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Contribution of NK cells to HBsAg seroconversion in inactive HBsAg carriers following pegylated IFN therapy

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Abstract

Our recent study showed a high rate of HBsAg seroconversion in inactive HBsAg carriers (IHCs) treated with pegylated IFN (PEG-IFN). To understand the immune-mediated component of the HBsAg seroconversion better, this study investigated the role of NK cells. A total of 44 IHCs were given 48 wk of PEG-IFN. Fifteen cases achieved HBsAg seroconversion (R group), whereas 29 failed (NR group). The proportion and activity (CD107α and IFN-γ production) of NK cells were measured before and during treatment. We found that the proportion of NK cells in the R group was higher than in the NR group at baseline and during PEG-IFN treatment, even when patients were matched for age, sex and treatment period. IFN- γ secretion and CD107α expression from NK cells in cases who achieved HBsAg seroconversion were significantly higher than patients matched for age, sex, HBsAg and treatment period in the NR group at baseline and during PEG-IFN treatment. We also found that in HBsAg seroconversion cases, NK cells activity increased after PEG-IFN treatment, especially before HBsAg seroconversion. These effects were not found in non-responders. In conclusion, we demonstrated that the increase of NK cells accompanied by enhanced activity during PEG-IFN treatment favoured HBsAg seroconversion for IHC, and that NK cells may play a role in HBV seroconversion.

Keywords

Pegylated IFN-α-2a, inactive HBsAg carriers, HBsAg, seroconversion, NK cells

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Introduction

Inactive HBsAg carriers (IHCs) are characterised by effective control of hepatitis B virus (HBV) replication and sustained HBeAg seroconversion. Patients in this phase have detectable circulating HBsAg, absence of HBeAg and the presence of anti-HBe, as well as undetectable or low levels of HBV DNA. Our recent study showed a high rate of HBsAg seroconversion for IHCs treated with pegylated IFN (PEG-IFN).[1] Li et al. also reported a similar finding in a small retrospective cohort study.[2] To understand the immune-mediated component to the HBsAg loss better, we analysed the changes in selected immune parameters. We found a significant increase of NK cells in patients who achieved HBsAg seroconversion. We hypothesised that NK cells may play a role in HBsAg seroconversion.

With the deepening of research, accumulating evidence supported that the liver is an immunological organ, enriched with innate immune cells, including Kupffer cells, NK cells and NK T cells.[3,4] In humans, almost 50% of all intra-hepatic lymphocytes are NK cells. NK cells have been proved to play an important role in chronic HBV infection by producing cytokines and by exerting cytotoxic functions against virus-infected cells.[5,6]

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PEG-IFN achieves sustained off-treatment responses in many chronic hepatitis B (CHB) patients because of its direct antiviral effects and regulation of the immune response. However, the exact mechanism of how PEG-IFN promotes HBsAg seroconversion remains unknown. One study found that NK cells increased markedly during PEG-IFN therapy in HBV control cases.[7] PEG-IFN could also induce NK activation in HIV-1/HCV co-infected patients, and is associated with HIV-1 DNA decline.[8] However, studies on NK cells of HBsAg seroconversion are still in the exploratory stage. We therefore explored the contribution of NK cells to HBsAg seroconversion in inactive HBsAg carriers following PEG-IFN therapy.

Methods

Patients

Patients were included if they met the following criteria: HBsAg-positive for >6 mo, serum HBsAg levels <1000 IU/ml, HBeAg negative, anti-HBe positive, anti-HBc positive, HBV DNA <20 IU/ml and the absence of previous antiviral therapy. Patients were excluded if they met any of the following criteria: HIV or hepatitis C virus co-infections, a history of autoimmune diseases, hepatic dysfunction, serious metabolic disease, cardiac, pulmonary or renal disease, evidence of liver carcinoma or other neoplastic diseases, or contraindication for IFN.

PEG-IFN-α-2a (135 μg/wk) was administered for 48 wk. At baseline and every 12 wk thereafter during the treatment, detection of HBsAg levels, liver function, routine blood tests and HBV DNA was performed, and PBMCs were separated and stored at –80°C. Patients were categorised into one of three groups: the responder (R) group who achieving seroconversion of HBsAg; the non-responder (NR) group who failed to achieve HBsAg seroconversion; and the healthy control (HC) group who were HBsAg negative, vaccinated against HBV and had anti-HBs levels >200 IU/ml.

