**TITLE PAGE**

**TITLE:** Hepatitis B virus replication influences levels of expression of Natural Killer cell ligands

**Short title:** Hepatitis B virus modulates Natural Killer ligands

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**AUTHOR CONTRIBUTIONS**

LK, Designed the study, performed the experiments, interpreted data, wrote the manuscript, revised the work for intellectual content, confirmed that the questions of the hypothesis were investigated and acted as corresponding author. TP, Contributed in the design of the experimental protocols and interpretation of data, supervised development of work and manuscript evaluation, revised the work for intellectual content, approved the final version of the manuscript, agreed that the questions of the hypothesis were investigated. GR, Supervised the development of work, approved the final version of the manuscript. NK, Obtained the informed consent details from all the patients, approved the final version of the manuscript. PK, Supervised the development of work, approved the final version of the manuscript. SK, Designed the study and experimental protocols, interpreted the data, supervised development of work, revised the study for intellectual content, approved the final version of the manuscript, confirmed that the questions of the hypothesis were investigated.

**ABSTRACT**

Hepatitis B virus (HBV) is one of the most fatal human pathogens accounting for over one million deaths annually due to immune-mediated chronic liver damage. Natural killer (NK) cells are abundant in the liver and play a key role in HBV persistence. The cytotoxic effects of NK cells are regulated by signals from multiple activating and inhibitory receptors. Cumulative evidence shows that HBV circumvents host antiviral immunity via the regulation of NK receptors and their ligands. In this study we investigated the effect of viral replication and HBeAg mutations on the expression of NK mediators both in a cohort of 19 chronic HBV pa tients and in a cell culture system. HBV monomers bearing hotspot mutations in the basal core promoter (BCP) and precore region were transfected into HepG2 cells using a plasmid-free assay. We determined the RNA expression of NKG2D ligands, B7H6, DNAX accessory molecule-1 (DNAM-1), lectin-like transcript 1 (LLT1), LFA-1 and TNF-related apoptosis-inducing ligand (TRAIL) in the livers of chronic patients and transfected cells. We found that in general high HBV replication upregulated mRNA of NK cell ligands, but specifically downregulated expression of MHC class-I polypeptide-related chain A (MICA) (p=0.02). Expression of the inhibitory NK cell ligand, LLT1, was increased both *in vivo* and *in vitro*. These data show that HBV replication has differential effects on NK cell ligands and suggest potential escape mechanisms through up-regulation of LLT1 and down-regulation of MICA.

**Key words**

Hepatitis B virus, NK cells, virus, LLT1, NKG2D, mutations, precore, viral replication

**INTRODUCTION**

Hepatitis B virus (HBV) is a hepatotropic DNA virus infecting humans for more than 1500 years[1]. To-date HBV is a leading cause of liver diseases worldwide, with more than 350 million people being persistently infected and at high risk developing liver failure, cirrhosis or hepatocellular carcinoma (HCC)[2,3]. An increased proportion of chronic disease is due to the emergence of viral variants that either abrogate or reduce HBeAg production[4]. One of the most critical mutations is the double A1762T/G1764A substitution in the basal core promoter (BCP) region and its appearance is usually followed by the development of the triple mutant A1762T/G1764A/C1766T[5]. The most common mutation that results in an HBeAg-negative phenotype is the G1896A variant of the precore region and is usually accompanied by a second G1899A mutation[6]. Variants with these mutations associate with more severe forms of liver damage and the development of HCC[7,8]. Studies on the effect of BCP mutations on viral replication remain controversial. Among other studies we have shown that they induce low viral transcription and replication[9-11] [Koumbi L, 2016, manuscript submitted]. Precore mutated variants associate with high viral replication or have no effect on viral load[11-13] [Koumbi L, 2016, manuscript submitted].

