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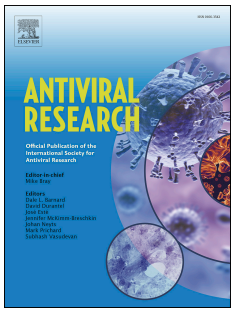

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NK cell phenotypic and functional shifts coincide with specific clinical phases in the natural history of chronic HBV infection

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Running title: Identification of unique NK cell compartments in HBV clinical phases

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Abstract:

Background: Chronic HBV infection can be divided into 4 distinct clinical phases: immune tolerant, immune active, inactive carrier, and HBeAg-negative hepatitis. Using a systems biology approach, we recently identified innate immune response components, specifically NK cells as a distinctive factor of specific HBV clinical phases. To expand on this study and identify the underlying immunological mechanisms, we performed a comprehensive profiling of NK cells in chronic HBV infection.

Methods: Peripheral blood from untreated chronic HBV patients was used to analyze phenotypic markers, as well as cytokine production and cytoxicity of NK cells.

Results: The overall composition, phenotype, and cytolytic activity of the NK cells remained constant across all clinical phases, with the exception of a few specific markers (KIRs, NKp46). $CD56^{bright}$ NK cells of chronic HBV patients differed in their ability to produce IFN-γ between the clinical phases pre- and post-HBeAg seroconversion.

Conclusion: This depicts a shift in NK cell characteristics between the immune active, under heavy viral or immune pressure, and inactive carrier phases, that coincides with HBeAg seroconversion. Although these changes in NK cells do not appear to be completely responsible for differences in liver damage characteristic of specific clinical phases, they could provide a step toward understanding immune dysregulation in chronic HBV infection.

Keywords: Hepatitis B, HBV clinical phases, natural killer cells, NK cells, cytotoxicity, IFN-γ

1. Introduction

Infection with the hepatitis B virus (HBV) leads to non-cytopathic infections of the hosts' hepatocytes. Control of viral replication and subsequent liver injury are believed to be the consequence of the activity of the host immune response to infection. Contrary to infections with the hepatitis C virus (HCV), chronic HBV infections are characterized by episodes with differentiating serum levels of HBV DNA, alanine transferase (ALT), a marker of liver damage, and HBV envelope antigen (HBeAg). Using these parameters, different clinical phases have been discerned to describe the dynamics of the natural history of chronic HBV infection over a period of many years, and determine the indication for antiviral treatment on the basis of rate of viral replication and ALT elevation. Chronic HBV patients have been categorized into 4 clinical phases: the HBeAg-positive immune tolerant (IT) and immune active (IA) phases, as well as the HBeAg-negative inactive carrier (IC) and hepatitis (ENEG) phases (European Association For The Study Of The, 2012). The nomenclature to describe the natural course of HBV infection has led to confusion on the underlying mechanisms, as the IT phase accurately describes the situation where high levels of HBV DNA are observed without elevated ALT levels in serum, but erroneously suggests that the immune response is more tolerant to the presence of virus than in other phases. This, however, is not the case, since normal HBV-specific T cell responses are observed and no distinctive HBV-specific or global T cell activity could be identified in any of the clinical phases (Bertoletti and Hong, 2014; Park et al., 2016).

Over the last decade the importance of natural killer (NK) cells has been extensively described in chronic viral hepatitis (Rehermann, 2013). Various studies, including our own, have shown that chronic HBV patients and healthy individuals have similar total numbers of $CD56^{+}CD3^{-}$ NK cells in peripheral blood (Tjwa et al., 2011; Tjwa et al., 2014), whereas the ability of NK cells from patients to produce interferon (IFN)-γ is impaired (Boni et al., 2015; Lunemann et al., 2014; Oliviero et al., 2009; Peppa et al., 2010; Tjwa et al., 2011). However, a myriad of studies have reported conflicting results, observing no difference or even higher levels of IFN-γ production by NK cells in HBV patients compared to healthy individuals (Conroy et al., 2015; Li et al., 2014; Sun et al., 2012; Zhang et al., 2011). Part of the variation in outcome of these studies may be attributed to the large variety in stimuli (e.g. interleukin (IL)-2, IL-12, IL-15, IL-18,

IFNα, PMA, and ionomycin) used alone or in combination to trigger IFN-γ production by NK cells. Also, the clinical and virological characteristics of the chronic HBV patients examined are likely to influence the features of the NK cell compartment, as many of the patient cohorts in these studies are an unsegregated mixture of all clinical phases (Lunemann et al., 2014; Oliviero et al., 2009; Peppa et al., 2010; Tjwa et al., 2011), whereas others examined either HBeAg-positive or HBeAg-negative patient groups (Boni et al., 2015; Conroy et al., 2015). This may be of particular relevance, as *in vitro* studies have demonstrated that exposure of NK cells to HBeAg affected IL-18 receptor signaling, and consequently reduced the capacity to produce IFN-γ (Jegaskanda et al., 2014). In addition, HBV infection may alter the activation potential of NK cells by modulating the balance of activating and inhibitory receptors on the cell surface. During viral challenge the balance shifts from inhibition to activation after a critical threshold of activation signals exceeds those of inhibition (Lanier, 2005; Vivier et al., 2011). We previously showed that chronic HBV patients express elevated levels of the inhibitory receptor NKG2A and downregulated expression of activating receptors CD16 and NKp30 (Tjwa et al., 2011), although in general reports of the phenotype of NK cells differ between studies with vast degree of conflicting results (Mondelli et al., 2010).

We recently performed a systems biology study of peripheral blood transcriptomes in chronic HBV infection to better identify the mechanisms that govern the distinct clinical phases. Besides enhanced activity of IFN-stimulated genes (ISG) in the IT phase and B cell-function related genes in the IA phase, we also observed that upregulation of cytotoxicity/NK cell activity-related genes clustered in the IA and ENEG phase, i.e. the clinical phases with elevated ALT levels (Vanwolleghem et al., 2015). In the current study, we hypothesized that differential NK cell functionalities contribute to the distinct features observed during the HBV clinical phases, including the fluctuations in liver damage markers and HBV replication. Numerical, phenotypical, and functional analysis of immune parameters, of NK cells obtained from the 4 clinical phases was conducted to obtain an in depth profiling of NK cells throughout the course of natural history of chronic HBV infection.