Tests of HBV infection

HBsAg and anti-HBs levels were detected using the HBsAg quantitative Elecsys (Roche Diagnostics GmbH, Mannheim, Germany). The lower limit of quantitative HBsAg determination was 0.05 IU/ml, and anti-HBs >10 IU/ml was defined as anti-HBs positive. HBV DNA levels were determined using the fluorescence quantitative PCR (FQ-PCR) method, with a lower detection limit of 20 IU/ml (Roche).

HBsAg synthetic peptides

HBsAg synthetic peptides (Sigma–Aldrich, St Louis, MO) were kindly donated by the MRC Human Immunology Unit, WIMM, University of Oxford. These polypeptides had all the aa sequences coded by the S gene region of HBV genotype C2/ACO053361, with each peptide containing 18 aa residues. Adjacent peptide fragments overlapped by 10 aa.

Flow cytometry

The mAbs were added to the separated PBMCs in this order: CD3-PerCP, CD56-APC and CD45-FITC. Isotype controls were also set up. FACS Calibur four-colour flow cytometry was performed, and the results were analysed using CELL Quest (Becton Dickinson, San Jose, CA). All mAbs were purchased from BD (San Diego, CA). NK cells were defined as $CD3^{-}CD56^{+}$, whereas NKT were $CD3^{+}CD56^{+}$ (Figure 1).

NK cell activity test

PBMCs were isolated from patients in the R and NR groups and co-cultured with HBsAg overlapping peptide pools and plasma samples from the patients (high anti-HBs level) at 37°C for 5 h with brefeldin A and monensin (Sigma–Aldrich). DMSO was added to the negative control, and combined SEB was added to the positive control. The addition of mAbs CD3-PerCP, CD56- FITC and CD107α-APC was performed with or without the Ab. After permeabilisation, IFN-γ-PE (BD Biosciences Pharmingen, San Jose, CA) was added to the samples. FACS Calibur four-color flow cytometry was performed, and the frequencies of IFN-γ and CD107α expression were analysed using CELL Quest (Becton Dickinson).

Statistical analysis

SPSS for Windows v11.5 (SPSS, Inc., Chicago, IL) was used for analysis. The data were normally distributed and are reported as the mean$\pm SD$. *t*-Tests were performed between two groups, and chi-square tests were performed between three groups. Two sided P values of <0.05 were considered statistically significant.

Results

Patients' clinical characterisation

A total of 44 IHC patients were given 48 wk of PEG-IFN. Fifteen cases achieved HBsAg seroconversion (R), whereas 29 failed (NR). There were no significant differences in sex, age, ALT, WBC and HBV DNA among the R, NR and HC groups. The HBsAg level

in the R group was lower than that in the NR group ($P < 0.001$). Patients in the R group experienced different periods of time to achieve HBsAg seroconversion. One case achieved it at 12 wk after PEG-IFN therapy, and seven, two and five cases achieved it at 24, 36 and 48 wk, respectively. The patients' demographic and clinical data are listed in Table 1.

Baseline proportion of NK cells in IHCs and HCs

The proportion of NK cells ($CD3^-CD56^+$) was analysed by flow cytometry. The proportion of NK cells was $12.07 \pm 0.65\%$ in 44 IHCs at baseline and $13.17 \pm 1.29\%$ in 12 HCs. There was no significant difference ($t = 0.78$, $P = 0.441$; Figure 2).

