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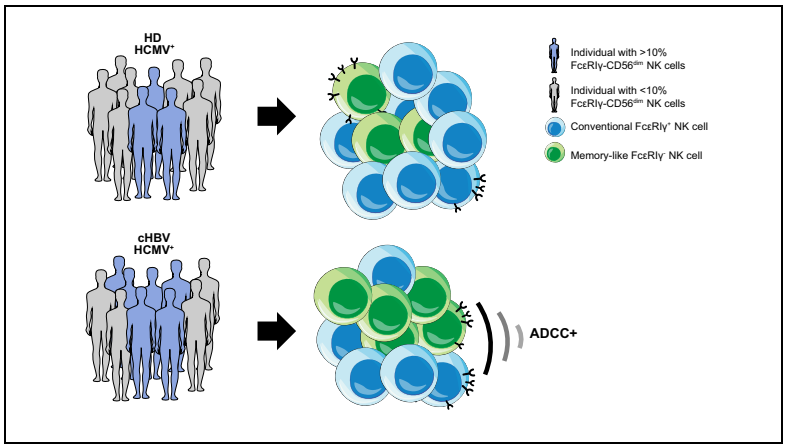
NK-cell responses are biased towards CD16-mediated effector functions in chronic hepatitis B virus infection

Graphical abstract



Highlights

- Frequent HBV/HCMV co-infection is associated with the expansion of memory-like NK cells.
- Memory-like NK cells are largely conserved in chronic hepatitis B virus infection.
- Memory-like NK cells determine the NK-cell response in chronically hepatitis B virus-infected patients.
- Adaptive antibody-dependent NK-cell response is increased in chronic hepatitis B virus infection.

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Lay summary

In chronic hepatitis B virus infection, NK-cell phenotype and function is altered. In this study, we demonstrate that these changes are linked to the emergence of a distinct NK-cell subset, namely memory-like NK cells. The emergence of these memory-like NK cells is associated with coinfection of HCMV that affects the majority of patients with chronic hepatitis B.

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NK-cell responses are biased towards CD16-mediated effector functions in chronic hepatitis B virus infection

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Background & Aims: Phenotypic and functional natural killer (NK)-cell alterations are well described in chronic hepatitis B virus (cHBV) infection. However, it is largely unknown whether these alterations result from general effects on the overall NK-cell population or the emergence of distinct NK-cell subsets. Human cytomegalovirus (HCMV) is common in cHBV and is associated with the emergence of memory-like NK cells. We aimed to assess the impact of these cells on cHBV infection.

Methods: To assess the impact of memory-like NK cells on phenotypic and functional alterations in cHBV infection, we performed in-depth analyses of circulating NK cells in 52 patients with cHBV, 45 with chronic hepatitis C virus infection and 50 healthy donors, with respect to their HCMV serostatus.

Results: In patients with cHBV/HCMV+, FcεRIγ- memory-like NK cells were present in higher frequencies and with higher prevalence than in healthy donors with HCMV+. This pronounced HCMV-associated memory-like NK-cell expansion could be identified as key determinant of the NK-cell response in cHBV infection. Furthermore, we observed that memory-like NK cells consist of epigenetically distinct subsets and exhibit key metabolic characteristics of long-living cells. Despite ongoing chronic infection, the phenotype of memory-like NK cells was conserved in patients with cHBV/HCMV+. Functional characteristics of memory-like NK cells also remained largely unaffected by cHBV infection with the exception of an increased degranulation capacity in response to CD16 stimulation that was, however, detectable in both memory-like and conventional NK cells.

Conclusions: The emergence of HCMV-associated memory-like NK cells shapes the overall NK-cell response in cHBV infection and contributes to a general shift towards CD16-mediated effector functions. Therefore, HCMV coinfection needs to be considered in the design of immunotherapeutic approaches that target NK cells in cHBV.

Lay summary: In chronic hepatitis B virus infection, natural killer (NK)-cell phenotype and function is altered. In this study, we demonstrate that these changes are linked to the emergence of a distinct NK-cell subset, namely memory-like NK cells. The emergence of these memory-like NK cells is associated with coinfection of human cytomegalovirus that affects the majority of patients with chronic hepatitis B.

