Clinical studies on hepatitis B, C, and E virus infection

Willemse, S.B.

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CHAPTER 1

Dynamics of the Immune Response in Acute Hepatitis B Infection


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ABSTRACT

Background
Acute hepatitis B virus (AHB) infection in adults is generally self-limiting but may lead to chronicity in a minority of patients.

Methods
We included 9 patients with acute HBV infection and collected longitudinal follow-up samples. NK cell characteristics were analysed by flowcytometry. HBV-specific T cell function was analysed by in vitro stimulation with HBV peptide pools, and intracellular cytokine staining.

Results
Median baseline HBV DNA load was 5.12 log IU/mL and median ALT was 2,652 U/mL. Of 9 patients, 8 cleared HBsAg within 6 months whereas one patient became chronically infected. Early timepoints after infection showed increased CD56\textsuperscript{bright} NK cells, and an increased proportion of cells expressing activation markers. Most of these had normalised at week 24, while the proportion of TRAIL-positive CD56\textsuperscript{bright} NK cells remained high in the chronically infected patient. In patients that cleared HBV, functional HBV-specific CD8\textsuperscript{+} and CD4\textsuperscript{+} responses could be observed, whereas in the patient who developed chronic infection, only low HBV-specific T cell responses were observed.

Conclusions
NK cells are activated early in the course of acute HBV infection. Broad and multispecific T cell responses are observed in patients who clear acute HBV infection, but not in a patient who became chronically infected.
INTRODUCTION

Infection with the hepatitis B virus (HBV) affects large numbers of individuals worldwide. As much as one third of the global population has encountered HBV at some point in their life. Infection with HBV at early age will lead to chronicity in the majority of cases (>95%), resulting in an estimated 240 million patients worldwide who are chronically infected with HBV.\textsuperscript{1,2} When an acute HBV infection is encountered later in life, the virus will be spontaneously cleared in most cases.\textsuperscript{3} Less than 5% of immunocompetent adult patients however, fail to clear the virus and become chronically infected with HBV. The mechanisms that lead to chronicity of hepatitis B infection are largely unknown. For clearance of the virus in the acute setting both the innate and the adaptive immune system are important.\textsuperscript{4–7} The innate immune system is responsible for early containment of the viruses and initial activation of adaptive immune responses. Although HBV has been shown to act as a ‘stealth’ virus in woodchucks and chimpanzees, evading early intrahepatic immune responses,\textsuperscript{8,9} it is uncertain whether these early innate responses are induced during acute HBV infection in man.\textsuperscript{10} Other players of the innate immune system, natural killer (NK) cells, are activated early during infection, before HBV-specific T cells arise.\textsuperscript{11,12} Later on during infection, functionally active HBV-specific T cells can be detected, which are thought to play an important role in viral clearance. In chimpanzees, depletion of CD8+ T cells at week 6 of infection lead to failure to clear the infection.\textsuperscript{5} During chronic infection, HBV-specific T cells are exhausted and their function is impaired.\textsuperscript{13} However, whether these HBV-specific T cells were functionally active during the initial phases of infection is unknown. In acute hepatitis C infection, patients with self-limited infection have significant T cell responses compared to little or no responses those who evolve to chronicity.\textsuperscript{14}

Acute hepatitis B infection is asymptomatic in the majority of cases which makes it difficult to study. However, previous studies have focused on blood donors that became HBsAg positive (n = 2) or a local outbreak (n = 5) for the initial phases of infection.\textsuperscript{11,15} Here we examined the early dynamics of NK and HBV-specific T cell responses in symptomatic patients with acute HBV infection who presented at our clinic.

PATIENTS AND METHODS

Patients

Patients were included at the gastroenterology and hepatology department of the Academic Medical Center in Amsterdam. Acute infection was diagnosed based on HBsAg and HBV DNA positivity, further serology, biochemistry and anamnesis reporting risk of acquiring HBV. Patients were assessed at the outpatient clinic at first visit (clinical onset) which was defined as baseline (BL). Follow-up was at week 1, 4, 12 and 24. All patients were HIV seronegative and were not co-infected with hepatitis C or hepatitis delta virus. The study was approved by the Ethical Review Board of the Academic Medical Center Amsterdam and all patients gave written informed consent. The study was conducted in accordance with Declaration of Helsinki, Good Clinical Practice guidelines, and local regulatory requirements.
Laboratory testing
Biochemical and virological analyses were carried out by local laboratories in accordance with good laboratory practice. Qualitative detection of serum hepatitis B surface antigen (HBsAg) and antibody to hepatitis B surface antigen (anti-HBs) was performed by enzyme immunoassay (AxSYM; Abbott Laboratories, Abbott Park, IL, USA). Quantitation of plasma HBV DNA levels was done by the Roche COBAS® TaqMan 48® assay (F. Hoffmann-La Roche Ltd, Diagnostics Division, Basel, Switzerland), with a dynamic range between 20 and $1.70 \times 10^8$ IU/mL. HBV genotype was determined by sequencing a part of the polymerase gene with dideoxynucleotide technology.