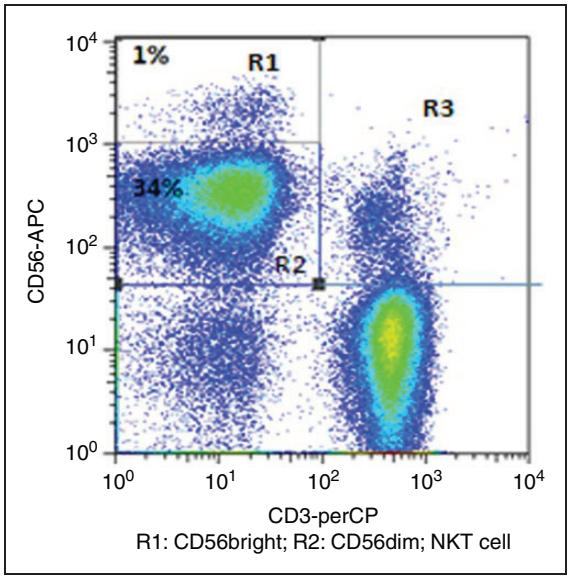

Figure 1. Analysis of NK cells and NKT cells. PBMCs stained with CD3-PerCP, CD56-APC and CD45-FITC. NK cells are $CD3^-CD56^+$; R1 is $CD3^-CD56^{bright}$; R2 is $CD3^-CD56^{dim}$. NKT cells are $CD3^+CD56^+$.

Baseline proportion of NK cells in R and NR groups

After PEG-IFN treatment of 44 IHCs, 15 cases achieved HBsAg seroconversion (R group) and 29 did not (NR group). The proportion of NK cells was $15.80 \pm 1.53\%$ in the R group, which was significantly higher than the NR group ($9.83 \pm 0.78\%$) at baseline ($t = 3.88$, $P < 0.001$; Figure 3).

Proportion of NK cells in patients matched for age, sex and treatment period during PEG-IFN treatment in R and NR groups

Because patients in the R group ($n = 15$) experienced different periods of time to achieve HBsAg

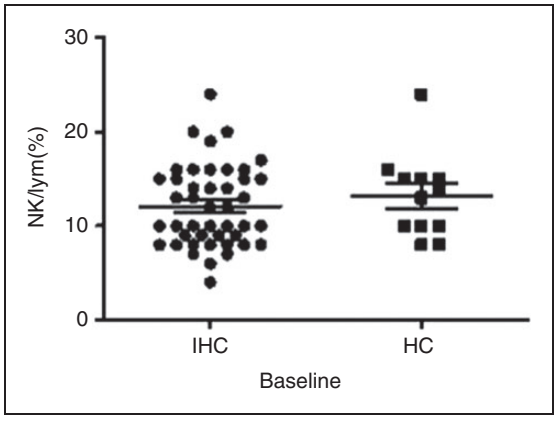

Figure 2. Comparison of NK cells in inactive HBsAg carriers (IHCs) and healthy control (HC) group at baseline. Comparison of proportion of NK cells between IHCs ($n = 44$) and healthy controls ($n = 12$) at baseline.

Table 1. Patient demographic and clinical data.

Parameter	R $n = 15$	NR $n = 15$	HC $n = 12$
Sex (M/F)	8/7	17/12	6/6
Age (yr), $M \pm SD$	34.47 ± 9.65	34.03 ± 10.03	30.75 ± 9.50
ALT (IU/l), $M \pm SD$	27.25 ± 9.26	25.87 ± 8.80	30.54 ± 6.19
AST (IU/l), $M \pm SD$	25.53 ± 5.44	23.49 ± 6.34	20.58 ± 6.31
Leucocytes (10^9/l), $M \pm SD$	5.72 ± 1.90	5.67 ± 1.78	5.69 ± 1.65
HBsAg (IU/ml), log10	1.33 ± 0.96**	2.24 ± 0.48**	UD
1st time achieved HBsAg seroconversion (n)			
12 wk	1	N	N
24 wk	7	N	N
36 wk	2	N	N
48 wk	5	N	N