2. Materials and Methods

2.1. Patient selection and characteristics

Prospectively collected peripheral blood mononuclear cell (PBMC) samples from 40 31 untreated chronic HBV patients attending the outpatient hepatology clinic of the Erasmus MC (Rotterdam, The Netherlands) were selected, if there were no concomitant HIV, HCV, or HDV infections or oncological/rheumatological diseases. In addition, patients were excluded if they were pregnant, had significant steatosis on liver ultrasound, other liver pathology on liver biopsy, or had received antiviral treatment within the previous year. Liver fibrosis, as determined by histology or transient elastometry, was restricted to a maximum F2 Metavir score or maximum elasticity of 7.0 kPa. Based on serum HBV DNA, ALT levels, and HBeAg presence at the time of sampling, patients were categorized into 4 clinical HBV phases according to international guidelines (European Association For The Study Of The, 2012). Immune tolerant (IT) patients had detectable serum HBeAg and repetitive normal ALT values (<40 U/L) for at least 1 year. The HBeAg-positive immune active (IA) and HBeAg-negative (ENEG) patients had repetitive or intermittent abnormal serum ALT (>40 U/L) values, and HBV DNA levels >2,000 IU/mL. Inactive carrier (IC) patients were HBeAg-negative and had both repetitive normal ALT values (<40 IU/L) and HBV DNA levels below 20,000 IU/ mL for at least 1 year. Serum ALT was measured on an automated analyzer, qualitative serum HBsAg and HBeAg levels were measured on an Architect Abbott analyzer, and serum HBV-DNA levels were measured using the COBAS AmpliPrep-COBAS Taq-Man HBVv2test (CAP-CTM; Roche Molecular Systems) (Chen et al., 2012; Feld et al., 2007; Papatheodoridis et al., 2012). Patient characteristics are presented in Table 1. PBMC were isolated from venous blood by ficoll separation (Ficoll-Paque^{TM} plus, Amersham), and stored at -150℃ until used for the various assays. Written informed consent was obtained from all participants. The study protocol was approved by the institutional ethics committee and conducted in accordance with the guidelines of the Declaration of Helsinki.

2.2. Expression of cell surface and intracellular molecules by flow cytometry

To determine the frequency and phenotype of peripheral blood NK cells, multi-color flow cytometry was performed on PBMC with anti-CD3-Alexa-Fluor700 (OKT-3, Beckman), anti-CD56-APC-eFluor780

(CMSSB, Beckman), anti-CD57-APC (HCD57, Biolegend), anti-KIR2D-Biotin (NKVFS1, Miltenyi biotech), anti-KIR3DL1/DL2-Biotin (5133, Miltenyi biotech), Streptavidin-eFluor450 (eBioscience), anti-NKG2A-PE (Z199, Beckman), anti-NKG2C-Alexa-488 (134591, R&D), anti-NKG2D-PerCP-Cy5.5 (1D11; BD), anti-NKp30-PE (Z25, Beckman), anti-NKp44-Biotin (P44-8, Biolegend), anti-NKp46-PE-Cy5 (BAB281, Beckman), anti-CD69-PacificBlue (FN50; Biolegend) and Live/Dead Aqua (Life Technologies). For intracellular expression of cytotoxic markers, PBMC ($0.5x10^6$ cells/200 µl) were fixed with 2% formaldehyde, and permeabilized for intracellular staining with anti-granzyme B-PE (GB11, eBioscience), anti-perforin-PerCP-eFluor710 (G9, eBioscience), and anti-TRAIL-Alexa488 (75402, R&D). Cells were again assessed by flow cytometry (FACS Canto II, BD) and analyzed using FlowJo version 10.1 (Tree Star Inc). Representative FACS plots for surface and intracellular molecules assessed that are not shown in the primary text/figures can be found in Supplementary Figure 1.

2.3. Stimulation and intracellular cytokine analysis of NK cells

PBMC were stimulated with IL-12 (0.25 ng/ml) and IL-18 (10 ng/ml) overnight for surface marker expression and intracellular cytokine production. After 18 hours, brefeldin A (10 µg/ml, Sigma) was added to the cultures and the cells were incubated for an additional 3 hours. Cellular activation and surface markers were measured using anti-CD3-PacificBlue (OKT3, eBioscience), anti-CD56-PE (MY31, BD), and anti-CD69-APC (L78, BD), followed by fixation with 2% formaldehyde, and permeabilization for intracellular staining with anti-IFN-γ-FITC (25723.11, BD). Activated and cytokine-producing NK cells were assessed by flow cytometry (FACS Canto II, BD) and analyzed using FlowJo version 10.1 (Tree Star Inc).

2.4. Statistical analysis

Data are expressed as the mean value ± SEM, unless indicated otherwise. The data were analyzed with Prism, version 5.0, software (GraphPad Software) using the Mann-Whitney U test to compare the variables between independent groups and the Spearman rank correlation coefficient test for nonparametric correlations. In all analyses, a 2-tailed $P < 0.05$ (95% confidence interval) was considered statistically significant.

3. Results

3.1. Baseline characteristics of HBV patient cohort

In order to study differential immune compartments in HBV clinical phases, we carefully selected a cohort of untreated chronically HBV-infected patients, without any other co-morbidities, attending our outpatient clinic. To rule out the impact of advanced liver fibrosis on any identified immune parameters, patients with more than an F2 fibrosis score were excluded (Table 1). Typical for the natural history of chronic HBV infections, patients in the IT phase were the youngest group, followed chronologically by IA, IC, and ultimately ENEG patients. In addition, more females were represented in the asymptomatic IT and IA groups, given that they are more likely to be referred after routine HBsAg testing during pregnancy (Table 1). Although different ethnicities were observed in our tertiary hospital, most of the patients are of Asian ancestry. This is reflected by the fraction of patients infected with HBV genotypes B or C, excluding the ENEG group (Table 1). Owing to the stringent definition criteria, differences in ALT and HBV-DNA levels were observed between the clinical phases, and undetectable or unreactive HBeAg were measured in all IC and ENEG patients (Figure 1A). Furthermore, similar to previous reports (Jaroszewicz et al., 2010), quantitative HBsAg levels were lowest in IC patients and with a subtle increase seen again in patients in the ENEG phase (Figure 1B).

3.2. The frequencies of circulating NK cells and cytolytic molecule-expressing NK cells do not differ between the HBV clinical phases

To understand the role NK cells play in the natural course of chronic HBV infection, we assessed the phenotype and function of NK cells in the 4 specific clinical phases by flow cytometry. As shown in Figure 2A, we observed no significant differences in the percentage of total $CD56^{+}CD3^{-}$ NK cells, or the ratio of $CD56^{bright}$ and $CD56^{dim}$ subsets in the peripheral blood between any of the clinical groups.

