti-HBs) were observed in 18.8% (38/434) of the HBV carriers; 21% (105/500) of healthy donors; 38.3% (118/309) of Spontaneous HBV resolvers and 5.5% of OBI (2/36). Reactivity to IgM anti-core was observed in 2.5% (11/434) and 5.5% (2/36) of HBV carriers and OBI, respectively. HBe antigen was observed in all blood donors with OBI and in HBV carriers, but not in healthy donors or Spontaneous HBV resolvers. However, 11.1% (4/36) of OBI and 12.5% of (54/434) HBV carriers were anti-HBe-positive.

### HBcAg-specific T cell response

The proliferative response of peripheral blood lymphocytes to nonspecific (PHA) and specific stimuli (e.g., HBV core peptide or HCV NS5 antigen) was analyzed in HBV carriers, healthy donors, OBI and spontaneous HBV resolvers. Healthy blood donors presented  $SI < 3.0$  in lymphoproliferation. The median SI obtained in HBV carriers and the OBI group was 13.6 and 12.2 respectively. Spontaneous HBV resolvers exhibited a large peripheral blood mononuclear cell (PBMC) response to HBcAg ( $SI=36.2$ ) compared to HBV carriers and OBI ( $P < 0.001$ ) (Figure 2).

### NK cell cytotoxicity assay

Higher NK cytotoxic activity was observed in spontaneous HBV resolvers ( $85 \pm 3.5\%$ ) compared to HBV carriers ( $56 \pm 2.7\%$ ), healthy blood donors ( $20 \pm 1.1\%$ ) and OBI ( $61 \pm 2.1\%$ ) (Figure 3).

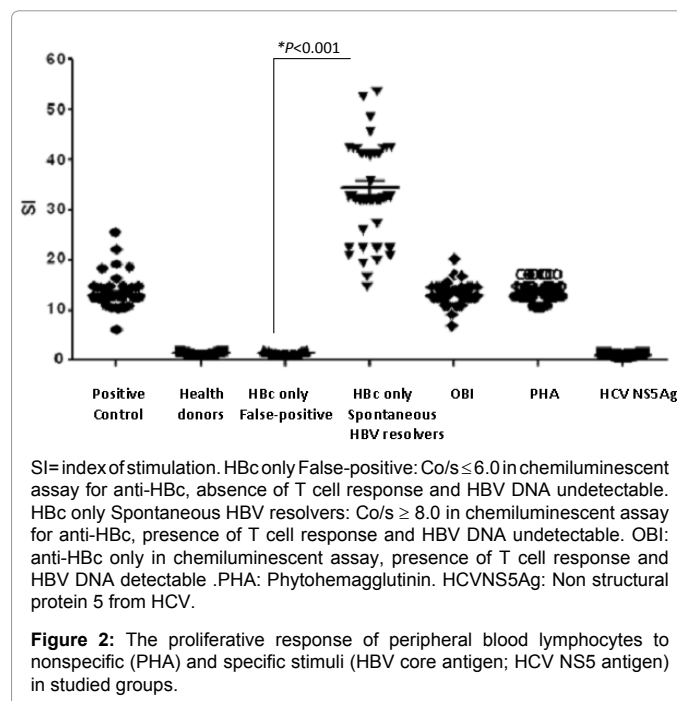
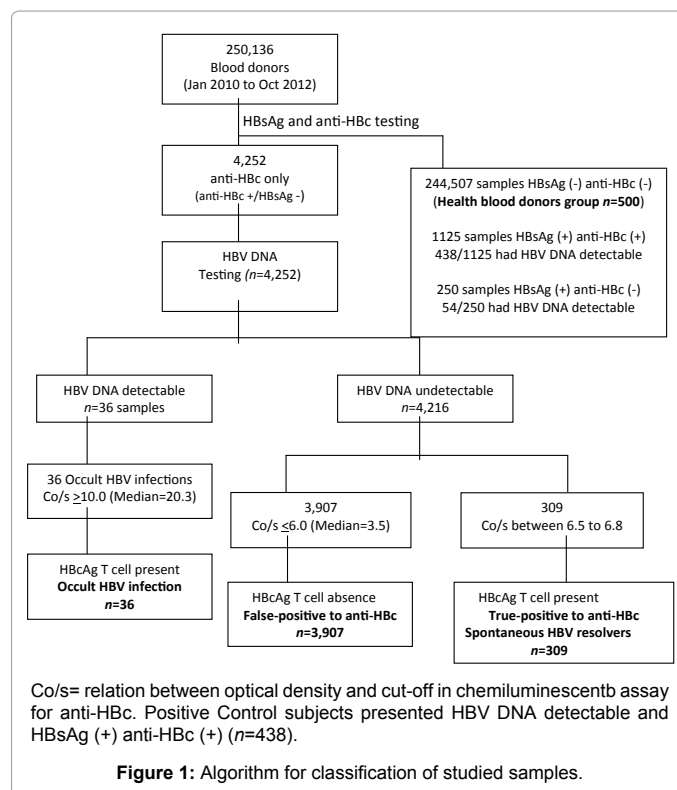
### Enzyme-linked immunospot (ELISpot) assay for IFN- $\gamma$