**$P < 0.01$; R vs. NR.
UD: undetected.

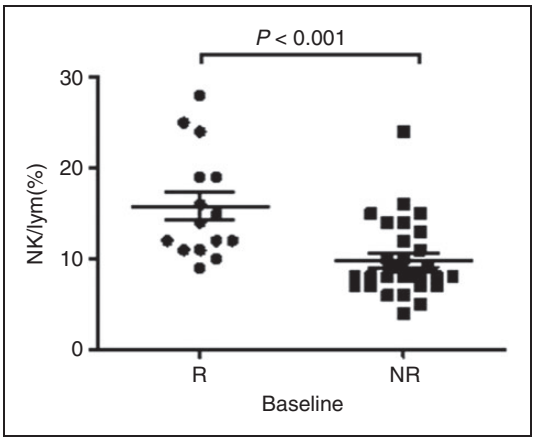

Figure 3. Comparison of NK cells in the R group and NR group at baseline. Comparison of NK cell levels between those who achieved seroconversion of HBsAg after 48 wk of treatment (R group; $n = 15$) and those who failed to achieve HBsAg seroconversion (NR group; $n = 29$) at baseline.

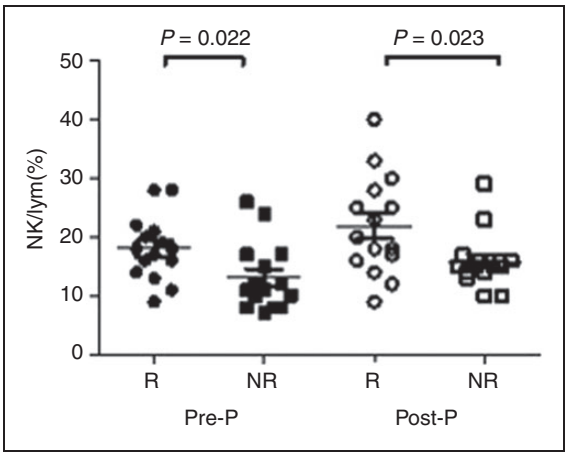

Figure 4. Comparison of NK cells in the R group and age-, sex- and treatment course–matched NR group during PEG-IFN treatment. Comparison of NK cell levels between those who achieved HBsAg seroconversion after 48 wk of treatment (R group; $n = 15$) and those who failed to achieve HBsAg seroconversion (NR group; $n = 15$) at the time point before HBsAg seroconversion was detected (Pre-P) and at the time point when seroconversion of HBsAg was detected (Post-P) after matching for age, sex and course of treatment.

seroconversion, the comparison time points were set as the last time point before HBsAg seroconversion was detected (pre-seroconversion point (Pre-P)) and the first time point when seroconversion of HBsAg was detected (post-HBsAg seroconversion point (Post-P)). Given the fact that age and sex significantly affect the distribution of NK cells, 15 patients in the NR group were compared to 15 patients in the R group after matching for age, sex and course of treatment. The results showed that the proportion of NK cells in the R group was significantly higher than in the NR group at Pre-P and Post-P (18.00±1.40 vs. 13.07±1.49, t=2.42, P=0.022; 21.87±2.18 vs. 15.87±1.22, t=2.41, P=0.023; Figure 4).

Proportion of NK cells in selected patients matched for age, sex and treatment period in R and NR groups

Considering the differences in clinical characterisation and length of time needed to achieve HBsAg seroconversion in the R group, we further selected seven patients who achieved HBsAg seroconversion at 24 wk and seven NR patients matched for age, sex, HBsAg at baseline and treatment course for comparison (Table 2). The results also showed that the proportion of NK cells in the R group was significantly higher than in the NR group at Pre-P (12 wk of PEG-IFN treatment) and Post-P (24 wk of PEG-IFN treatment; 19.00±1.34 vs. 11.29±1.30, t=4.12, P=0.001; 24.14±2.58 vs. 14.71±0.42, t=3.61, P=0.004; Figure 5).

NK cell activity in patients matched for age, sex, HBsAg level at baseline and treatment period in R and NR groups

To evaluate NK cells activity for the 14 patients described previously, we used overlapping peptides spanning the entire HBsAg protein of the HBV C2 genotype, which is one of the most common genotypes in China. Example FACS data are shown in Figure 6. This patient (number 5 in R group, table 2) achieved HBsAg seroconversion at 24 wk after PEG-IFN treatment. At baseline, the HBsAg level was 13.56 IU/ml, and anti-HBs was negative (1.29 IU/ml). At 12 wk, although the HBsAg level was still positive (8 IU/ml), anti-HBs was also positive (22.13 IU/ml). At 24 wk, HBsAg loss was achieved, and the anti-HBs level rose to 72.9 IU/ml.