Next, we performed intracellular analysis of the cytolytic mediators perforin, granzyme B, and TNF-related apoptosis-inducing ligand (TRAIL) (Figure 2B). No differences were observed in the expression of any of these cytotoxic markers in NK cells, with all expression levels remaining stable across the 4 clinical phases. In line with these findings, also K562-induced degranulation of NK cells did not differ (data not

shown). Collectively, these results show no discerning factors in the total NK cell and NK cell subset frequencies, as well as expression of cytolytic mediators of NK cells in the different chronic HBV clinical phases.

3.3. The frequencies of IFN-γ+/CD56bright expressing NK cells differ in IC patients when compared to the preceding clinical phases

To explore if the functionality of NK cells is correlated to a specific HBV clinical phase, we investigated the activation and cytokine-producing capacity of NK cells upon stimulation with IL-12 and IL-18 in the aforementioned chronic HBV patient cohort. As shown in Figure 3A and 3B, the percentages of CD69 and IFN-γ expressing total NK cells after stimulation were consistent across all 4 groups. However, when analyzing NK cell subsets a significant (1.3 fold) increase in the frequencies of IFN-γ-producing $CD56^{bright}$, but not $CD56^{dim}$ NK cells was observed in patients in the IC phases relative to those in the preceding IT and IA clinical phases (Figure 3C). Differential capacities for particular effector functions have long been described for the $CD56^{bright}$ and the $CD56^{dim}$ NK cell subsets, with $CD56^{bright}$ being able to produce higher levels of cytokine under certain conditions (Campbell et al., 2001; Cooper et al., 2001; Jacobs et al., 2001). This differential IFN-γ production observed in the $CD56^{bright}$ populations of IT and IA patients could therefore attribute to the distinct elevated viral load levels or expression of additional viral antigens (HBeAg) observed in these phases.

3.4. NK cells of chronic HBV patients in the HBeAg-positive phases are characterized by reduced KIR and increased NKp46 expression as compared to the ENEG phase

NK cell activity is tightly regulated by a balance of activating and inhibitory receptors, and during viral infection the balance shifts from inhibition, the steady-state condition, toward activation (Lanier, 2005; Vivier et al., 2011). C-type lectin-like inhibitory receptor (CD94-NKG2A) and killer-cell immunoglobulin-like receptors (KIRs) comprise two of the main classes of inhibiting NK cell receptors, while NCR (e.g. NKp30, NKp46) and C-type lectin-like receptors (e.g. NKG2D) are two primary classes of activating NK receptors (Lanier, 2005). We therefore chose to investigate the expression of these receptors on NK cells in the setting of the natural history of HBV infection. Although the expression of activating NCR NKp30

remained stable across all 4 clinical phases, NK cells of ENEG patients also express significantly lowers levels of activating NCR NKp46 than the IT group (Figure 4A). No differences were observed in the expression of inhibitory NKG2A, as well as activating NKG2D, C-type lectin receptors on NK cells between any of the IT, IA, IC, and ENEG phases (Figure 4C). The expression of KIRs, however, was significantly reduced (1.25 fold) on NK cells from patients in the HBeAg-positive IT and IA clinical phases relative to patients with ENEG hepatitis (Figure 4C).

Concurrently to investigating the activation and inhibitory potential of the NK cells in the varying HBV clinical phases, we chose to investigate the developmental phenotype of these cells using the specific differentiation-associated markers CD16, CD57, and NKG2C (Beziat et al., 2010; Bjorkstrom et al., 2010; Luetke-Eversloh et al., 2013). During analysis of these differentiation-associated receptors, only significant differences were observed when comparing CD57 in the two HBeAg-positive phases, with a significant reduction of CD57 expression seen in HBV patients in the IA clinical phase relative to the IT phase (Figure 4B). Additional analysis for all phenotypic markers was also performed on both the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets (Supplementary Figures 2-3). An increase in expression of activation receptor expression observed in $CD56^{bright}$ NK cells of IC patients, specifically NKp30 and NKG2D expression in respect to the earlier HBeAg-postive IT and IA phases (IT only for NKG2D). Collectively these results suggest an increased inhibitory potential and reduced activation potential in the NK cells of ENEG patients compared to both HBeAg-positive phases, and a reduction of differentiated $CD57^{+}$ NK cells during the transition from the IT phase to the IA phase.

4. Discussion

Previous modular transcriptome analysis of the whole blood of HBV patients identified NK cell/cytotoxicity activities as a distinctive marker in the elevated ALT phases, IA and ENEG, when compared to the IT phase (Vanwolleghem et al., 2015). To build on this study and to obtain a more detailed insight into the heterogeneity among chronic HBV patients, we examined, in the detail, NK cells throughout the course of the natural history of chronic HBV infection, specifically their potential causal role in the fluctuations of liver damage markers and HBV replication observed in these clinical phases. We demonstrate that (1) the overall composition, phenotype, and cytolytic activity of the NK peripheral cell compartment remains relatively constant across all clinical phases, with the exception of a few specific markers (e.g. KIRs, NKp46, and CD57), (2) $CD56^{bright}$ NK cells of chronic HBV patients differ in their ability to produce IFN-γ between the clinical phases pre- and post-HBeAg seroconversion.

The IA and ENEG clinical phases are characterized by elevated levels of ALT and liver damage. We hypothesized that this could partially be attributed to increased direct cytolytic activity and killing of infected hepatocytes by NK cells, but were surprised to find no measurable differences in the expression of cytotoxic mediators perforin, granzyme, and TRAIL across any clinical phases. In line with this, overall similarities were observed by flow cytometry in expression of NK cell activation (NKG2D, NKp30) and inhibitory receptors (NKG2A) when comparing the inflammatory (IA/ENEG) and non-inflammatory (IT/IC) phases. Furthermore, the lack of differences observed in the total percentage of NK cells in the lymphocyte compartment, as well as subset composition ($CD56^{bright/dim}$), suggests no increase in the proportion of NK cells or a phenotypic skewing toward the more cytolytic $CD56^{dim}$ subset. However, the significant changes observed in the proportion of NK cells expressing NKp46 and KIRs suggest an altered activation potential of NK cell compartments during specific clinical phases, markedly the ENEG phase. NK cells from patients in the terminal phase of the natural history of HBV infection, ENEG, expressed decreased levels of activating NCR NKp46 and enhanced levels of inhibitory, and markers of NK cell differentiation, KIRs, potentially resulting a decreased potential for activation for NK cells during this phase. Additionally, NKp46 has been shown to trigger NK cell activation and cytotoxicity upon ligation, but is expressed on mature NK cells irrespective of their activation in humans (Kruse et al.,

2014). Thus, the functional consequences of these observations are complex in nature, and could not be determined in the present study, since the characteristic NK cell parameters (granzyme, perforin, degranulation and TRAIL expression) were stable during the natural history of chronic HBV.