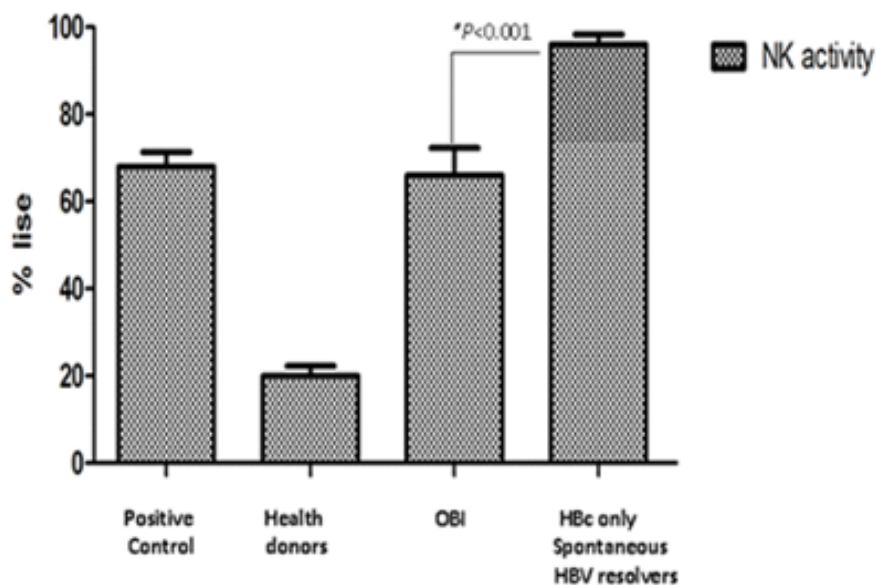
ELISPOT-IFN- $\gamma$  assays were positive in OBI, spontaneous HBV resolvers, healthy blood donors and HBV carriers. The magnitude of T cell responses to HBcAg in spontaneous HBV resolvers ranged from 1,020 to 1,628 ISCs/106 PBMCs, whereas they ranged from 132 to

548 ISCs/106 PBMCs among OBI, from 160 to 582 ISCs/106 PBMCs, among HBV carriers and from 8 to 15 ISCs/106 PBMCs among healthy blood donors. In spontaneous HBV resolvers, the frequency of positive IFN- $\gamma$  ELISPOT responses indicated higher sustained T cell responses compared to HBV carriers ( $P < 0.001$ ) and OBI ( $P < 0.001$ ) (Figure 4).

### Cytokine patterns

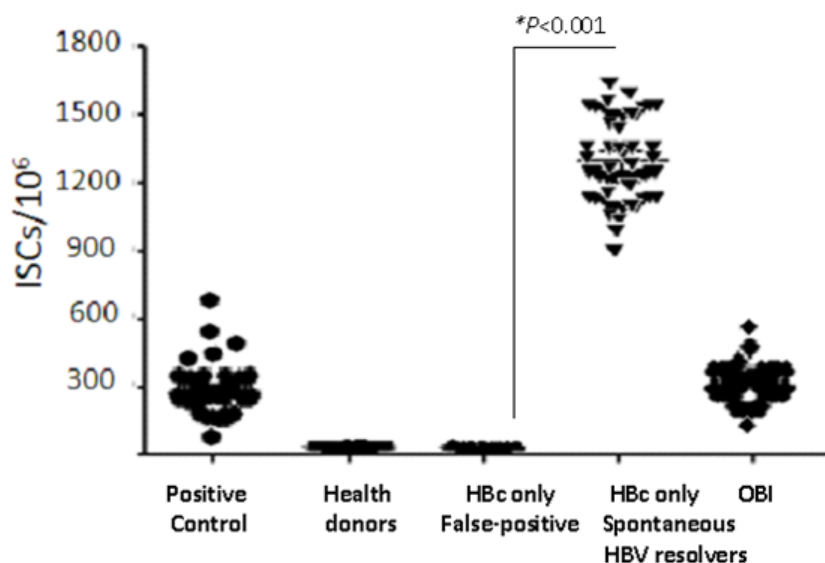
IL-8 and IL-12 serum levels increased significantly ( $P < 0.001$ )





