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SHORT COMMUNICATION

KIR and a specific *HLA-C* gene are associated with susceptibility and resistance to hepatitis B virus infection in a Brazilian population

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BRIEF REPORT

The activity of natural killer (NK) cells is partially regulated by killer cell immunoglobulin-like receptors (KIRs) interacting with human leukocyte antigen C (HLA-C) ligands.[1] The ligands of several inhibitory (2DL and 3DL) and activating (2DS and 3DS) *KIR* have been described.[2] Clinical observations of hepatitis B virus (HBV) infection reveal that some cases progress to liver failure, fibrosis, cirrhosis and hepatocellular carcinoma, whereas infection resolution occurs in other cases. However, the mechanisms involved in the susceptibility or resistance to HBV are not completely understood.

Study subjects were recruited from a blood bank, COLSAN-Associação Beneficente de Coleta de Sangue (São Paulo, Brazil), during the period between January 2010 and December 2010. We selected 20 occult HBV infection (OBI) cases and 40 spontaneous HBV resolvers (SHRs). As control subjects, we enrolled 80 healthy blood donors and 42 HBV carriers ($HBsAg^+$, $anti\text{-}HBc^+$ and detectable HBV DNA). The study was approved by the Ethics Committee of the Federal University of Sao Paulo, Brazil, and all study participants signed an informed consent. Sera were screened for anti-HBc and HBsAg using a commercial chemiluminescent microparticle immunoassay (Abbott, Wiesbaden, Germany). A commercial test was used for the detection of HBV DNA (HBV Monitor; Roche, Nutley, NJ, USA). An HBcAg-specific T-cell response, *ex vivo* NK cell activity assay and enzyme-linked immunospot assay for interferon-gamma were performed as previously described.[3] The cytokines tumor-necrosis factor-alpha (TNF-α), IL-1, IL-6, IL-8, IL-10 and IL-12 were evaluated using cytometric bead array assays (human Th1/Th2 cytokine kit; BD Biosciences, San Diego, CA, USA). IL-2, IL-4 and IL-18 assays were performed using an enzyme immunoassay (BioLegend, San Diego, CA, USA). *HLA-C* and *KIR* genotyping was performed using a Luminex MultiAnalyte profiling system (One Lambda, Inc., Canoga Park, CA, USA) with the LABType SSO OneLambda typing kit (One Lambda, Inc.). *KIR* locus typing was performed to detect the presence or absence of 15 known *KIR* genes, including 2DL1-5, 2DS1-5, 3DL1-3, 3DS1 and the pseudogene *KIR*3DP1.

The T-cell response is thought to be a key factor determining the outcome of infection, and it has been shown that chronic HBV is associated with the presence of dysfunctional immune responses.[4] Within this context, we observed that SHRs presented a higher magnitude of HBcAg-specific T-cell responses (Stimulation Index (SI)=SI=62.3, $P<0.0001$) in comparison to HBV carriers (SI=25.4) and OBI cases (SI=26.2).

The role of NK cells in viral clearance during acute HBV infection is also supported by previous reports showing that early high interferon-gamma production by NK cells may contribute to initial control of the infection and allow the timely development of an adaptive immune response.[1,5,6] In this study, INF-γ production by T cells was measured to observe the regulatory effect of T cells on NK cell activity *via* INF-γ. Higher NK cell activity (93%±3.1%, $P=0.005$) was observed in SHRs compared to HBV carriers (58%±2.5%) and OBI cases (57%±2.3%), and INF-γ production by T cells was higher in SHRs (1852±2.2 Intracytoplasmic cytokine staining (ISCs)/10^6 PBMCs, $P<0.0001$) compared to OBI cases (653±2.0 ISCs/10^6 PBMCs) and HBV carriers (685±2.3 ISCs/10^6 PBMCs). Higher T-cell responses and INF-γ production correlated with higher NK cell activity ($P<0.0001$). However, the

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Table 1A Immune parameters results in health blood donors, HBV carrier, OBI and spontaneous HBV resolvers