Although the NK cell compartment composition and expression of cytolytic mediators did not differ between clinical phases, one key distinction was the ability of $CD56^{bright}$ NK cells to produce IFN-γ upon cytokine stimulation. IFN-γ has been shown to be a trigger for the process of non-cytolytic HBV clearance and recruitment of inflammatory immune cells in both the innate and adaptive immune response to HBV infection (Bertoletti and Ferrari, 2016; Maini and Gehring, 2016). We demonstrate a differential capacity to produce IFN-γ in NK cells of chronic HBV patients pre- and post-HBeAg seroconversion, specifically between the IT/IA and IC phases. This differential production of IFN-γ observed could be the result of enhanced immune pressure due to high viral and antigen load present during the HBeAg-positive clinical phases, relieved after seroconversion and the subsequent viral suppression. However, we cannot say if this is a direct result of the high levels of viral particles and antigens present, or an indirect effect due to the coinciding immune activity in these phases.

Collectively, by conducting a detailed analysis of the phenotype and function of NK cells using clinically well-defined patient cohorts, we were able to characterize distinctive NK cell compartmental alterations during the progression of the chronic HBV natural history. Our data, obtained using peripheral blood, provide no evidence for higher NK cell activities during the IA and ENEG phase, thereby limiting the likelihood that NK cells are responsible for observed liver damage during these specific phases as reflected by fluctuating serum ALT levels. However, our findings show subtle changes indicative of a shift in NK cell phenotype and function between the IA phase under heavy viral or immune pressure, and the IC phase, that coincides with the process of HBeAg seroconversion. These observations shed light on the differences in the NK cell compartment during the natural history of chronic HBV, which may help better understand the extensive heterogeneity of immune responses observed in chronic HBV patients.

Conflict of Interest: The authors of this manuscript have no conflicts of interest to declare.

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References

Bertoletti, A., Ferrari, C., 2016. Adaptive immunity in HBV infection. J Hepatol 64, S71-83.

Bertoletti, A., Hong, M., 2014. Age-Dependent Immune Events during HBV Infection from Birth to Adulthood: An Alternative Interpretation. Front Immunol 5, 441.

Beziat, V., Descours, B., Parizot, C., Debre, P., Vieillard, V., 2010. NK cell terminal differentiation: correlated stepwise decrease of NKG2A and acquisition of KIRs. PLoS One 5, e11966.

Bjorkstrom, N.K., Riese, P., Heuts, F., Andersson, S., Fauriat, C., Ivarsson, M.A., Bjorklund, A.T., Flodstrom-Tullberg, M., Michaelsson, J., Rottenberg, M.E., Guzman, C.A., Ljunggren, H.G., Malmberg, K.J., 2010. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. Blood 116, 3853-3864.

Boni, C., Lampertico, P., Talamona, L., Giuberti, T., Invernizzi, F., Barili, V., Fisicaro, P., Rossi, M., Cavallo, M.C., Vecchi, A., Pedrazzi, G., Alfieri, A., Colombo, M., Missale, G., Ferrari, C., 2015. Natural killer cell phenotype modulation and natural killer/T-cell interplay in nucleos(t)ide analogue-treated hepatitis e antigen-negative patients with chronic hepatitis B. Hepatology 62, 1697-1709.

Campbell, J.J., Qin, S., Unutmaz, D., Soler, D., Murphy, K.E., Hodge, M.R., Wu, L., Butcher, E.C., 2001. Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. J Immunol 166, 6477-6482.

Chen, Y.C., Huang, S.F., Chu, C.M., Liaw, Y.F., 2012. Serial HBV DNA levels in patients with persistently normal transaminase over 10 years following spontaneous HBeAg seroconversion. J Viral Hepat 19, 138-146.

Conroy, M.J., Mac Nicholas, R., Grealy, R., Taylor, M., Otegbayo, J.A., O'Dea, S., Mulcahy, F., Ryan, T., Norris, S., Doherty, D.G., 2015. Circulating CD56dim natural killer cells and CD56+ T cells that produce interferon-gamma or interleukin-10 are expanded in asymptomatic, E antigen-negative patients with persistent hepatitis B virus infection. J Viral Hepat 22, 335-345.

Cooper, M.A., Fehniger, T.A., Turner, S.C., Chen, K.S., Ghaheri, B.A., Ghayur, T., Carson, W.E., Caligiuri, M.A., 2001. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood 97, 3146-3151.

European Association For The Study Of The, L., 2012. EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. J Hepatol 57, 167-185.

Feld, J.J., Ayers, M., El-Ashry, D., Mazzulli, T., Tellier, R., Heathcote, E.J., 2007. Hepatitis B virus DNA prediction rules for hepatitis B e antigen-negative chronic hepatitis B. Hepatology 46, 1057-1070.

Jacobs, R., Hintzen, G., Kemper, A., Beul, K., Kempf, S., Behrens, G., Sykora, K.W., Schmidt, R.E., 2001. CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. Eur J Immunol 31, 3121-3127.

Jaroszewicz, J., Calle Serrano, B., Wursthorn, K., Deterding, K., Schlue, J., Raupach, R., Flisiak, R., Bock, C.T., Manns, M.P., Wedemeyer, H., Cornberg, M., 2010. Hepatitis B surface antigen (HBsAg) levels in the natural history of hepatitis B virus (HBV)-infection: a European perspective. J Hepatol 52, 514-522.

Jegaskanda, S., Ahn, S.H., Skinner, N., Thompson, A.J., Ngyuen, T., Holmes, J., De Rose, R., Navis, M., Winnall, W.R., Kramski, M., Bernardi, G., Bayliss, J., Colledge, D., Sozzi, V., Visvanathan, K., Locarnini, S.A., Kent, S.J., Revill, P.A., 2014. Downregulation of interleukin-18-mediated cell signaling and interferon gamma expression by the hepatitis B virus e antigen. J Virol 88, 10412-10420.