The outcome of HBV infection depends on the intensity and type of anti-viral immunity produced by the host. In acute infection the adaptive immune system mounts a strong, multi-specific response, but its effectiveness depends on the quality of the earlier innate immune response. NK cells are important effectors of the innate antiviral immunity. In addition to the direct killing of viral-infected cells without antigen-specific priming, they regulate the adaptive immune response by producing IFN-γ, TNF-α and immunoregulating cytokines. NK cells are enriched markedly in the liver, the site of HBV replication, implying that HBV has to evade NK cell-mediated immune responses to establish a persistent infection. The high NK activity early in infection and during the incubation time suggests that they are key plays in viral clearance[14-17]. NK cells, however, can also negatively regulate specific antiviral immunity in CHB infection by contributing in the liver inflammation through TNF-related apoptosis-inducing ligand (TRAIL)- and Fas-mediated death[17,18] and by the direct killing of HBV-specific CD8+ T cells, which triggers the recruitment of inflammatory cells that sustain and amplify the hepatic damage[19,20].

The effector functions of NK cells are tightly regulated by the dynamic and coordinated balance of activating and inhibitory signals that are delivered by a diverse array of cell surface receptors. The main NK cell activating receptors studied in this respect include C-type lectin-like NK group 2D (NKG2D) receptor and immunoglobulin-like receptor NKp30. NKG2D is a potent activating receptor expressed in all NK cells, NKT cells and activated CD8+ T lymphocytes[21,22]. It binds to a family of ligands (NKG2DL) including the MHC class-I polypeptide-related chain (MIC) protein family that contains MICA and MICB; and the cytomegalovirus UL16-binding proteins (ULBP) family, which consists of five members, ULBP1-4 and RAET1G[23]. Another activating receptor involved in the direct regulation of NK cell-mediated recognition of cellular stress is the DNAX accessory molecule-1 (DNAM-1, CD226) that is constitutively expressed on NK cells, T cells, macrophages and a small subset of B cells[24]. Similar to NKG2D, DNAM-1 not only promotes adhesion and activation of NK cells and CTLs but also greatly enhances their cytotoxicity toward ligand-expressing targets[24]. Furthermore, lectin-like transcript 1 (LLT1, gene *clec2d*) is a type II transmembrane receptor that belongs to the C-type lectin like (CTL) superfamily of NK cell receptors. Six alternatively spliced transcripts of *clec2d* exist, with the isoform 1 (designed as LLT1) being the only one presented on the cell surface and hence the only isoform able to participate in the cell-to-cell transmission[25]. LLT1 is expressed mainly on activated lymphocytes, including NK, T cells and B cells, as well as on antigen presenting cells (APC)[26]. However, a decade ago, LLT1 was also identified as a physiological ligand of the NKR-P1 (CD161), which is expressed on all NK cells and subsets of T cells and represents the only member of the human NKR-P1 subfamily[27,28]. NK cell cytotoxicity and IFN-γ production is inhibited upon engagement of NKR-P1 on NK cell with LLT1 on target cell[27,28]. Upon viral infection, LLT1 expression is up-regulated on surface of epithelial cells, while IFN-γ has been shown to increase LLT1 expression on B cells and APC[29]. Hence LLT1-mediated signaling is thought to represent a system that regulates both innate and adaptive responses. For activated NK cells to achieve successful lysis, strong adhesion to target cells that is required is mediated by the β2 integrin LFA1[30].

The objective of this study was to investigate the modulation of ligands for cell surface NK receptors in chronic HBV patients and HepG2 cells transfected with BCP and precore mutants. Furthermore, we aimed to correlate patient’s viral replication and the differential viral capacity of hotspot mutants with the expression of NK receptors and their ligands at the RNA level.

**METHODS**

*Patients*

Nineteen patients (11 men and 8 women; mean age 44.5 ± 12 years) with chronic hepatitis B were studied (Table 1). Tissue samples were obtained by percutaneous needle biopsy, snap-frozen and stored in liquid nitrogen. From all the individuals included in the study a serum sample was collected at the time of liver biopsy, stored at -80oC and HBV DNA levels were measured using real-time PCR (qPCR). Individuals with a history of excess alcohol, or positive for HCV, HIDV or HIV were excluded. Informed consent was obtained from all the patients (The department of Hepatology St Mary’s Liver Tissue Bank, 06/Q0509/39). 1mg of liver tissue was processed using the gentleMACS Dissociator (Miltenyi Biotec SAS, Paris, France) prior to RNA extraction.