Introduction

Hepatitis B virus (HBV) is a non-cytopathic DNA virus that triggers immune-mediated liver pathology. It is estimated that 257 million people worldwide are suffering from chronic HBV (cHBV) infection and are therefore at high risk of developing progressive liver disease. The capacity of the immune system to control HBV infection provides a rationale for immunotherapeutic approaches. Direct and indirect roles of natural killer (NK) cells in mediating anti-HBV immunity have been described.[1,2] For example, in a hydrodynamic injection model of acute HBV infection, a direct antiviral effect of NK cells has been reported.[3] In that model, NK cells can also indirectly support HBV clearance by positively affecting HBV-specific T-cell responses via interferon γ (IFNγ) secretion.[4] However, in cHBV infection NK cells exhibit an impaired IFNγ production, consequently leading to reduced non-cytolytic antiviral potential and diminished support of T-cell responses.[5,6] A landmark study has also shown that despite their reduced cytokine production, NK cells obtained from patients with cHBV displayed a conserved cytotoxic function. This phenomenon has been termed functional dichotomy.[5]

Keywords: NK cells; Chronic hepatitis B virus infection; Human cytomegalovirus; CD16; Memory-like; Adaptive.
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The repertoire of human NK cells is altered in human cytomegalovirus seropositive (HCMV+) compared to HCMV seronegative (HCMV-) patients.[7–11] In particular, expansion of phenotypically and functionally distinct NK-cell subsets can be observed for long periods of time, giving these cells memory-like properties.[8,12–16] The expanded memory-like NK-cell subsets are characterized by the expression of NKG2C, or CD2, and in cases of homozygous *NKG2C* (*KLRC2*) deletion, by the lack of the adaptor protein FcεRIγ and by distinct epigenetic profiles that are similar to CD8 T cells.[12,13,17,18] Especially the lack of FcεRIγ marks memory-like NK cells that provide superior effector function in response to antibody triggering mediated by the FcγRIII (CD16).[12,13]

HCMV is universally distributed among human populations.[19] In cHBV patients HCMV coinfection is common[20] and HCMV-associated expansions of NK-cell populations are also evident.[21,22] However, to date it is not clear whether HCMV infection and its associated expansion of memory-like NK cells promotes NK-cell alterations observed in cHBV infection. To address this, we focused on CD56^{dim} NK cells, the majority of circulating NK cells, and performed comparative analyses of conventional FcεRIγ+ *vs.* memory-like FcεRIγ- subsets obtained from the blood of patients with cHBV and healthy donors (HD). We detected memory-like FcεRIγ- NK-cell subsets in the majority of patients with cHBV/HCMV+, and with a higher prevalence compared to HD/HCMV+. Comprehensive phenotypic, functional, metabolic and epigenetic analyses further revealed that the distinct characteristics of conventional FcεRIγ+ and memory-like FcεRIγ- NK cells were largely conserved, except for a generally increased CD16-mediated degranulation/cytotoxicity in patients with cHBV/HCMV+ compared to HD/HCMV+. Thus, cHBV-associated CD56^{dim} NK-cell alterations directly correlated with the frequency of the memory-like FcεRIγ- subsets present in patients with cHBV/HCMV+. In summary, an increase in CD16-mediated CD56^{dim} NK-cell effector function linked to the pronounced expansion of FcεRIγ- memory-like NK cells that have *per se* a superior CD16 responsiveness revealed that the NK-cell response in cHBV infection is biased towards CD16-mediated effector functions.

Materials and methods

Study cohorts

A total of 52 patients with cHBV, 45 with chronic hepatitis C virus (cHCV) and 50 HD were recruited at the Department of Medicine II of the University Hospital Freiburg, Germany. All cHBV patients were positive for anti-HBc and HBV surface antigen for at least 1 year. Patients with cHCV had detectable serum HCV RNA for at least 1 year. Viral loads and aminotransferases were determined as part of the clinical diagnostics at the University Hospital Freiburg. Patients with liver cirrhosis were excluded from the study. Detailed patient characteristics are listed in (Tables S1–3). Written informed consent was obtained in all cases and the study was conducted according to federal guidelines, local ethics committee regulations (Albert-Ludwigs-University, Freiburg, HBUF 474/14 and 299/01), Germany) and the Declaration of Helsinki (1975).