Kruse, P.H., Matta, J., Ugolini, S., Vivier, E., 2014. Natural cytotoxicity receptors and their ligands. Immunol Cell Biol 92, 221-229.

Lanier, L.L., 2005. NK cell recognition. Annu Rev Immunol 23, 225-274.

Li, Y., Wang, J.J., Gao, S., Liu, Q., Bai, J., Zhao, X.Q., Hao, Y.H., Ding, H.H., Zhu, F., Yang, D.L., Zhao, X.P., 2014. Decreased peripheral natural killer cells activity in the immune activated stage of chronic hepatitis B. PLoS One 9, e86927.

Luetke-Eversloh, M., Killig, M., Romagnani, C., 2013. Signatures of human NK cell development and terminal differentiation. Front Immunol 4, 499.

Lunemann, S., Malone, D.F., Hengst, J., Port, K., Grabowski, J., Deterding, K., Markova, A., Bremer, B., Schlaphoff, V., Cornberg, M., Manns, M.P., Sandberg, J.K., Ljunggren, H.G., Bjorkstrom, N.K., Wedemeyer, H., 2014. Compromised function of natural killer cells in acute and chronic viral hepatitis. J Infect Dis 209, 1362-1373.

Maini, M.K., Gehring, A.J., 2016. The role of innate immunity in the immunopathology and treatment of HBV infection. J Hepatol 64, S60-70.

Mondelli, M.U., Varchetta, S., Oliviero, B., 2010. Natural killer cells in viral hepatitis: facts and controversies. Eur J Clin Invest 40, 851-863.

Oliviero, B., Varchetta, S., Paudice, E., Michelone, G., Zaramella, M., Mavilio, D., De Filippi, F., Bruno, S., Mondelli, M.U., 2009. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. Gastroenterology 137, 1151-1160, 1160 e1151-1157.

Papatheodoridis, G.V., Manolakopoulos, S., Liaw, Y.F., Lok, A., 2012. Follow-up and indications for liver biopsy in HBeAg-negative chronic hepatitis B virus infection with persistently normal ALT: a systematic review. J Hepatol 57, 196-202.

Park, J.J., Wong, D.K., Wahed, A.S., Lee, W.M., Feld, J.J., Terrault, N., Khalili, M., Sterling, R.K., Kowdley, K.V., Bzowej, N., Lau, D.T., Kim, W.R., Smith, C., Carithers, R.L., Torrey, K.W., Keith, J.W., Levine, D.L., Traum, D., Ho, S., Valiga, M.E., Johnson, G.S., Doo, E., Lok, A.S., Chang, K.M., Hepatitis, B.R.N., 2016. Hepatitis B Virus-Specific and Global T-Cell Dysfunction in Chronic Hepatitis B. Gastroenterology 150, 684-695 e685.

Peppa, D., Micco, L., Javaid, A., Kennedy, P.T., Schurich, A., Dunn, C., Pallant, C., Ellis, G., Khanna, P., Dusheiko, G., Gilson, R.J., Maini, M.K., 2010. Blockade of immunosuppressive cytokines restores NK cell antiviral function in chronic hepatitis B virus infection. PLoS Pathog 6, e1001227.

Rehermann, B., 2013. Pathogenesis of chronic viral hepatitis: differential roles of T cells and NK cells. Nat Med 19, 859-868.

Sun, C., Fu, B., Gao, Y., Liao, X., Sun, R., Tian, Z., Wei, H., 2012. TGF-beta1 down-regulation of NKG2D/DAP10 and 2B4/SAP expression on human NK cells contributes to HBV persistence. PLoS Pathog 8, e1002594.

Tjwa, E.T., van Oord, G.W., Hegmans, J.P., Janssen, H.L., Woltman, A.M., 2011. Viral load reduction improves activation and function of natural killer cells in patients with chronic hepatitis B. J Hepatol 54, 209-218.

Tjwa, E.T., Zoutendijk, R., van Oord, G.W., Biesta, P.J., Verheij, J., Janssen, H.L., Woltman, A.M., Boonstra, A., 2014. Intrahepatic natural killer cell activation, but not function, is associated with HBsAg levels in patients with HBeAg-negative chronic hepatitis B. Liver Int 34, 396-404.

Vanwolleghem, T., Hou, J., van Oord, G., Andeweg, A.C., Osterhaus, A.D., Pas, S.D., Janssen, H.L., Boonstra, A., 2015. Re-evaluation of hepatitis B virus clinical phases by systems biology identifies unappreciated roles for the innate immune response and B cells. Hepatology 62, 87-100.

Vivier, E., Raulet, D.H., Moretta, A., Caligiuri, M.A., Zitvogel, L., Lanier, L.L., Yokoyama, W.M., Ugolini, S., 2011. Innate or adaptive immunity? The example of natural killer cells. Science 331, 44-49.

Zhang, Z., Zhang, S., Zou, Z., Shi, J., Zhao, J., Fan, R., Qin, E., Li, B., Li, Z., Xu, X., Fu, J., Zhang, J., Gao, B., Tian, Z., Wang, F.S., 2011. Hypercytolytic activity of hepatic natural killer cells correlates with liver injury in chronic hepatitis B patients. Hepatology 53, 73-85.

Figure Legends

Figure 1. Baseline characteristics of chronic HBV patients separated into the four clinical phases based on HBV DNA, ALT, HBeAg, and HBsAg levels. (A) Serum samples of 31 chronic HBV patients were assessed for their HBV DNA, ALT, (B) HBeAg, and HBsAg levels.

Figure 2. The frequency of circulating NK cells and cytolytic molecule-expressing NK cells do not differ between the clinical phases. (A) Representative dot-plot showing the gating strategy for $CD56^{+}CD3^{-}$ NK cells in peripheral blood (B) The collective frequencies of $CD56^{+}CD3^{-}$ NK cells within the lymphocyte population of patients in the four different clinical phases, and the frequencies of $CD56^{bright}$ and $CD56^{dim}$ NK cells within the total NK cell population in PBMC of patients in the different clinical phases. (C) Intracellular perforin, intracellular granzyme B, and TRAIL expression were measured within the total NK cell compartment of the aforementioned groups. IT (n=7), IA (n=6), IC (n=8), and ENEG (n=10), all samples were evaluated by flow cytometry and data are shown as mean ± SEM. * denotes $p<0.05$ and ** $p<0.01$ (Mann-Whitney U test).