%. Percentage of lise of K562 cells by NK cells. HBc only False-positive: Co/s  $\leq$  6.0 in chemiluminescent assay for anti-HBc, absence of T cell response and HBV DNA undetectable. HBc only Spontaneous HBV resolvers: Co/s  $\geq$  8.0 in chemiluminescent assay for anti-HBc, presence of T cell response and HBV DNA undetectable. OBI: anti-HBc only in chemiluminescent assay, presence of T cell response and HBV DNA detectable.

**Figure 3:** NK cell activities in HBV carriers, health blood donors, spontaneous HBV resolvers and OBI.



ISCs: index spot-forming cells index. HBc only False-positive: Co/s  $\leq$  6.0 in chemiluminescent assay for anti-HBc, absence of T cell response and HBV DNA undetectable. HBc only Spontaneous HBV resolvers: Co/s  $\geq$  8.0 in chemiluminescent assay for anti-HBc, presence of T cell response and HBV DNA undetectable. OBI: anti-HBc only in chemiluminescent assay, presence of T cell response and HBV DNA detectable.

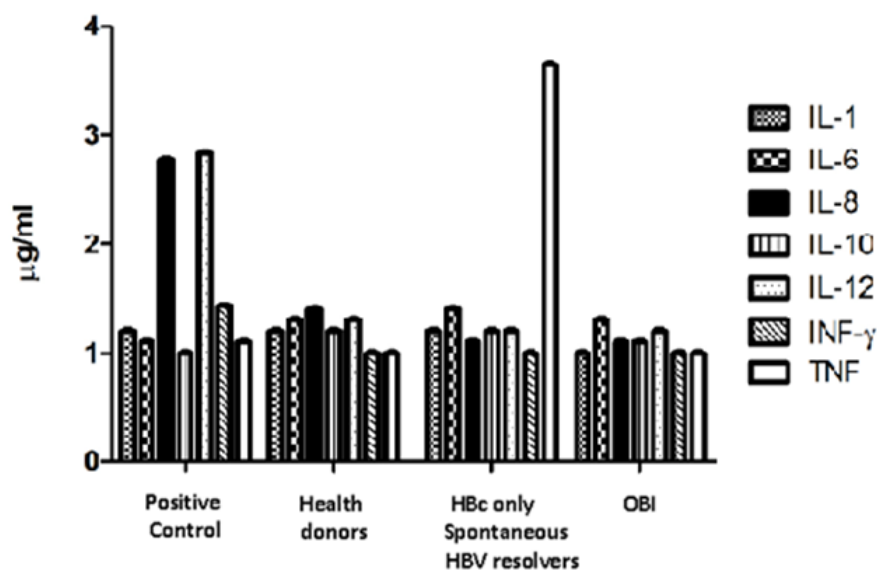
**Figure 4:** ELISPOT-INF- $\gamma$  assays in HBV carriers, health blood donors, spontaneous HBV resolvers and OBI.