Multiparametric flow cytometry

Flow cytometry protocols are specified in the supplementary materials and methods and the supplementary CTAT table.

Assessment of NK-cell function

Degranulation, assessed by CD107a staining of NK cells, and cytokine production were determined upon overnight cytokine stimulation or CD16-crosslinking. Detailed information is provided in the supplementary materials and methods.

Metabolic analyses

To assess metabolic requirements of NK-cell subsets, glucose uptake and mitochondrial characteristics were analyzed. Detailed information is provided in the supplementary materials and methods.

DNA methylation analyses

CD14-, CD19-, CD3- CD56^{dim} lymphocytes were FACS-sorted according to their FcεRIγ/Helios expression with a BD FACS Aria Fusion Cell Sorter. DNA methylation analyses were performed by EpigenDx (Hopkinton, MA, USA) applying bisulfite sequencing. Analyzed CpG sites are listed in the supplementary materials and methods.

Statistics

Statistical analysis was performed with GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Statistical tests used are indicated in the figure legends. Levels of significance are indicated as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

For further details regarding the materials used, please refer to the CTAT table and supplementary information.

Results

Increased frequencies of memory-like FcεRIγ- NK cells in the peripheral blood of patients with cHBV/HCMV+

To assess the presence of memory-like NK cells in cHBV infection, we first analyzed FcεRIγ expression (Fig. S1) in circulating CD56^{dim} NK cells obtained from cHBV patients compared to patients with cHCV and HD (Table S1-3). As depicted in Fig. 1A, FcεRIγ- CD56^{dim} NK cells were present in patients with cHBV, cHCV and HD. FcεRIγ- CD56^{dim} NK cells were only detectable in patients who were HCMV+, clearly demonstrating linkage to HCMV infection. In contrast to patients with cHCV/HCMV+, the frequency of circulating FcεRIγ- CD56^{dim} NK cells in patients with cHBV/HCMV+ was significantly increased compared to HD/HCMV+. In addition, the prevalence of HCMV+ individuals harboring FcεRIγ- CD56^{dim} NK cells (defined as >10% FcεRIγ- of CD56^{dim} NK cells) was also increased in patients with cHBV compared to HD (Fig. 1B). Thus, memory-like FcεRIγ- CD56^{dim} NK cells are more common in patients with cHBV/HCMV+ both in frequency and prevalence. Yet, we did not find any correlation in the frequency of FcεRIγ- CD56^{dim} NK cells with viral load, serum aminotransferase levels or treatment in patients with cHBV and frequencies remained stable for years during nucleos(t)ide analog therapy (Fig. S2).

Key characteristics of memory-like FcεRIγ- CD56^{dim} NK cells are conserved in cHBV infection

To evaluate whether FcεRIγ- CD56^{dim} NK cells share key characteristics in patients with cHBV/HCMV+ and HD/HCMV+, we comparatively analyzed expression of the signaling molecules EAT2 and Syk, the transcriptional regulators PLZF and Helios and of the maturation/differentiation markers CD57 and CD7. EAT2, Syk, PLZF, Helios and CD7 are marker molecules that are