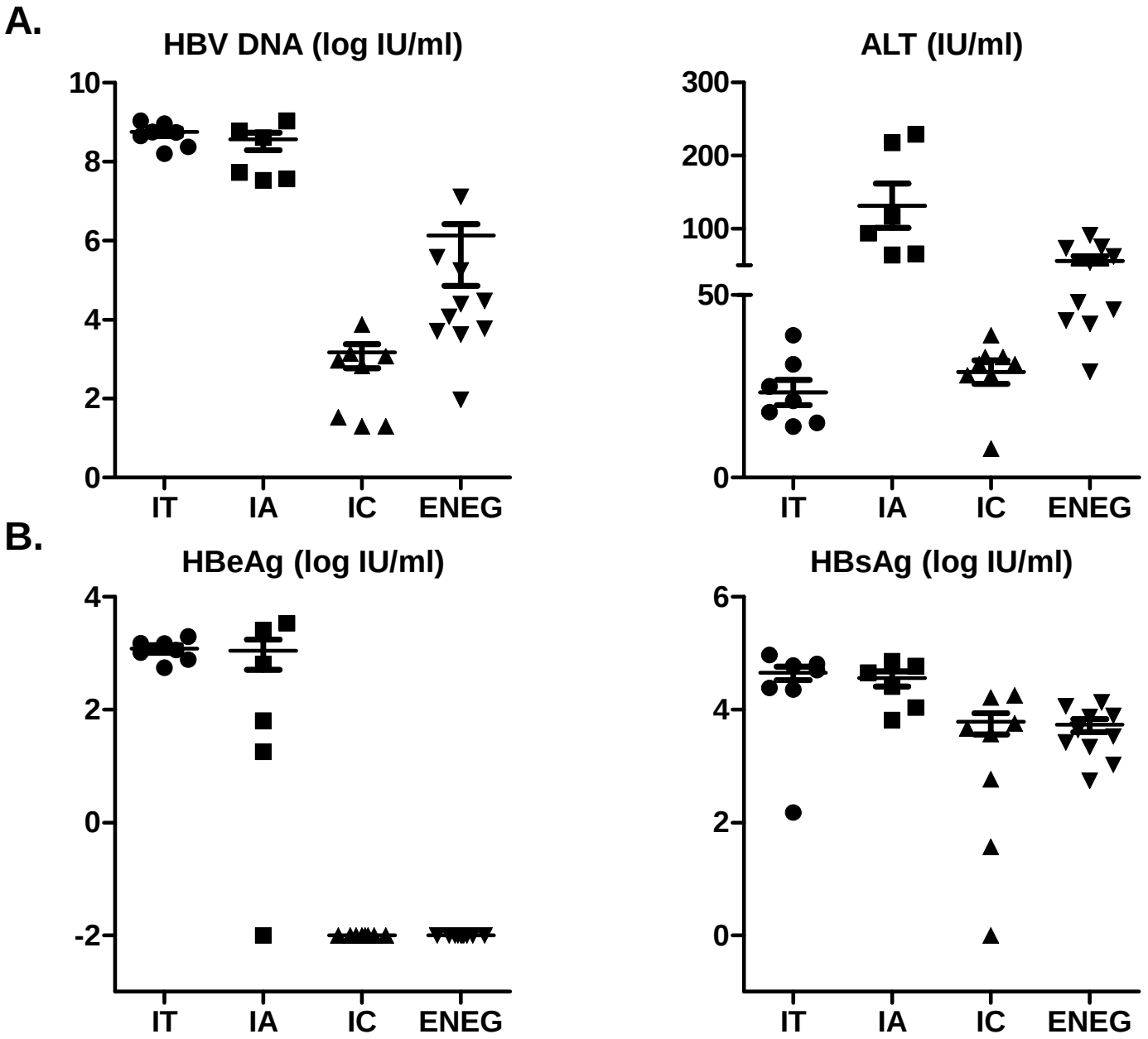
Figure 3. The frequency of IFN-γ^{+}/$CD56^{bright}$ expressing NK cells differs in IC patients when compared to the preceding clinical phases. (A) Representative dot-plots for the CD69 expression and (B) intracellular IFN-γ expression of NK cells, for PBMC either left unstimulated or in the presence of IL-12/IL-18 overnight, of chronic HBV patients in the IT, IA, IC and ENEG clinical phases. Collective results of multiple patients in each group shown in far right panels. (C) The frequency of IFN-γ-expressing NK cells was further divided into $CD56^{bright}$ and $CD56^{dim}$ NK subsets. IT (n=7), IA (n=6), IC (n=8), and ENEG (n=10), all samples were evaluated by flow cytometry and data are shown as mean ± SEM. * denotes $p<0.05$ and ** $p<0.01$ (Mann-Whitney U test).