Sampling
Peripheral blood samples were obtained at baseline (BL) and during follow-up (week 1, 4, 12 and 24). Sampling included plasma as well as peripheral blood mononuclear cells (PBMCs) which were isolated using standard density gradient centrifugation and subsequently cryopreserved until the day of analysis. Eight healthy blood donors were included for comparison.

Cytokine measurements IP-10 and IL-18
Levels of IP-10 and IL-18 were measured in available plasma samples with a DuoSet ELISA (R&D Systems, Minneapolis, MN, USA). Values were extrapolated from standard curves (IP-10 range: 62.6–4000 pg/ml, IL-18 range: 11.7–750 pg/ml). Plasma samples from 6 healthy controls were included in the analyses.

Immune phenotyping by flow cytometry
PBMCs were washed in PBA (PBS containing 0.01 % (w/v) NaN3, 0.5 % (w/v) bovine serum albumin and 2 mM EDTA) and 1.0 × 10^6 cells were incubated for 30 min in the dark at 4°C with different combinations of fluorescent label-conjugated mouse monoclonal antibodies (mAbs). For phenotypic analysis, the following mAbs were used: CD3 V500, CD56 BUV395, CD16 BV786, CD16 BV421, CD27 BUV737, HLA-DR FITC, CD38 PE-Cy7, PD-1 BV421, CD14 PE-CF594, CD19 PE-CF594 (BD Biosciences, San Jose, USA), CD8 BV711 CD8 BV785, CD57 Alexa Fluor 647 (Biolegend), life/dead fixable red stain (Invitrogen, Camarillo, USA), NKP46 PerCP-efluor 710, CD45RA efluor 605 (eBioscience, San Diego, USA), NKG2A PE (Beckman Coulter, Fullerton, CA, USA) and TRAIL APC (Miltenyi Biotec, Bergisch Gladbach, Germany). For intracellular staining, cells were fixed after surface staining with FACS Lysing Solution (BD) and subsequently permeabilized (FACS Permeabilizing Solution 2 (BD)). Cells were incubated for 30 min in the dark at 4°C with one or more of the following antibodies: perforin FITC (BD Biosciences), granzyme B PE (Sanquin, Amsterdam, The Netherlands), Ki67 BV711 (Biolegend, San Diego, CA, USA), Eomes PerCP-efluor 710, and T-bet Pe-Cy7 (eBioscience, San Diego, USA). Measurements were done using LSR Fortessa flow cytometer (BD Biosciences, Europe) and FACS Diva Software. Analysis was done using FlowJo v10 (FlowJo LLC, Ashland, USA).

Intracellular staining HBV-specific T cells
PBMC were cultured for 10 days with 5 pools of in total 315 15-mer peptides (10 overlapping residues) covering all proteins of HBV genotype A (Chiron Mimotopes, Victoria, Australia) at 1 µg/ml. PBMC were restimulated for 6 hours at day 10 in the presence of
CD107a PE (BD Biosciences, San Jose, CA, USA), Brefeldin A, and monensin. The production of cytokines was evaluated by intracellular staining with IFN-γ BV421, MIP-1β Pe-Cy7, TNF-α AF700, and IL-2 APC monoclonal antibodies (BD Biosciences, San Jose, CA, USA) after staining with surface markers as described above. Measurements and analyses were done as described above.

**Statistical analyses**

The two-tailed Mann-Whitney U test was used for analysis of differences between groups. For longitudinal analysis in individual patients the Wilcoxon signed rank test was used. P values < 0.05 were considered statistically significant. GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla, CA, USA) was used for analyses.