in HBV carriers (920.8 $\pm$ 1.1 pg/mL) and OBI (803.7 $\pm$ 1.0 pg/mL). IL-6 serum levels increased (P <0.05) in spontaneous HBV resolvers (177.8 $\pm$ 1.0 pg/mL) compared to HBV carriers and OBI (90.1 $\pm$ 1.1 pg/mL and 94.4 $\pm$ 1.3 pg/mL). INF- $\gamma$  serum levels increased (P <0.05) in spontaneous HBV resolvers (75.7 $\pm$ 1.0 pg/mL) compared to HBV carriers and OBI (22.3 $\pm$ 1.1 pg/mL and 34.6 $\pm$ 1.3 pg/mL). TNF- $\alpha$  serum levels increased significantly (P <0.001) in spontaneous HBV resolvers (1465.3 $\pm$ 1.0 pg/mL) compared to HBV carriers and OBI (78.0 $\pm$ 1.1 pg/mL and 67.2 $\pm$ 1.3 pg/mL). In spontaneous HBV resolvers (stimulation

index >36,2), TNF- $\alpha$  levels (30.0 $\pm$ 1.0 pg/mL) correlated with both HBcAg-specific T cell responses (r=0.00002) and NK cell activity (r=0.00009). The serum levels of IL-1 and IL-10 were not significantly different among HBV carriers, OBI and spontaneous HBV resolvers (Table 1).

## Discussion

Determining the frequency of true HBc alone profiles in blood



Ug/ml: Concentration of cytokine analysed by flow cytometry. Hbc only Spontaneous HBV resolvers: Co/s  $\geq$  8.0 in chemiluminescent assay for anti-HBc, presence of T cell response and HBV DNA undetectable. OBI: anti-HBc only in chemiluminescent assay, presence of T cell response and HBV DNA detectable.

**Figure 5:** Cytokine patterns in HBV carriers, health blood donors, spontaneous HBV resolvers and OBI.

Cytokines pg/ml	M-HBV carrier	M-Health Blood Donor	M-Spontaneous HBV resolvers	M - OBI	P value
IL-1	98.7	11.3	95.2	97.9	No
IL-6	90.1	10.5	167.8	94.3	<0.05
IL-8	920.8	12.1	114.3	803.7	<0.001
IL-10	68.4	19.8	61.5	66.9	No
IL-12	924.8	10.4	100.8	844.1	<0.001
INF- $\gamma$	22.3	8.6	75.7	34.6	No
TNF- $\alpha$	78.0	12.8	1465.3	67.2	<0.0001

Pg/ml: Concentration of cytokine analysed by flow cytometer. HBV carrier: HBsAg (+) / anti-HBc (+) and HBV DNA detectable. Spontaneous HBV resolvers: Co/s  $\geq$  8.0 in CMIA for anti-HBc, Subsequent reactivity for anti-HBc and HBV DNA undetectable, **OBI**: anti-HBc only in CMIA and HBV DNA detectable. M = median of cytokine concentration. P value = calculated between HBV carrier and OBI versus Spontaneous HBV resolvers. No = no statistically significant.

**Table 1:** Cytokine patterns in HBV carriers, health blood donors, spontaneous HBV resolvers and OBI.

donors is critical to blood safety strategies and for identifying resolved infections, escape mutants and OBI. Algorithms including multiple successive assays are necessary, but not sufficient, to identify true reactivity to anti-HBc39. To meet this challenge, Japanese blood banks have set up an anti-HBc cut-off agglutination title of  $\geq$  32; below this level, donations are considered non-infectious (presumably false positive or with undetectable anti-HBs) [24].

In this study, 91.8% of all results were false positives for anti-HBc via CMIA. This frequency is extremely high, but it was confirmed by the absence of a response to HBcAg in lymphoproliferation assay and a lack of INF- $\gamma$  production. These data suggest that antibody titers indeed correlated with HBcAg-T cell response and ELISPOT-INF-. The high frequency of false-positive results could be the result of loss of blood units, donor stigma and interference in elucidating the meaning of the anti-HBc alone profile in blood donors.

The anti-HBc alone profile can be masked by OBI in blood donors. In this study, we identified 1 OBI present among 118 HBc alone profile blood donors. During the study period, we found OBI in 1 of 6948 blood donors, corroborating the results of a previous study in Brazil [25,26].

Surprisingly, we found anti-HBs in OBI and HBV carriers. In vaccinate OBI, Stramer SL, Zou S and Notari EP et al. [27], observed

low levels of anti-HBs (3–100 IU/L). HBV strains isolated in these vaccinated donors were wild type, quasi-species and escape mutations. It has been concluded that low anti-HBs levels induced by vaccination protect against hepatitis B disease and chronic infection but favor OBI [28]. The generation of new hepatitis B vaccines that are protective against escape mutants might provide a solution.