	Study groups			
Immune parameter	*Health blood donors*	*HBV carrier*	*OBI*	*Spontaneous HBV resolvers*
HBc specific T-cell response	SI<3.0	Median SI=25.4	Median SI=26,2	Median SI=62,3***
NK cell activity	20%±1.2%	58%±2.5%	57%±2.3%	93%±3.1***
INF-γ production by ELIspot	8–21 ISCs/10^6	187–685 ISCs/10^6	141–653 ISCs/10^6	1110–1852 ISCs/10^6***
IL-1 levels	0.5±0.2 pg/ml	3.7±1.5 pg/ml	3.8±1.4 pg/ml	4.9±1.2 pg/ml
IL-2 levels	1.7±0.2 pg/ml	8.2±1.7 pg/ml	7.7±1.3 pg/ml	15.2±1.9 pg/ml*
IL-4 levels	2.3±0.8 pg/ml	108.3±1.8 pg/ml*	102.3±1.1 pg/ml*	84.7±1.6 pg/ml
IL-6 levels	0.7±0.2 pg/ml	28.4±1.3 pg/ml**	27.1±1.1 pg/ml**	7.8±1.0 pg/ml
IL-8 levels	0.8±0.3 pg/ml	920.8±1.1 pg/ml**	803.7±3.0 pg/ml**	369.4±8.0 pg/ml
IL-10 levels	0.2±0.2 pg/ml	4.1±1.8 pg/ml	3.5±1.2 pg/ml	5.3±1.7 pg/ml
IL-12 levels	0.5±0.4 pg/ml	1008±10.1 pg/ml***	1110±13.0 pg/ml***	471.2±5.0 pg/ml
IL-18 levels	98.1±10.2 pg/ml	245.6±8.3 pg/ml	301.8±6.7 pg/ml	489.6±10.2 pg/ml*
INF-γ levels	1.3±0.8 pg/ml	58.3±2.8 pg/ml	62.3±1.1 pg/ml	175.7±1.0 pg/ml**
TNF-α levels	0.5±0.3 pg/ml	652.3±11.3 pg/ml	753.1±8.9 pg/ml	1465±11.0 pg/ml***

Abbreviations: ELIspot: enzyme-linked immunospot assay for IFN-γ; IFN-γ : interferon-gamma; IL: interleukin; OBI: occult hepatitis B infection; TNF-α: tumor-necrosis factor-alpha.
HBV carrier and OBI patients *vs.* spontaneous HBV resolvers: ***$P<0.0001$; **$P<0.001$; *$P<0.05$.

opposite scenario was observed in cases of HBV persistence: low T-cell responses, INF-γ production and NK cell activity ($P<0.0001$).

We then assessed whether cytokines other than INF-γ have an influence on this immunological scenario and found that IL-8 and IL-12 serum levels were significantly increased in HBV carriers (920.8±1.1 pg/ml and 1008±10.1 pg/ml, respectively) and OBI cases (803.7±1.0 pg/ml and 1110±13.0 pg/ml, respectively) compared to SHR (471.2±5.0 pg/ml, $P<0.0001$, respectively). In a recent study, it has been demonstrated that IL-12 can facilitate immune evasion and maintain the persistence

Table 1B Frequency of KIR genes and HLA-C1 and HLA-C2 in health blood donors, HBV carrier, OBI and spontaneous HBV resolvers