*Transient transfection experiments*

The G1896A, G1899A, G1896A/G1899A, A1762T/G1764A and A1762T/G1764A/C1766T mutations were generated by splice extension mutagenesis using as a template a plasmid (p3.8II) containing a 1.2 genome length of HBV subtype *adr* (genotype C)[31] in a pBluescript II KS (+) background (Stratagene, CA, USA) (Table 2). Full-length HBV genomes were amplified from WT or mutant HBV genomes according to the method described by Günther, using the P1 sense (HBV positions: nt1821-1841) and P2 antisense primers (HBV positions: nt1823-1806) modified to contain the *HindIII/SapI* sites and the *SacI/SapI* sites, respectively (Table 2)[32]. Polymerase chain reactions (PCR) were performed using the FastStart High Fidelity PCR System (Roche Applied Science, Germany) according to the manufacturer's instructions. Each of the 3.2kb amplified HBV fragments were purified from agarose gels using the GeneJET Gel Extraction Kit (Thermo Scientific, Life Science Research, Lutterworth, UK) and were cloned into a pCRII TA-vector (Invitrogen, Paisley, UK). All constructs were sequenced to confirm the presence of the mutations and to ensure that no additional mutations were introduced in the process.

Linear HBV monomers were released by *SapI* restriction digestion (New England Biolabs, Beverly, MA), gel purified, and then used for transient transfection of HepG2 by lipofection. HepG2 cells were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 2mM L-Glutamine, 100IU penicillin ml-1 and 100ug streptomycin ml-1. Cells were seeded at a density of 4x105 cells in 60mm diameter dishes (Corning, Flintshire, UK) and the following day were transfected with 500ng HBV linear monomers using Lipofectamine Plus (Invitrogen) according to manufacturer’s instructions. The culture medium was changed 24hours after transfection, and cells were harvested at 48 and 72hrs.

All transfections included 1μg of reporter plasmid expressing a green fluorescent protein (GFP) to monitor transfection efficiency by fluorescence-activated cell sorting (FACS) analysis and ranged 43-50% while the empty pUC19 vector used as a negative control.

**RNA quantification**

*Northern Blot analysis*

For Northern blot analysis, 10μg of total RNA per sample was separated on a 1% formaldehyde-agarose gel and blotted onto a Zeta-Probe GT membrane (BioRad, Bio-Rad Laboratories, Hercules, CA). Radioactive probes were prepared by random priming, using either full-length HBV DNA or 18S cDNA template and 32P labelled αdCTP (Amersham). After hybridization the membrane was washed and exposed to X-Omat film (Kodak, Rochester, NY) at -80oC.

*Relative quantification by Real Time PCR*

Total RNA was extracted from HepG2 cells 48 hours after transfection with *SapI*-digested HBV DNA monomers and liver biopsies using Trizol reagent (Invitrogen) as recommended by the manufacturer. The RNA samples were treated with DNase (Qiagen, Crawley, UK) for 10 min at 25oC and were then purified using the RNeasy mini kit (Qiagen). RNA integrity was confirmed by agarose gel electrophoresis under UV, RNA concentrations were monitored by using the nanodrop and samples were stored at -80oC until further use.

Two micrograms RNA from each transfection and 500ng RNA from each liver biopsy were reversed transcribed in 20ul reactions using the RT2 First Strand kit (Qiagen). There were three biological replicates in each group for each transfection experiment and duplicates for each liver biopsy samples. The resulting cDNA samples were stored at -80oC until further use.

The relative levels of total HBV RNA transcripts concentrations were analysed by real time PCR with a TaqMan (Life technologies Paisley, UK) in a Light-Cycle (Roche Diagnostic, West Sussex, UK). Each PCR reaction was performed in a 75ul reaction volume containing 5μl cDNA, 0.5 mmol/L forward and reverse primers, 0.2 mmol/L 3' FL-labelled probe, and 0.4mmol/L LC-labelled probe. The forward and reverse primers used were 5’-CTCGTGGTGGACTTCTCTC-3’and 5’-cagcaggatgaagaggaa-3’, and the probes were 5’-LC640-TGTCCTGGTTATCGCTGGATGTGTCT-PH-3’ and 5’-CACTCACCAACCTCCTGTCCTCCAA-FL-3’. The h-GAPDH housekeeping gene Light Cycler Set (Roche DNA control kit, Roche Diagnostics) was used to normalize RNA expression.