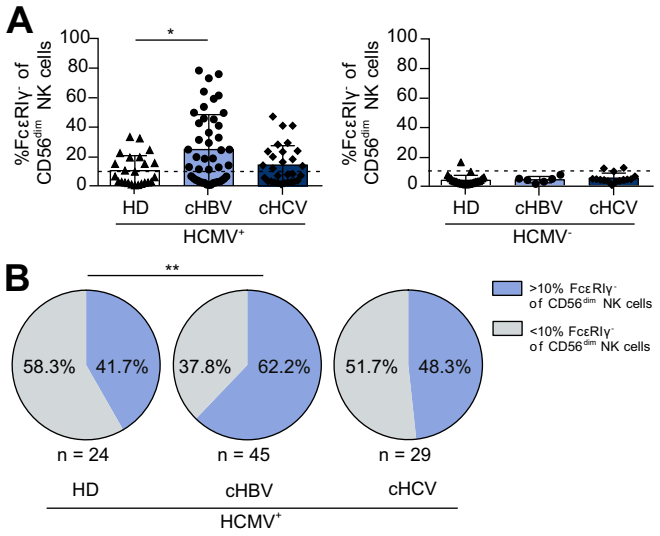

Fig. 1. Increased frequencies of FcεRIγ- CD56^{dim} NK cells in cHBV infection. (A) Frequencies of FcεRIγ- cells among CD56^{dim} NK cells in HD, patients with cHBV and those with cHCV according to HCMV serostatus. (B) Proportion of HCMV+ individuals with memory-like NK cells (FcεRIγ- cells among CD56^{dim} NK cells >10%; indicated in blue) and without memory-like NK cells (FcεRIγ- cells among CD56^{dim} NK cells <10%; depicted in grey). Each dot represents an individual. Bars indicate the median with IQR. The following statistical analyses were performed: Kruskal-Wallis test (A), parts of whole analysis (B). cHBV, chronic hepatitis B virus; cHCV, chronic hepatitis C virus; HCMV, human cytomegalovirus; HD, healthy donors; NK, natural killer.

downregulated whereas CD57 is upregulated in memory-like FcεRIγ- compared to conventional FcεRIγ+ CD56^{dim} NK cells.[12,13] As shown (Fig. 2A), FcεRIγ- CD56^{dim} NK cells obtained from patients with cHBV/HCMV+ expressed similar levels of EAT2 as FcεRIγ- CD56^{dim} NK cells obtained from HD/HCMV+. Furthermore, frequencies of FcεRIγ- CD56^{dim} NK cells expressing Syk, PLZF, Helios, CD7 and CD57 were similar in patients with cHBV/HCMV+ and HD/HCMV+ (Fig. 2B-F). Taken together, key characteristics of FcεRIγ- CD56^{dim} NK cells from cHBV/HCMV+ patients and HD/HCMV+ are similar, suggesting a conserved profile of this memory-like NK-cell subset in cHBV/HCMV+.

Frequencies of memory-like FcεRIγ- subsets determine NK-cell receptor expression on CD56^{dim} NK cells in cHBV infection

In cHBV infection, an altered NK-cell phenotype has been reported.[23] Therefore, in a next set of experiments, we addressed the question of whether these phenotypic alterations are affected by the frequency of the memory-like FcεRIγ- NK-cell subsets that exhibit a changed NK-cell receptor pattern compared to conventional NK cells.[12] For this, we analyzed NK-cell receptor expression on FcεRIγ-based CD56^{dim} NK-cell subsets from patients with cHBV/HCMV+ and HD/HCMV+. To take potential cHBV-associated FcεRIγ subset-specific effects into account, we first analyzed the NK-cell receptor expression on FcεRIγ- and FcεRIγ+ CD56^{dim} NK cells, respectively, and compared cells obtained from patients with cHBV/HCMV+ and HD/HCMV+. Expression of CD2, NKG2C, NKG2A, NKG2D, NKp30 and Siglec-7 were similar on FcεRIγ+ NK cells (Fig. 3A-C and Fig. S3A). NKp46 expression was only slightly increased (Fig. 3D) in conventional FcεRIγ+ CD56^{dim} NK cells from patients with cHBV/HCMV+ compared to HD/HCMV+. In addition, conserved expression of all tested NK-cell receptors was evident for memory-like FcεRIγ- CD56^{dim} NK cells from patients with cHBV/HCMV+ and from HD/HCMV+ (Fig. 3A-F). Thus, NK-cell receptor expression of CD2, NKG2C, NKG2A, NKG2D, NKp30, NKp46 and Siglec-7 was barely altered in cHBV infection with respect to FcεRIγ-based CD56^{dim} NK-cell subsets. However, with the exception of NKG2D, expression of these NK-cell receptors was different between memory-like FcεRIγ- and conventional FcεRIγ+ CD56^{dim} NK cells, irrespective of whether the cells were derived from cHBV/HCMV+ patients or from HD/HCMV+ (Fig. 3A-F and Fig. S3A/B). Consequently, expression of CD2, NKG2C, NKG2A, NKp30, NKp46 and Siglec-7 on bulk