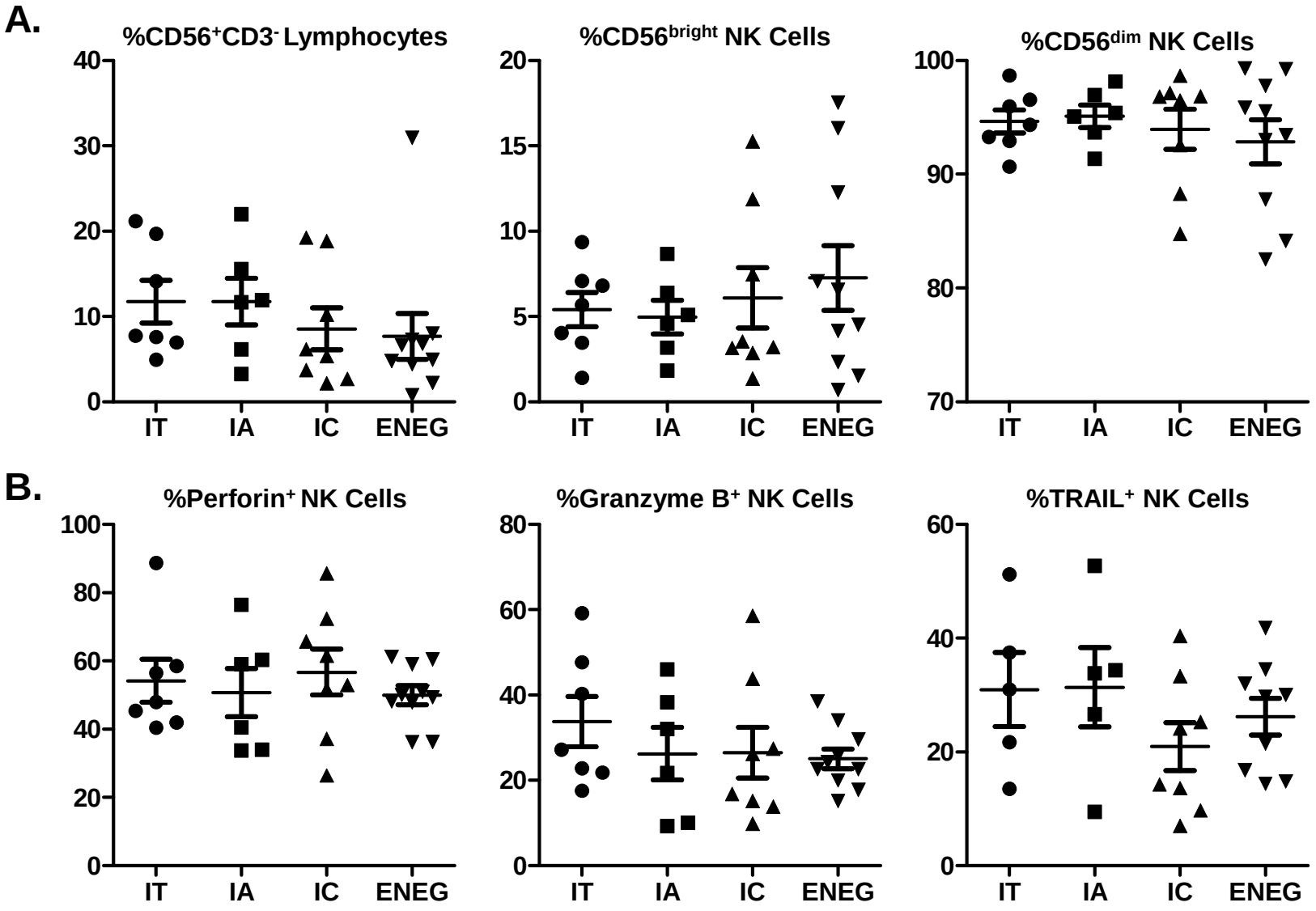
Figure 4. NK cells of chronic HBV patients in the HBeAg-positive phases are characterized by reduced KIR and increased NKp46 expression as compared to the ENEG phase. Flow cytometric analysis was performed on the PBMC of chronic HBV patients in different clinical phases to determine the

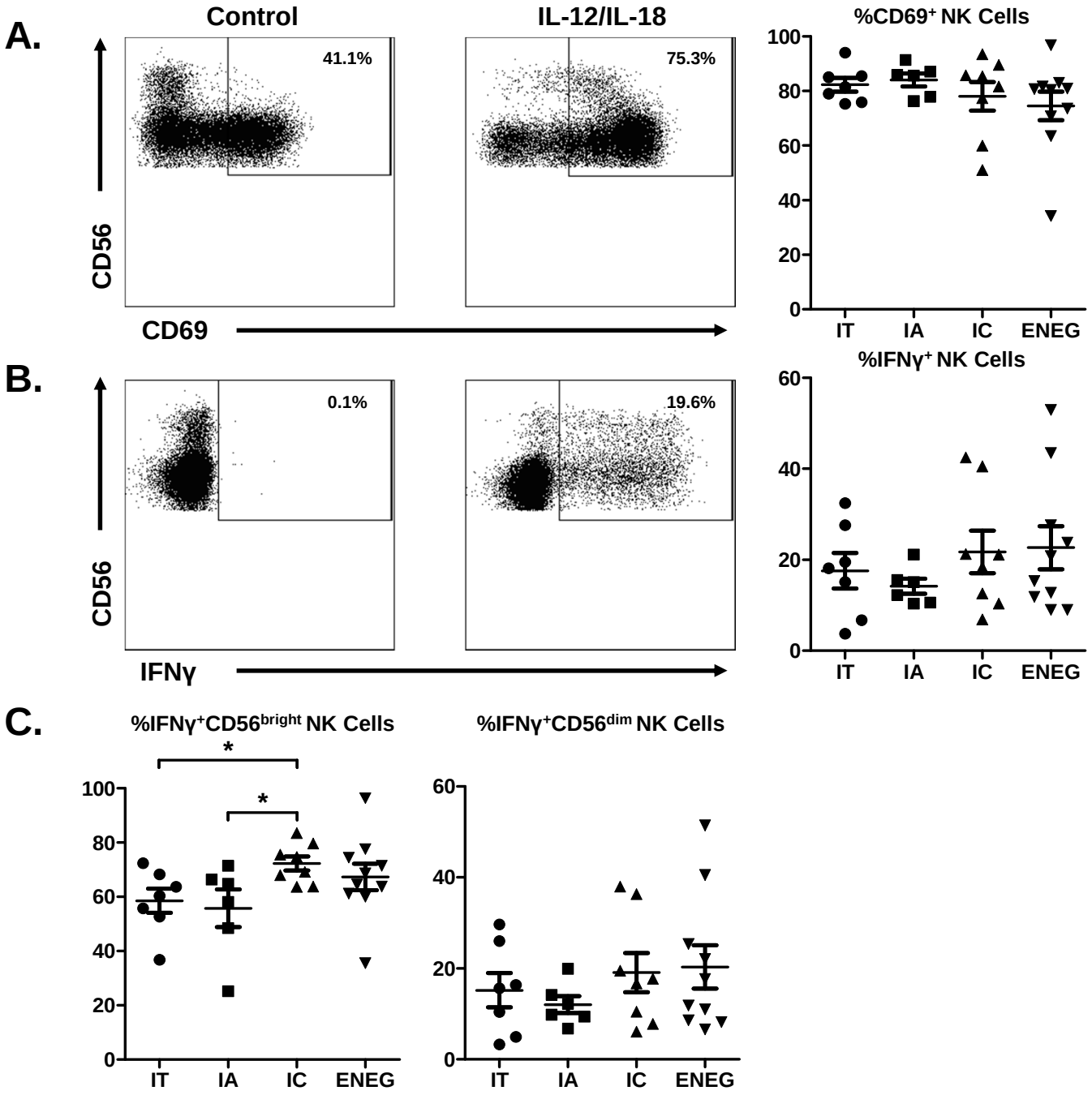
expression of (A) NCRs and activating receptors NKp30 and NKp46, (B) maturation-associated markers CD16 and CD57, (C) as well as a panel of KIRs and c-type lectin activating and inhibitory receptors NKG2A, NKG2C, and NKG2D on total NK cells. IT (n=7), IA (n=6), IC (n=8), and ENEG (n=10), all samples were evaluated by flow cytometry and data are shown as mean ± SEM. * denotes $p<0.05$ and ** $p<0.01$ (Mann-Whitney U test).