For the detection of MICA, ULBP2, ULBP3, B7H6, CD226 (DNAM-1), CLEC2D (LLT1), ITGAL (LFA-1) and TNFSF10 (TRAIL) we performed RT PCR arrays with customized RT2 custom arrays containing pre-dispensed primer assays on a StepOne Plus Real Time PCR (AB Applied Biosystems, Thermo Scientific, Loughborough, UK) using RT2 SYBR Green/qPCR Master Mix (Qiagen). The custom-made array included two housekeeping genes (GAPDH and β-Actin) and three internal controls (human genomic DNA contamination control, reverse transcription control and positive PCR control). Each PCR reaction contained cDNA synthesized from 125ng of total RNA. The thermocycler parameters were 95oC for 10min, followed by 45 cycles of 95oC for 15 s and 60oC for 1 min.

Relative changes in gene expression for both viral and cell RNA transcripts were calculated using ΔΔCt (threshold cycle) method. Threshold cycle numbers (Ct)2 above 35 were considered below detection level. The β-ACTIN housekeeping gene was used to normalize the RNA amounts. Results are expressed relative to β-ACTIN with NK markers as 2-ΔΔCT, where ΔΔCt = ΔCT-sample - ΔΔCT-control and where ΔCT = CT-target gene  - ΔCT-ACT.

**Statistical Analysis**

Two group comparisons of continuous variables were performed using the non-parametric Mann-Whitney test with two tailed values (GraphPrism 6). P values below 0.05 were considered statistical significant.

**RESULTS**

**Liver and serum viremia in CHB patients**

We determined the expression of total HBV RNA in the liver of infected patients by RT PCR and compared this to the levels of serum viraemia. It is well known that intrahepatic viremia does not correlate with serum viremia since the liver sampled cannot be representative. Indeed the levels of intrahepatic HBV RNA did not correlate with serum HBV viremia (r = 0.34, p>0.1) (Figure 1). We therefore divided the patients into four groups: 1) Low Serum Viremia patients, included those with serum viremia 1x101 – 1x104 viral copies/ml; 2) High Serum Viremia group, included patients with serum viremia 1x106 – 1x1010 viral copies/ml; 3) Low Liver HBV RNA patients, included those liver samples with viral RNA levels 1x101- 1x103 HBV RNA levels relative to β-ACTIN expression; 4) High Liver HBV RNA samples with 1x104- 1x105 HBV RNA levels (Table 1). Twelve out of 19 patients were HBeAg-negative and HBeAb-positive; all patients had minimal inflammation levels; 1/19 patient was cirrhotic and 8/19 patients had elevated ALT levels.

**Expression of NK regulatory molecules**

*Chronic patients*

We examined the expression of the key NK cells ligands MICA, ULBP2, ULPB3, B7H6, the NK receptors DNAM-1, LFA-1, TRAIL and LLT1 in the livers of CHB patients (Figure 2). In general individuals with higher liver viral loads had higher expression of NK cell ligands and receptors (ULBP2, ULBP3, LLT1, B7H6, LFA-1, and DNAM-1). The exception to this was MICA, which was significantly downregulated in patients with high intrahepatic HBV RNA levels (p=0.02) and decreased in high viremic patients (p=0.05). We also compared the gene expression in 7 HBeAg(+) patients and 12 HBeAg(-) patients (Figure 3). The expression of ULBP2, ULBP3, LLT1, LFA-1, and DNAM-1 was upregulated in HBeAg(+) patients, the majority of which had increased serum and liver HBV levels, while MICA was downregulated in this group of patients. Interestingly LLT1 is a ligand for an inhibitory receptor, CD161, expressed on NK cells and intrahepatic T cells. Thus this, together with the down-regulation of MICA, represent potential escape mechanisms for HBV, counteracting the trends observed for the other NKG2D ligands and also B7H6 the ligand for NKp30. Consistent with the lack of correlation between serum and liver HBV viraemia, we observed no consistent trends of NK cell ligand expression and serum viraemia.