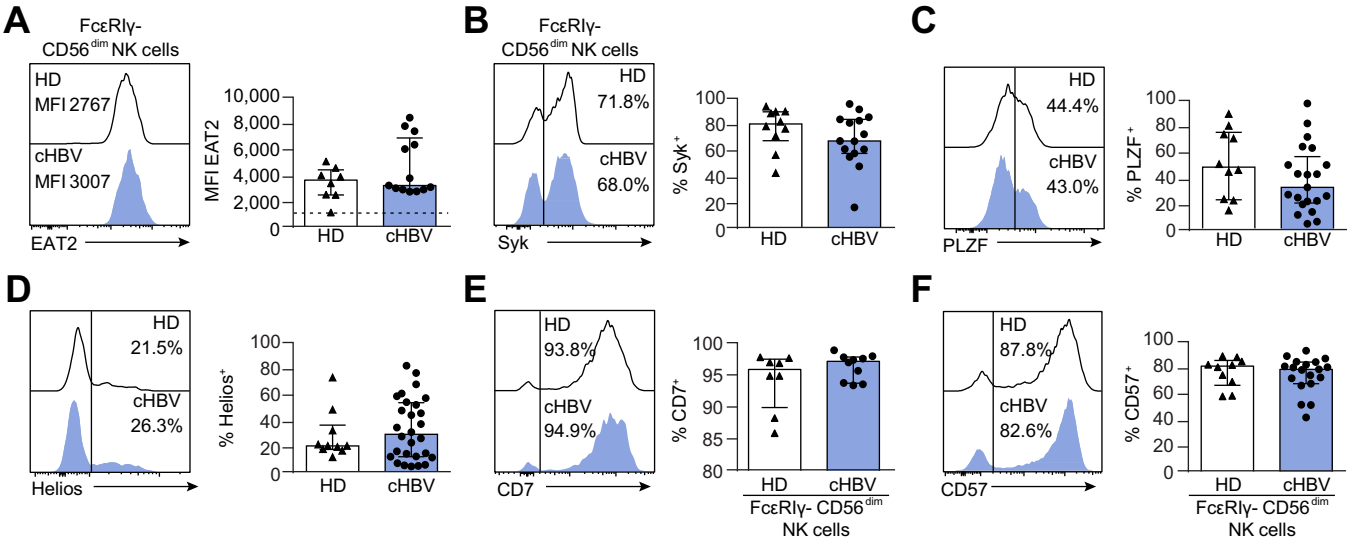

Fig. 2. Memory-like signature of FcεRIγ- CD56^{dim} NK cells in cHBV infection. (A) EAT2, (B) Syk, (C) PLZF, (D) Helios, (E) CD7 and (F) CD57 expression on FcεRIγ- CD56^{dim} NK cells of HD/HCMV+ and patients with cHBV/HCMV+. Bars indicate the median with IQR. Each dot represents an individual. Dotted line represents MFI of EAT2 expression on CD3+ cells that served as reference population. The following statistical analyses were performed: unpaired *t* test with Welch's correction (A-C, E, F) Mann-Whitney test (D). cHBV, chronic hepatitis B virus; cHCV, chronic hepatitis C virus; HCMV, human cytomegalovirus; HD, healthy donors; MFI, mean fluorescence intensity; NK, natural killer.