IFN-γ secretion from NK cells in the R group was 2.29±0.44%, 3.80±0.46% and 3.39±0.44% at baseline, Pre-P (12 wk) and Post-P (24 wk), respectively, which was significantly higher than in seven matched patients in the NR group (1.05±0.22%, 1.30±0.24%, 1.32±0.27%; t=2.52, P=0.027; t=4.83, P<0.001; t=4.00, P=0.002; Figure 7a). Similarly, CD107α expression was significantly higher in the R group at baseline, Pre-P (12 wk) and Post-P (24 wk; 3.62±0.69% vs. 1.72±0.33%, t=2.50, P=0.028; 6.02±0.89% vs. 1.61±0.31%, t=4.69, P=0.005; 5.37±0.69 vs. 1.49±0.35, t=5.00, P<0.001; Figure7b).

Table 2. Clinical characterisation of seven patients who achieved HBsAg seroconversion at 24 wk and seven NR cases matched for age, sex, HBsAg level at baseline and treatment course.

	Patient ID	Age	Sex	ALT	HBsAg	1st time point achieved seroconversion
R (n=7)	1	32	M	32	239.3	24 wk
	2	39	M	29	0.693	24 wk
	3	39	M	35	2.85	24 wk
	4	17	F	33	0.326	24 wk
	5	40	F	31	13.56	24 wk
	6	32	F	30	85	24 wk
	7	41	M	28	70	24 wk
NR (n=7)	1	38	M	28	230.4	NA
	2	35	M	29	1.424	NA
	3	40	M	31	6.7	NA
	4	20	F	34	0.592	NA
	5	36	F	29	12.9	NA
	6	32	M	30	89.4	NA
	7	40	F	34	78	NA

NA: not achieved.

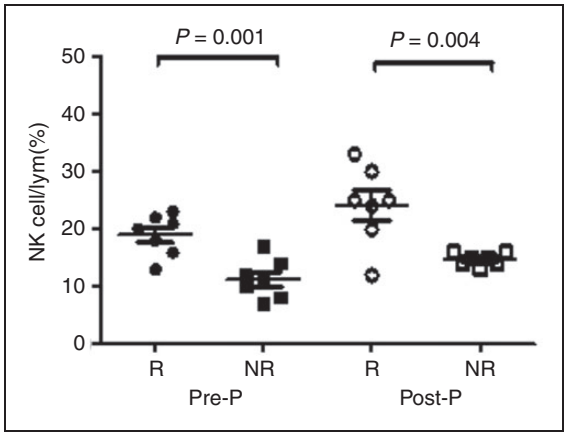

Figure 5. Comparison of NK cells proportion in R group and those matched for age, sex, HBsAg level at baseline and treatment course in the NR group during PEG-IFN treatment. Comparison of proportion of NK cells between IHCs who achieved seroconversion of HBsAg at 24 wk of PEG-IFN treatment (n=7) and those patients who failed to achieve seroconversion of HBsAg (n=7) after matching for age, sex, HBsAg level at baseline and course of treatment.

IFN-γ secretion and CD107α expression were increased from baseline to Pre-P, and then decreased in Post-P, but still higher than baseline in the R group. It is suggested that the function of NK cells was enhanced during Peg-IFN treatment with the removal of HBsAg, although there were no significant differences among the three points (F=3.12, P=0.069; F=2.64, P=0.099), which might be related to the small sample size (Figure 8a and c). In contrast, IFN-γ secretion and CD107α expression did not change much in patients in the NR group after PEG-IFN therapy (Figure 8b and d).

Discussion

In our recent study, we observed a high proportion of cases of HBsAg seroconversion in IHC patients with low HBsAg levels treated with Peg-IFN.[1] The exact mechanism of how PEG-IFN promotes HBsAg clearance remains unknown, and studies on predictors of HBsAg loss are still in the exploratory stage.[9–11] However, there is accumulating evidence that PEG-IFN predominantly expands the NK cells compartment without boosting virus-specific T cells in HBV.[12,13] NK cells respond quickly to stimulants. They can gather in large quantities to attack virus-infected or malignant cells without prior stimulation.[14] Aside from the strong cytotoxic activity against tumour cells and viruses, NK cells secrete a variety of cytokines (IFN-γ, TNF-α, GM-CSF and chemokines), participate early in the innate immune response, modulate the acquired immune response and act as a bridge linking the two immune responses.[15–17] Here, we first performed longitudinal analysis of lymphocyte subsets in IHCs undergoing PEG-IFN treatment, focusing on NK cells.