	Study groups			
	Health blood donors	*HBV carrier*	*OBI*	*Spontaneous HBV resolvers*
KIR gene and HLA-C	n/*total (frequency%)*	n/*total (frequency%)*	n/*total (frequency%)*	n/*total (frequency%)*
2DL1	39/80 (48.7%)	20/42 (47.6%)	9/20 (45.0%)	18/40 (450%)
2DL2	41/80 (51.2%)	21/42 (50.0%)	10/20 (50.0%)	19/40 (47.5%)
2DL3	41/80 (51.2%)	29/42 (52.4%)	9/20 (45.0%)	37/40 (92.5%)**$^{\psi}$
2DL4	37/80 (46.2%)	19/42 (45.2%)	10/20 (50.0%)	20/40 (50.0%)
2DL5	38/80 (47.5%)	20/42 (47.6%)	9/20 (45.0%)	19/40 (47.5%)
2DP1	40/80 (50.0%)	22/42 (52.4%)	11/20 (55.0%)	21/40 (52.5%)
2DS1	42/80 (52.5%)	39/42 (92.8%)**$^{\psi}$	18/20 (95.0%)**$^{\psi}$	14/40 (35.0%)
2DS2	39/80 (48.7%)	21/42 (50.0%)	10/20 (50.0%)	22/40 (55.0%)
2DS3	37/80 (46.2%)	28/42 (66.6%)	13/20 (65.0%)	25/40 (62.5%)§
2DS4	35/80 (43.7%)	20/42 (47.6%)	11/20 (55.0%)$^{¥}$	20/40 (50.0%)
3DL1	32/80 (40.0%)	18/42 (42.8%)	9/20 (45.0%)	21/40 (52.5%)§
3DL2	36/80 (45.0%)	22/42 (52.3%)	12/20 (60.0%)$^{¥}$	19/40 (47.5%)
3DL3	33/80 (41.2%)	19/42 (45.2%)	9/20 (45.0%)	19/40 (47.5%)
3DP1	41/80 (51.2%)	21/42 (50.0%)	11/20 (55.0%)	25/40 (62.5%)§
3DS1	36/80 (45.0%)	25/42 (59.5%)$^{\psi}$	9/20 (45.0%)	21/40 (52.5%)
HLA-C1C2	39/80 (48.7%)	21/42 (50.0%)	9/20 (45.0%)	19/40 (47.5%)
HLA-C1 homozygous	51/80 (63.7%)	39/42 (92.8%)**	19/20 (95.0%)**	14/40 (35.0%)
HLA-C2 homozygous	53/80 (66.2%)	37/42 (88.1%)$^{\psi}$	16/20 (80.0%)	38/40 (95.0%)*

Abbreviations: HBV, hepatitis B virus; HLA-C, human leukocyte antigen C; KIR, killer cell immunoglobulin-like receptor; OBI: occult hepatitis B infection.
HBV carrier and OBI patients *vs.* spontaneous HBV resolvers: **$P<0.001$; *$P<0.05$.
Health blood donors *vs.* HBV carrier: $^{\psi}P<0.05$.
Health blood donors *vs.* OBI: $^{¥}P<0.05$.
Health blood donors *vs.* spontaneous HBV resolvers: $^{\S}P<0.05$.

of HBV,[7] and it has also been observed that IL-8 may influence the inflammatory process during the pathological stage of hepatitis B infection.[8] Both studies correlated IL-8 and IL-12 with the persistence of HBV DNA, corroborating the findings reported in the current study. IL-4 production in OBI cases (102.3±1.1 pg/ml) and HBV carriers (108.3±1.8 pg/ml) compared to SHRs (84.7±1.6 pg/ml) were significant, agreeing with previous studies that reported a relationship between IL-4 production and viral persistence in hepatitis B infection.[9]

Serum levels of IL-6 were also increased in OBI cases (17.1±1.1 pg/ml) and HBV carriers (18.4±1.3 pg/ml) compared to SHRs (7.8±1.0 pg/ml) ($P<0.05$). Serum IL-6 levels have been reported to be elevated in individuals with chronic hepatitis B infection, cirrhosis and hepatocellular carcinoma,[10] and serum IL-6 levels were in agreement with this reported relationship between this cytokine and the progression of HBV infection. INF-γ and IL-2 serum levels increased in SHRs (175.7±1.0 pg/ml and 15.2±1.9 pg/ml, respectively) compared to HBV carriers (58.3±2.8 pg/ml and 8.2±1.7 pg/ml, respectively) and OBI cases (62.3±1.1 pg/ml and 7.7±1.3 pg/ml, respectively).