**RESULTS**

**Patients**

Nine patients with acute hepatitis B infection were included in the study (for baseline characteristics, see Table 1). Patients were infected with genotype A (n = 5), or genotype B, D, E, or F (all n = 1). At BL, median HBV DNA load was 5.12 log IU/mL (iqr: 3.80–6.64, Figure 1A) and median ALT was 2,652 U/mL (iqr: 1,554–3,390 U/mL Figure 1B). Six months after infection, 8 of 9 patients had spontaneously cleared the virus, of which 6 had formation of anti-HBs antibodies. One patient, infected with HBV genotype A, did not clear HBsAg within 6 months. At 6 months after initial presentation the viral load in this patient was > 1.7x10^8 IU/mL (upper limit of quantification) and ALT was 454 U/mL (Figure 1A,B). In a subset of patients, IP-10 and IL-18 levels were measured. At baseline, the plasma levels of IP-10 were increased in patients with acute HBV infection as compared to healthy controls (median 1613.0 and 39.4 pg/mL respectively, p = 0.0007, Figure 1C). In addition, baseline IL-18 levels were significantly increased in patients with AHB infection (median 1182 pg/mL) as compared to healthy controls (median 124.3 pg/ml, p=0.002, Figure 1D).

At week 24, IP-10 levels had normalised, while IL-18 levels were still increased as compared to healthy controls (median 306.0 and 124.3 pg/mL respectively, p = 0.007 Figure 1D).

Early time points after infection (BL and week 1) showed an increase in the proportion of CD56^{bright} NK cells (BL median 7.4%) as compared to healthy controls (median 1.4%, p = 0.0028, Figure 2A). Furthermore, the proportion of CD56^{dim} NK cells was decreased (Figure 2B) early during infection, while the proportion of total NK and total CD8+ T cells was not significantly different from that of healthy controls (Figure 2C,D). There was no significant change in the proportion of effector and memory CD8+ T cells. However, the memory T cell population was activated, as demonstrated by the significant increase in PD-1, Ki67, HLA-DR/CD38, perforin and granzyme B positive memory T cells (Supplementary Fig 1).

**Early NK cell activation**

To investigate the role of NK cells at different time points during acute infection we measured the expression of several markers of NK cell activation. At baseline, the proportion of CD56^{bright} NK cells expressing CD38 was significantly increased (median 44.0%) as
### Table 1. Baseline characteristics

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**Figure 1.** Course of HBV DNA (A), ALT (B), IP-10 (C) and IL-18 (D) in patients with acute HBV infection. Not all patient samples were available for IP-10 (n=5) and IL-18 (n=4) measurements. Statistical testing: Mann-Whitney U test. lloq; lower limit of quantification, uloq; upper limit of quantification; ns, non-significant; ***, p < 0.001; **, p < 0.01.
compared to healthy controls (median 30.3 %, p = 0.01, Figure 3A). Similarly, CD38 was expressed on 56.2 % of CD56<sup>dim</sup> NK cells in AHB patients at baseline as compared to 42.0 % of HC CD56<sup>dim</sup> NK cells (p = 0.03, Figure 3A). At baseline, HLA-DR was expressed on an increased proportion of CD56<sup>dim</sup> NK cells (median 18.3 %) in AHB patients as compared to healthy controls (median 4.6 %, p = 0.0008). The proportion of HLA-DR+ CD56<sup>dim</sup> NK cells was still increased 1 week after presentation of AHB infection (Figure 3B). Ki67, a marker for proliferation, was not differentially expressed between baseline NK cells from AHB patients and healthy controls. At week 4 and week 12 however, the proportion of Ki67+ CD56<sup>dim</sup> NK cells was increased as compared to healthy controls (Figure 3C). The differentiation status of NK cells, as measured by CD57 and NKG2A expression, was not different in patients with acute hepatitis B infection as compared to healthy controls (Supplementary Fig 2).

**Figure 2.** Proportion of CD56<sup>bright</sup> (A) and CD56<sup>dim</sup> (B) NK cells, total NK cells (C), and total CD8<sup>+</sup> T cells (D) in 9 patients with acute HBV infection as well as in healthy controls (HC). Statistical testing: Mann-Whitney U test. ns, non-significant; **, p < 0.01.
Long term increase in effector markers
The proportion of CD56\textsuperscript{bright} NK cells expressing NKp46, an activating receptor, was significantly increased at all time points during acute HBV infection, as compared to healthy controls (AHB baseline median 96% and HC 81.6%, p < 0.0001, Figure 4A). This was similar for CD56\textsuperscript{dim} NK cells (Figure 4A). The TNF related apoptosis inducing ligand TRAIL is generally expressed by a minority of CD56\textsuperscript{bright} NK cells. At baseline acute HBV