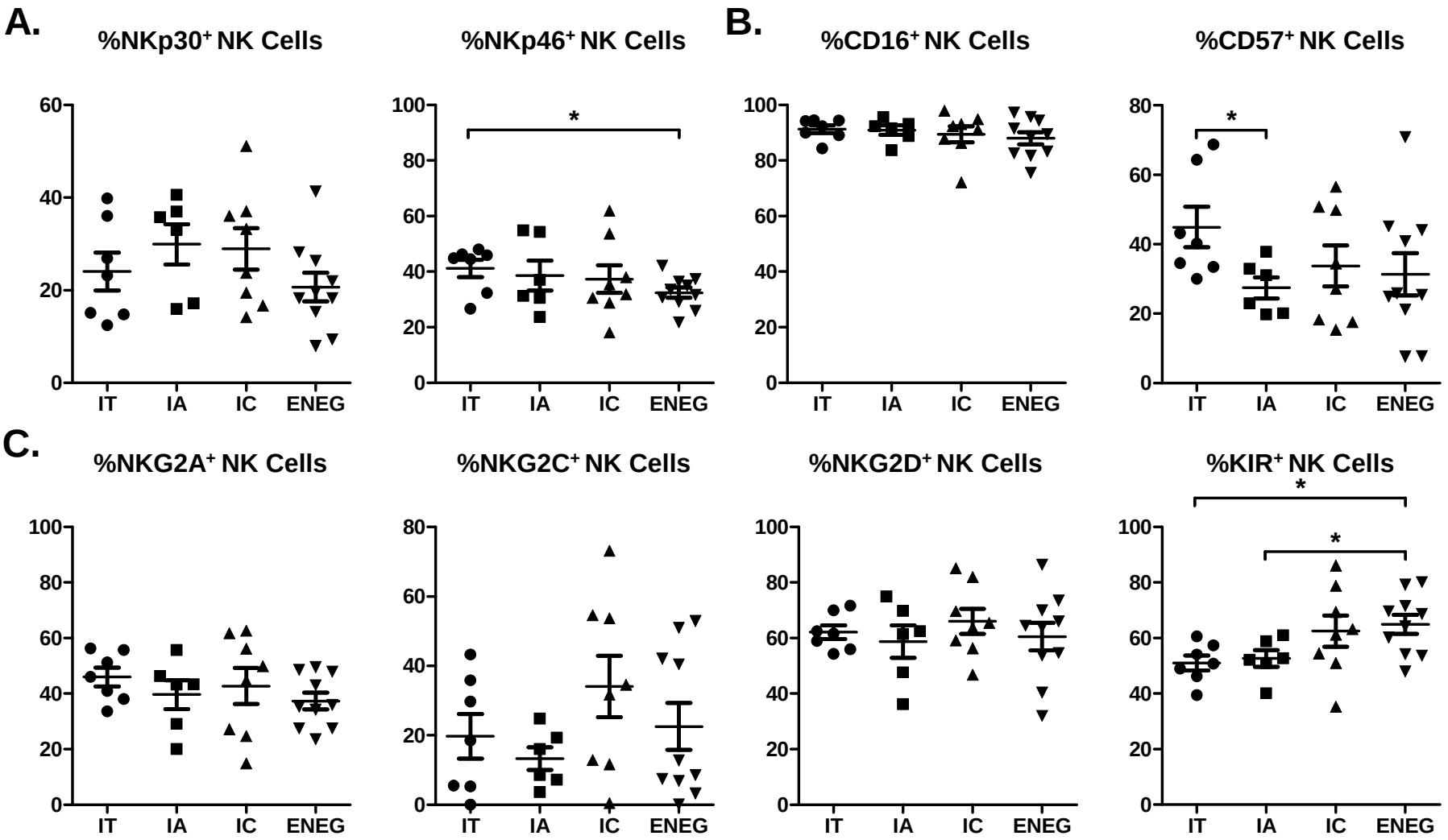
Table 1: HBV patient characteristics

	Immune Tolerant (IT)	Immune Active (IA)	Inactive Carrier (IC)	HBeAg-Hepatitis (ENEG)
Sex (M/F)	3/4	4/2	5/3	9/1
Age (Years)	29.7 (24-37)	36.8 (18-49)	38.0 (30-48)	40.9 (29-47)
Ethnicity:				
Asian	7	5	5	4
African	0	0	3	3
Other	0	1	0	3
ALT	23.3 (14-39)	131.3 (64-229)	28.9 (8-39)	56.3 (29-73)
HBV DNA (IU/ml)	$5.7x10^{8}$ ($1.6x10^{8}$ -$1.1x10^{9}$)	$3.8x10^{8}$ ($3.4x10^{7}$ -$1.1x10^{9}$)	$1.5x10^{3}$ ($2.0x10^{1}$ -$7.6x10^{3}$)	$1.3x10^{6}$ ($9.4x10^{1}$ -$1.3x10^{7}$)
HBsAg (IU/ml)	52930 (150-93730)	36628 (6495-72570)	6152 (1-17970)	5439 (543-13466)
HBeAg	Positive	Positive	Negative	Negative
HBV Genotype:				
A	0	1	0	3
B/C	5	5	4	2
D	0	0	0	2
E	0	0	1	0
Unknown	2	0	3	3
Fibrosis:				
F0-F1	6	2	8	8
F1-F2	1	4	0	2






A.
Control
IL-12/IL-18
41.1%
75.3%
CD56
CD69
%CD69+ NK Cells
100
80
60
40
20
0
IT
IA
IC
ENEG
B.
0.1%
19.6%
CD56
IFNγ
%IFNγ+ NK Cells
60
40
20
0
IT
IA
IC
ENEG
C.
%IFNγ+CD56bright NK Cells
%IFNγ+CD56dim NK Cells
*
*
100
80
60
40
20
0
60
40
20
0
IT
IA
IC
ENEG
IT
IA
IC
ENEG



Highlights

- The frequency of circulating NK cells is stable during the course of chronic HBV
- Between the clinical phases of chronic HBV IFNγ production by NK cells differs
- The transition to the IA phase is characterized by a reduction of $CD57^+$ NK cells