In our study, we found that the proportion of total NK cells in patients with HBsAg seroconversion was higher than in those who failed to achieve seroconversion. Even when patients were matched for age, sex and treatment period, the conclusion was the same. We directly measured NK cell activity by using overlapping peptides spanning HBsAg, and we detected degranulation and IFN-γ secretion. We found IFN- γ secretion

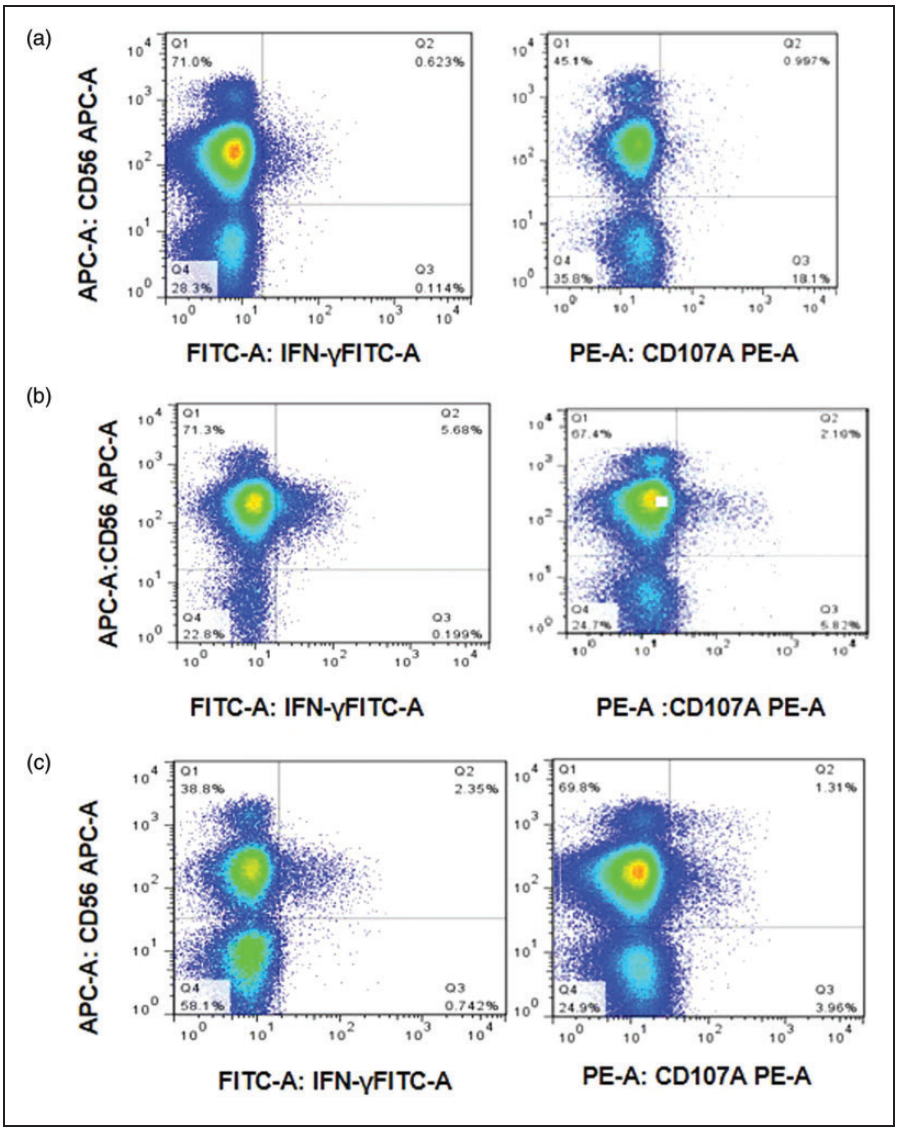

Figure 6. NK cells activity in a patient who achieved HBsAg seroconversion at 24 wk after PEG-IFN treatment. IFN-γ secretion and CD107α expression detected by flow cytometry analysis at (a) baseline, (b) the time point before HBsAg seroconversion was detected (Pre-P) and (c) the time point when seroconversion of HBsAg was detected (Post-P).