Both INF-γ and IL-2 may be a stimulus for the activation of NK cells and higher levels of T-lymphocyte activation, and both cytokines correlate with control of HBV infection. Another major cytokine, IL-18, has also been reported to be involved with resistance to HBV infection.[11] In this study, SHR showed higher IL-18 serum levels (489.6±10.2 pg/ml) compared to OBI cases (301.8±6.7 pg/ml) and HBV carriers (245.6±8.3 pg/ml). IL-18 is mediated by INF-γ production, which is also high in the serum of SHRs. TNF-α serum levels were significantly increased in SHRs (1465±11.0 pg/ml) compared to HBV carriers and OBI cases (652.3±11.3 pg/ml and 753.1±8.9 pg/ml, respectively; $P<0.0001$).

TNF-α is an extremely critical cytokine for host immune responses to viral infection. Indeed, circulatory TNF-α levels increase during HBV infection,[10] and increased hepatic TNF-α production is associated with the suppression of HBV replication in transgenic mice expressing HBV in the liver.[12]

Altogether, 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 (Tables 1A and 1B). In SHRs, a significant HBcAg-specific T-cell response and high TNF-α levels correlated with high NK cell activity (Odds Ratio (OD)=2.65, $P<0.0001$) and the absence of viral DNA (OR=2.03, $P<0.0001$). In OBI cases and HBV carriers, high IL-8 and IL-12 levels correlated with low NK cell activity and HBcAg-specific T-cell responses (OR=1.99, $P<0.001$) and the presence of viral DNA (OR=2.78, $P<0.001$). Serum levels of IL-1 and IL-10 were similar in the presence (OBI cases and HBV carriers) or absence (SHRs) of HBV DNA, a result that is in contrast to what has been previously described. A previous study demonstrated a key role for IL-1 in the progression of HBV-mediated disease due to an IL-1 polymorphism.[13]

With regard to the mechanism accounting for this immunological pattern in the presence or absence of HBV DNA, it is surprising that the immunological profile (T-cell response, NK cell activity and cytokine production) obtained in the HBV DNA-positive (OBI cases and HBV carriers) and HBV DNA-negative groups (SHR) were associated with the frequency of a specific pattern of *KIR* and *HLA-C* (OR=2.87, $P=0.0001$). The frequency of blood donors with *HLA-C2* homozygosity was higher in OBI cases (50.6%) compared to SHRs (35.8%); a reciprocal association of *HLA-C1* homozygosity with SHRs (92% in resolved *versus* 80.2% in persistent infection) was also observed. The presence of *KIR2DL1* and *KIR2DL2* (both inhibitory receptors) was observed in all the studied groups, and also, the *KIR2DL3* activation receptor was found at a high frequency (92.8%) in SHRs in the current study. In SHRs, the interaction between *KIR2DL3* and *HLAC1* homozygosity (high affinity) was associated with high NK cell activity, HBcAg T-cell responses and significant TNF-α production (OR=2.91, $P=0.0001$).

The interaction between *KIR2DS1* and *HLAC2* homozygosity was related to persistent infection[17], as we observed an association of HBV carriers and OBI cases with low NK cell activity and HBcAg T-cell responses and high production of IL-6, IL-8 and IL-12. *HLA-C1C2* was present in all of the studied groups and did not demonstrate a specific relationship. It has been reported that *KIR2DL3:HLA-C1* homozygosity was protective in a Chinese population,[14] whereas *KIR2DL1:HLA-C2* was associated with HBV infection susceptibility according to subsequent findings.[15,16] Unfortunately, studies of *KIR/HLA-C* interactions in Brazilian populations are focused on autoimmune diseases and infectious diseases other than hepatitis B.

In summary, the interaction between *KIR* genes and *HLA* is important for determining antiviral immunity and contributes to the protection against or susceptibility to HBV infection. Further research is required to describe other immunological and molecular mechanisms related to susceptibility to or protection from hepatitis B infection.

COMPETING FINANCIAL INTEREST

No conflict of interest to declare.

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