*In vitro correlation of HBV and NK cell ligands*

We had previously constructed using site directed mutagenesis, the replication-competent plasmids bearing the BCP mutations 1762/64 and 1762/64/66 and the precore 1896, 1899 and 1896/1899 [Koumbi L, 2016, manuscript submitted] (Table 2). Using the same HBV constructs and plasmid free transfection system, we have previously demonstrated that compared to the WT and precore variants, BCP mutations result in lower viral replication capacity while the precore variants had similar replication fitness to the WT. BCP mutants consistently produced the decreased accumulation of HBV RNA transcripts (Northern blotting)(Figure 3); replicative intermediates including cccDNA (Southern blotting); and culture HB surface antigen (HBsAg) levels (ELISA) [Koumbi L, 2016, manuscript submitted].

To investigate whether the differential replication of HBV induces changes in the expression of ligands for NK cell receptors on transfected HepG2 we performed RT qPCR (Figure 4). Transfection of HepG2 cells induced up-regulation of all NK cell ligands tested. MICA, ULBP3 and B7H6 transcript levels were not significantly altered after exposure to the WT and HBeAg mutant variants examined (Figure 4). However we observed a weak trend towards an association of ULBP2 with viraemia in that there was reduce levels of ULBP2 mRNA with the BCP mutants as compared to WT and increased expression with the pre-core variants. However, expression of LLT-1 in HepG2 transfected with the WT genome was significantly decreased as compared to 1762/74 (P=0.01) and 1762/64/66 (P=0.04). Thus LLT-1 correlated with the BCP mutations and their low replication efficiency. This is consistent with our findings for intrahepatic LLT1 RNA levels.

**DISCUSSION**

Accumulating evidence suggest that activation of the NKG2D-ligand pathway contributes to the outcome of HBV infection. The unpredictable natural history of chronic HBV infection makes it difficult to sample the immune correlates of viral replication activity longitudinally. The level of HBV DNA in serum is commonly used as a surrogate marker of HBV replication in the clinical setting but it does not reflect the levels of HBV in the liver[33]. To monitor HBV levels, in addition to serum viremia we assessed HBV RNA expression in the same liver sample that we determined NK ligand RNA expression. We found that MICA transcripts were decreased in highly viremic liver samples of CHB patients whereas there was a trend towards increased expression of ULBP2 and ULBP3 with HBV liver viremia. Thus a general trend towards upregulating expression of NK cell ligands, could be counteracted by decreasing expression of MICA and hence weakening NK surveillance of the infected cells. These patients had minimal inflammation on their liver biopsies and therefore downregulation of MICA could be both harmful by inhibiting NK-induced viral clearance but also protective by preventing the infected hepatocytes from becoming targets for NK cell-mediated killing and hence preventing the development of liver injury. Furthermore the NK markers B7H6, DNAM-1, LFA-1 and TRAIL were also slightly elevated in high viremic liver samples. Our results indicate that high HBV replication induces the upregulation of gene expression of these NK ligands. A recent study by Lunemann showed lower levels of NKG2A, DNAM-1 and TRAIL on the NK cells of CHB patients as compared to acute patients [34]. It has been previously shown that higher expression of NKG2D ligands on cancer cells is associated with down-regulation of NKG2D on NK cells related to soluble NKG2D ligand secretion. We therefore propose that a similar process occurs in CHB.

In the course of CHB infection, HBeAg-negativity concurs with the emergence of the precore mutation 1896 and 1899 while the BCP mutation 1762/64 and 1762/64/66 reduces HBeAg production. We have previously showed that the BCP mutations result in lower viral replication capacity, whereas the major precore mutations have no effect on viral replication. The expression of all NKG2DL was increased when HepG2 cells were transfected by HBV suggesting that HBV induces NKG2DL upregulation. This finding is consistent with our observation that NK cell ligands, with the exception of MICA, are increased in HBV infection. However consistent trends for the ligands for activating receptors were not observed between *in vivo* and *in vitro* assays nor between HBeAg(+) and HBeAg(-) patients, with the possible exception of ULBP2. In general NKG2D is a system thought to be important for clearing viral infection, and especially CMV which has developed multiple mechanisms to down-regulate different NKG2D ligands[35]. Consistent with previous studies we found that MICA expression is reduced in the liver of CHB patients[36,37]. Transfection of HepG2 cells with all HBV constructs induced its expression suggesting that early in infection MICA is upregulated but in the persistent infection HBV weakens the immune surveillance of NK cells by inhibiting MICA expression.