and CD107α expression from NK cells in patients who achieved HBsAg seroconversion were all significantly higher than in patients matched for age, sex and treatment period in the NR group at baseline, and at the last time point before HBsAg seroconversion and the first time point when seroconversion of HBsAg was detected. This result suggests that NK cells from HBsAg seroconversion patients are more functional. In addition, we also found that in HBsAg seroconversion cases, NK cells activity (IFN-γ secretion and CD107α expression) increased after PEG-IFN treatment, especially at the time point before HBsAg seroconversion. It is suggested that the function of NK cells was enhanced during PEG-IFN treatment with the removal of HBsAg, although there was no significant difference, which might be related to the small sample size. In contrast, there were no such trends in the NR group.

Our findings suggest that the elevation of NK cells is associated with response and that it favours HBsAg clearance. It has been reported that in chronic HBV infection patients, increased NK cell function is associated with HBsAg seroconversion following structured NA cessation.[18] CD56 bright NK cells induce HBsAg reduction via cytolysis and cccDNA decay in long-term entecavir-treated patients switching to PEG-IFN.[19] Combination therapy significantly influences NK cell phenotype and function. Differences between patients with CHB with HBsAg clearance and non-responders suggest that NK cells play a role in the clearance of

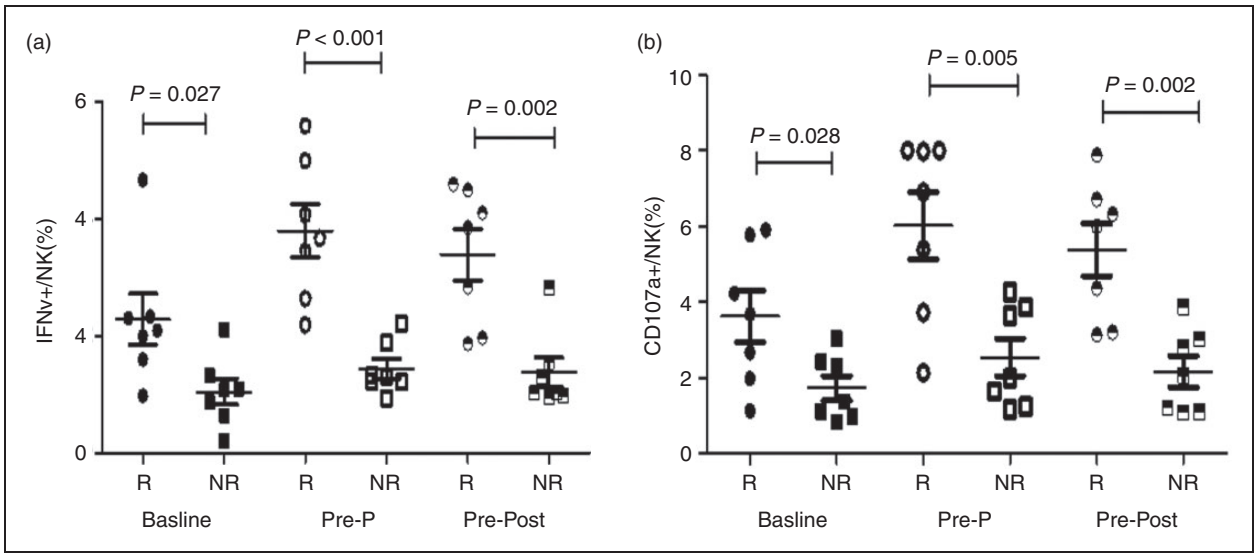

Figure 7. IFN-γ secretion and CD107α expression of NK cells in the R group and NR group at baseline and during PEG-IFN treatment. (a) Comparison of IFN-γ secretion of NK cells between IHCs who achieved seroconversion of HBsAg at 24 wk of PEG-IFN treatment (n=7) and the patients who failed (n=7) after matching for age, sex, HBsAg level at baseline and course of treatment. (b) Comparison of CD107αexpression of NK cells between IHCs who achieved seroconversion of HBsAg at 24 wk of PEG-IFN treatment (n=7) and those who failed (n=7) after matching for age, sex, HBsAg level at baseline and course of treatment.