Figure 3. Markers of NK cell activation CD38 (A), HLA-DR (B), Ki67 (C) in CD56\textsuperscript{bright} (left) and CD56\textsuperscript{dim} (right) NK cells. Statistical testing: Mann-Whitney U test. ns, non-significant; ***, p < 0.001; *, p < 0.05.
infection, the proportion of TRAIL+ CD56\textsuperscript{bright} NK cells was significantly increased as compared to healthy controls (median 11.7 and 2.2 % respectively, \( p = 0.0028 \), Figure 4B). Furthermore, TRAIL expression in the patient that was chronically infected was still elevated at week 24 after initial presentation (29.5% of CD56\textsuperscript{bright} NK cells, Figure 4B). During acute HBV infection the proportion of granzyme B and perforin positive CD56\textsuperscript{dim} NK cells was significantly increased compared to healthy controls (granzyme B baseline AHB 71.7 and HC 48.6 %, \( p = 0.04 \). Perforin baseline AHB 82.4 % and HC 63.3 %, \( p = 0.01 \), Figure 4C,D). In addition, granzyme B was expressed by a considerate proportion of CD56\textsuperscript{bright} NK cells, which are generally thought to play a lesser role in cytotoxicity (Figure 4C). In particular, a high proportion of granzyme B expressing CD56\textsuperscript{bright} NK cells remained present in the patient who evolved to chronic infection (Figure 4C).

**HBV-specific T cell responses**

HBV-specific T cell responses, measured by cytokine production in response to HBV peptide pools, were analysed in 5 patients who were infected with HBV genotype A. Even though intra-individual differences were observed in the specificity and timing of the response, HBV-specific T cell responses could be observed in all patients that spontaneously cleared the HBV infection during the 6 months follow-up (Figure 5A-D). In the patient who became chronically infected however, very low HBV-specific T cell responses were observed at all time points (Figure 5E). Patients who cleared the HBV infection could have an early peak of the HBV-specific CD8+ response (patient 4 and 6) or a later peak (patient 5 and 8). The specificity of the T cell response also differed between patients. In patients 4 and 5 the highest observed sum of cytokine responses was observed in response to the polymerase peptide pool, whereas in patient 6 the highest cytokine production was against the envelope pool, and in patient 8 against the core peptide pool. CD4+ T cell responses mostly followed the same patterns (Supplementary Figure 3).
Continuation of Figure 4.
Figure 5. HBV-specific CD8+ T cell functionality was assessed in patients who were infected with HBV genotype A. TNFα, IL-2, MIP-1β and IFNγ production by HBV-specific CD8+ T cells in 4 patients who cleared AHB infection (A-D) and 1 patient who evolved to chronicity (E).
DISCUSSION

Here we show the temporal dynamics of NK cells and T cells in nine patients during acute hepatitis B infection, of which one developed chronicity. HBV is a non-cytopathic virus, however, immune responses mounted by the host can cause serious liver damage. Whereas viral clearance can occur without clinical symptoms or cell destruction,\textsuperscript{4,11} in our patient cohort hepatocyte damage was apparent by ALT elevations. As we observed a significant increase in the proportion of total peripheral memory T cells expressing makers of activity and cytolytic proteins, HBV-nonspecific bystander T cells could have played a role in hepatocyte injury,\textsuperscript{16,17} as seen in mouse models of acute fulminant HBV infection.\textsuperscript{18} At the time of presentation at the clinic, plasma levels of IP-10, an interferon stimulated gene (ISG), were significantly increased suggesting broad innate immune activation via the interferon pathway. Previous studies have shown no significant induction of type I interferons in the initial phases of acute HBV infection in chimpanzees\textsuperscript{8} or woodchucks.\textsuperscript{9}
Also in man, type I interferon production was not observed early during infection,\textsuperscript{10} still, it has not been ruled out that other interferon types such as IFN-\textlambda{} are induced.\textsuperscript{10} As we included patients who already were symptomatic, it is more likely that local danger signals or production of interferons by lymphocytes have activated the ISG pathway.\textsuperscript{8}