Resistance or susceptibility to HBV reflects a complex interplay between the virus and immune response. The T cell response is considered a key factor in determining the outcome of infection, and it has been shown that chronic HBV (susceptibility) is associated with the presence of dysfunctional immune responses [29-31]. In this context, we observed that spontaneous HBV resolvers presented a higher magnitude of HBcAg-specific T cell response compared with HBV carriers and OBI.

However, in addition to the T cell response, the role of NK cells in viral clearance during acute HBV infection was also supported by previous reports showing that early large quantities of INF- $\gamma$  production by NK cells may contribute to the initial control of infection and allow the timely development of an adaptive immune response [19-21]. In this study, we did not measure INF- $\gamma$  production by NK cells, but we only investigated the cytotoxicity capacity in the presence or absence of HBV DNA. INF $\gamma$  production by T cells was measured to observe the

regulatory effect of T cells on NK activity via INF- $\gamma$ . Similar to the T cell responses, higher NK cytotoxic activity was observed in spontaneous HBV resolvers compared to HBV carriers and OBI. INF- production by T cells was higher in spontaneous HBV resolvers compared with OBI. Higher T cell responses and INF- $\gamma$  production correlated with the higher cytotoxic activity of NK cells. In cases of HBV persistence, we observed the opposite scenario: low T cell responses, INF- $\gamma$  production and NK cell activity.

Could other cytokines influence this immunological scenario? The serum levels of IL-1 and IL-10 were similar in the presence (OBI and HBV carriers) or absence (spontaneous HBV resolvers) of HBV DNA. This result contrasts the findings of Saxena R, Chawla YK, Verma I, Kaur J [32], who observed a key role for IL-1 in the progression of HBV-mediated disease in the Indian population, due to an IL-1 polymorphism. Unfortunately, we do not have an analogous evaluation in the Brazilian population to allow a comparison of the studies.

IL-8 and IL-12 serum levels were increased significantly in the presence of HBV DNA (OBI and HBV carrier). In a recent study, He D, Yan G, Wang Y. [33] demonstrated that IL-12 can facilitate immune evasion and maintain the persistence of HBV. Xue Qin, Yan Deng, Xiang-Cheng Liao, et al. [34] observed that IL-8 may influence the inflammatory process during the pathological stage of hepatitis B infection. Both studies correlated IL-8 and IL-12 with the persistence of HBV DNA, corroborating our findings.

IL-6 is a pleiotropic cytokine with pivotal functions in the regulation of the biological responses of several target cells, including hepatocytes. Serum IL-6 levels have been reported to be elevated in patients with chronic hepatitis B infection, cirrhosis and hepatocellular carcinoma, and serum IL-6 was identified as the best marker of HBV-related clinical progression compared with several other cytokines [35]. In this study, IL-6 serum levels were high ( $P < 0.05$ ) in spontaneous HBV resolvers compared with HBV carriers and OBI, in agreement with the literature.

TNF- $\alpha$  is an extremely critical cytokine in host immune responses to viral infection. Circulatory TNF- $\alpha$  levels increase during HBV infection. Increased hepatic TNF- $\alpha$  production is associated with the suppression of HBV replication in transgenic mice that express HBV in the liver. In fact, the regulation of the TNF- $\alpha$  gene and the possibility of the production of TNF- $\alpha$  variants have attracted significant attention. Polymorphisms are reported in different populations but have not yet been reported in Brazilian populations; however, contradictory results were found regarding the relationships of the polymorphism with the progression of HBV infection [36,37]. In HBc alone blood donors and undetectable HBV DNA (spontaneous HBV resolvers), we observed extremely high TNF- $\alpha$  serum levels ( $P < 0.0001$ ) compared with the HBc alone profile with detectable HBV DNA, similar to OBI and HBV carriers.

Together, the NK cell activity, HBcAg-specific T cell responses and cytokine patterns observed in this study suggest that a balance of the magnitudes of these immune responses determines susceptibility versus resistance to HBV. Furthermore, the high levels of TNF- $\alpha$  in spontaneous HBV resolvers suggest that TNF- $\alpha$  might influence this balance.

What mechanism accounts for these findings? It is not yet clear, but defects in various intrinsic cell signaling pathways, such as killer immunoglobulin-like receptors (KIR) and human leukocyte antigen (HLA), have been suggested to play the role of "maestro" in viral control.

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