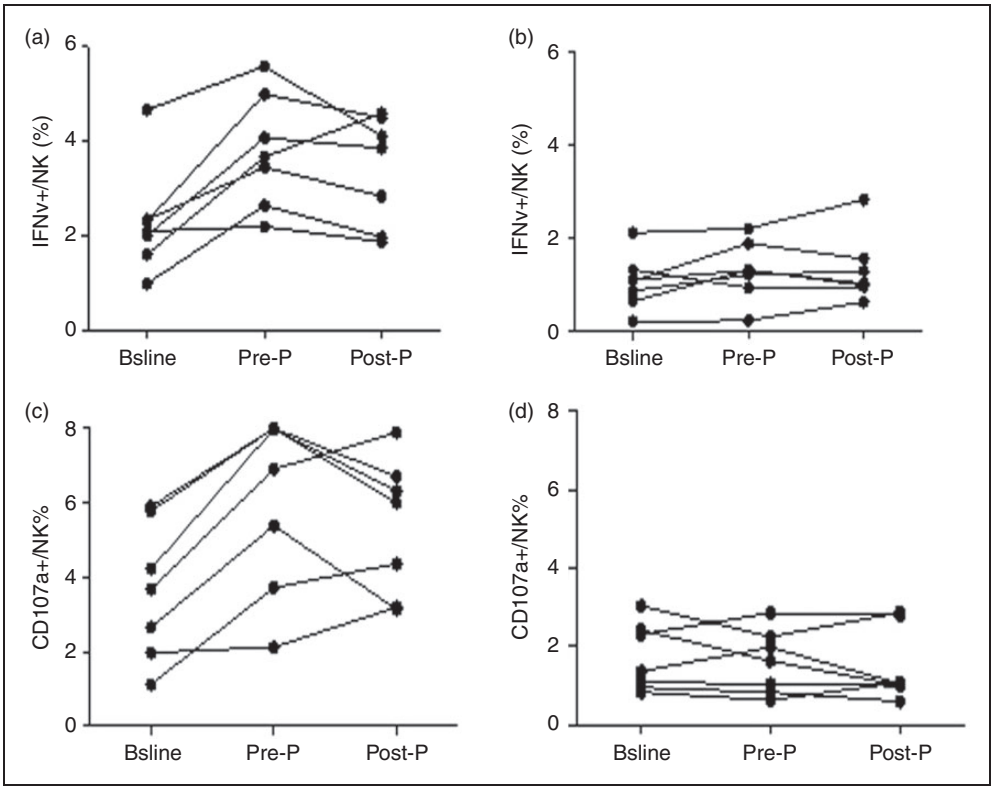

Figure 8. Longitudinal analysis of IFN-γ secretion and CD107αexpression of NK cells in the R group and NR group at baseline and during PEG-IFN treatment. (a) IFN-γ secretion in the R group (n=7). (b) IFN-γ secretion in the NR group (n=7). (c) CD107α expression in the R group (n=7). (d) CD107α expression in the NR group (n=7).

HBsAg during IFN-based combination therapy.[20] All of these data indicate that NK cell levels are closely correlated with the control of HBV infection. In agreement with the previously mentioned reports, we found that PEG-IFN can increase NK cells levels and function, which favours the seroconversion of HBsAg.

In conclusion, we demonstrated that for IHCs, the increase of NK cells during PEG-IFN treatment favoured HBsAg seroconversion, and NK cells may play a role in HBV seroconversion. Because it is very difficult to acquire HBsAg seroconversion for chronic HBV infection patients, our sample size was small. Of course, larger studies are therefore needed.

Declaration of conflicting interests

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