Early during the infection, the proportion of CD56\textsuperscript{bright} NK cells was significantly increased as compared to healthy controls. Furthermore, we observed a striking increase in the proportion of CD38, HLA-DR and Ki67 positive NK cells, indicating activation of these cells. Previously, it was shown that in patients with symptomatic infection, NK cell function in inhibited before peak viremia, and NK cells only become activated after peak viremia.\textsuperscript{10} As we only included patients after peak viremia, we may have missed the described inhibition of NK cells in the preclinical phase of infection. The proportion of NK cells expressing perforin and granzyme B was increased in patients with an acute HBV infection. While in mice, hepatocytes infected with a recombinant adenovirus have been suggested to be resistant to perforin mediated killing,\textsuperscript{19} the increase in the proportion of NK cells expressing cytotoxic molecules suggests they could have a role in hepatocyte damage. As such, an increase in perforin expression by HBV-specific T cells has been observed in acute HBV infection.\textsuperscript{20}

As the proportion of TRAIL expressing CD56\textsuperscript{bright} NK cells was significantly increased, TRAIL mediated killing of infected hepatocytes could also be responsible for ALT elevations.\textsuperscript{21} However, in two previously described patients who cleared the infection without any symptoms or ALT elevations, NK cells were activated early in the course of acute HBV infection, emphasizing that these cells can be important in non-cytopathic elimination of HBV.\textsuperscript{11} While Ki67, HLA-DR, and CD38 normalised after clearance of the virus, other NK cell markers were still increased at week 24 as compared to healthy controls, including NKp46, TRAIL, perforin, and granzyme B. In line with this, baseline elevated levels of IL-18, which is associated with NK cell activation,\textsuperscript{22} were still significantly elevated at week 24. Interestingly, in patients with chronic hepatitis B, TRAIL expression is significantly elevated.\textsuperscript{23} Similarly, in the patient who evolved to chronic infection, TRAIL remained expressed on a significant proportion of CD56\textsuperscript{bright} NK cells. Whether this TRAIL positive population is a cause or a result of chronic infection remains unanswered. During chronic infection, TRAIL has been suggested to play a role in killing of infected hepatocytes,\textsuperscript{21} as well as in NK cell mediated clearance of HBV-specific T cells.\textsuperscript{24}

Even though HBV-specific T cells seem to be inhibited by IL-10 and arginase early during infection,\textsuperscript{10, 25} their presence is highly associated with viral clearance.\textsuperscript{5, 10, 26} From acute HCV infection, we know that evolution to chronicity is associated with weak and transient responses of HCV-specific T-cells.\textsuperscript{14, 27} Clearance of the infection on the other hand, is associated with the appearance of multi-specific HCV-specific CD8+ T-cell responses against multiple epitopes.\textsuperscript{27} However, in two blood donors with acute HBV infection who were followed during asymptomatic infection, HBV-specific T cell responses reached their peak when HBV DNA was already declining, emphasizing the importance of other immune mechanisms for antiviral activity.\textsuperscript{11} Early T cell responses in these patients were directed against envelope and polymerase proteins.\textsuperscript{11} In 2 other patients, early responses were directed against polymerase and the X protein, followed by responses against core and envelope.\textsuperscript{10} Here we observed broad reaction to all HBV proteins in 2 patients at the earliest time point (patient 4 and 6). A more delayed and
A narrow HBV-specific T cell response was observed in 2 other patients, while they already showed ALT elevation and viral load decline (patient 5 and 8). Previously, a lack of T cell responses was associated with persistently high HBV DNA levels and the need for treatment in an immunosuppressed patient. In the one patient who did not clear HBV infection, the observed narrow T cell response may have led to chronic infection. This is in line with evolution of chronicity in woodchucks infected with the woodchuck hepatitis virus, as well as observations in acute hepatitis C patients.

Acute HBV infection can present in many different ways. With or without ALT elevations, and with or without symptoms. Therefore, the underlying mechanisms may also differ between cases. As one of the patients in this study developed chronic infection we had the unique opportunity to analyse early events that could be associated with failure to clear HBV. Our data suggests that the absence of a HBV-specific T cells response at all time points could play a role in progression to chronic infection. Furthermore a substantial population of CD56bright NK cells expressing TRAIL, as seen in chronic HBV infection, was present at all time points in this patient. A better understanding of the early course of acute infection and the evolution to chronicity may help the development of novel therapeutics targeting chronic hepatitis B virus infection.

SUPPLEMENTARY DATA
Supplementary data are available on request.
REFERENCES