Conversely, expression of LLT1 was increased in patients with high serum and intrahepatic viremia. Similarly, in HepG2 transfections we found low but detectable levels of LLT1 that were positively correlated with the differential viral replication of the HBeAg mutated variants. Upregulation of LLT1 and its subsequent engagement to their CD161 receptor expressed on NK cells have been shown to inhibit NK-mediated cytotoxity and IFN-γ production by T cells[27]. LLT1 expression has been reported to be absent in normal liver tissue but upregulated in HCC. Here we show for the first time that LLT1 is expressed in HBV infected liver and its levels associate with viral replication in HBV infection. Thus HBV may circumvent the immunity of the host by inhibiting NK cytotoxicity and IFN-γ production via the upregulation of LLT1.

A detailed characterization of the molecular players that link the HBV stimuli to the regulation of NK cells will be critical to advance our knowledge on how HBV circumvents the host’s immunity. The manipulation of ligand expression can provide new insight on chronic HBV immunopathogenesis and can lead to possibilities of developing effective treatment strategies.

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**SUMMARY BOX**

* Natural killer (NK) cells play a key role Hepatitis B virus (HBV) persistence.
* HBV circumvents host’s immunity by modulating NK receptors and their ligands.
* MICA is an activating NK ligand and is decreased in chronic infection.
* LLT1 is an inhibitory NK molecule, expressed on NK cell and on target cells.
* MICA is increased in transient transfection while is downregulated in chronic patient’s livers.
* LLT1 positively correlates with HBV replication both *in vivo* and *in vitro*.
* The expression of ULBP2, ULPB3, B7H6, DNAM-1, LFA-1 and TRAIL is elevated in high HBV replication *in vivo*.
* To establish chronicity, HBV is weakening NK activity via the up-regulation of LLT1 and down-regulation of MICA.

**TABLES**

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**Table 1**. Clinical characteristics of the studied patients. Cirrhosis was measured with according to the Ishak scoring system and necro-inflammation with the Knodel system. Expression of liver HBV RNA was normalized with h-GAPDH and data represents the mean ± SEM from 3 RT PCR experiments. Patients were divided into four groups according to their serum viremia and intrahepatic HBV RNA levels.



**Table 2:** Primers used full-length HBV genome generation by splice extension mutagenesis. Mutated nucleotides are shown in bold.

**FIGURES LEGENDS**

**Figure 1.** Serum HBV DNA levels by qPCR and the relative expression of intrahepatic HBV RNA relative expression. Each dot represents a patient and data for HBV RNA relative expression is shown as the mean ± SEM from 2 RT PCR experiments.

**Figure 2.** RNA levels of NK regulators in liver biopsies from CHB patients. Gene expression was normalized with β-Actin levels and data represents the mean ± SEM from 3 independent experiments of the relative gene expression between patients. The ALT levels are shown as the mean ± SEM.

**Figure 3.** The RNA expression of NK regulators in liver biopsies from 7 HBeAg(-) and 12 HBeAg(+) CHB patients. Gene expression was normalized with β-Actin levels and data represents the mean ± SEM from 3 independent experiments of the relative gene expression between patients. The ALT levels are shown as the mean ± SEM.

**Figure 4.** Northern blot analysis of HBV transcripts. 10μg RNA were extracted from HepG2 cells after 48h of transfection with 500ng linear HBV DNA monomers carrying BCP or precore mutations.

**Figure 5.** RNA expression of MICA, ULBP2, ULBP3 and B7H7 was assessed at 48h post transfection by RT PCR normalized to GAPDH or β-Actin expression. WT indicated wild-type virus, 1762/64 and 1762/64/66 are BCP variants and 1896, 1899 and 1896/99 are precore variants. Data is presented from 3 independent transfection experiments of HepG2 cells and values are expressed as mean ± SEM.

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