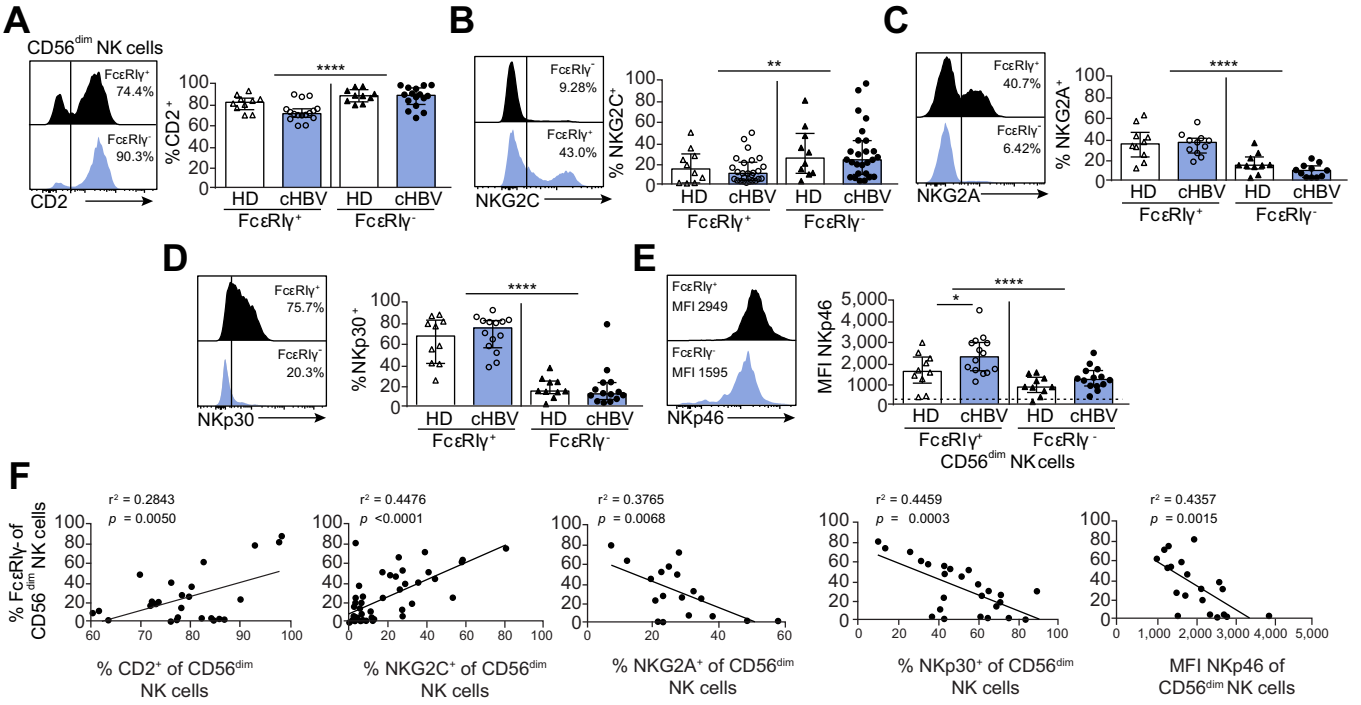

Fig. 3. CD56^{dim} NK-cell phenotype in cHBV infection is affected by memory-like FcεRIγ- subsets. Expression of (A) CD2, (B) NKG2C, (C) NKG2A, (D) NKp30 and (E) NKp46 of FcεRIγ+ and FcεRIγ- CD56^{dim} NK cells obtained from HD/HCMV+ and patients with cHBV/HCMV+ and (F) correlation analyses of marker expression and frequencies of FcεRIγ- cells among CD56^{dim} NK cells. Bars indicate the median with IQR. Each dot represents an individual. The following statistical analyses were performed: two-way ANOVA (A-E) and linear regression analyses (F). cHBV, chronic hepatitis B virus; cHCV, chronic hepatitis C virus; HCMV, human cytomegalovirus; HD, healthy donors; NK, natural killer.

CD56^{dim} NK cells from patients with cHBV correlated with the frequency of memory-like FcεRIγ- CD56^{dim} NK cells (Fig. 3F). In summary, HBV-associated alterations of NK-cell receptor expression on CD56^{dim} NK cells was linked to the HCMV-associated expansion of memory-like FcεRIγ- CD56^{dim} NK cells in cHBV infection.

Increased homeostatic proliferation of memory-like FcεRIγ- and conventional FcεRIγ+ CD56^{dim} NK cells in cHBV infection

Based on the observation that the profiles of FcεRIγ-based CD56^{dim} NK-cell subsets in cHBV infection are largely conserved, we wondered whether the homeostatic characteristics remain unaffected by the presence of chronic infection. As

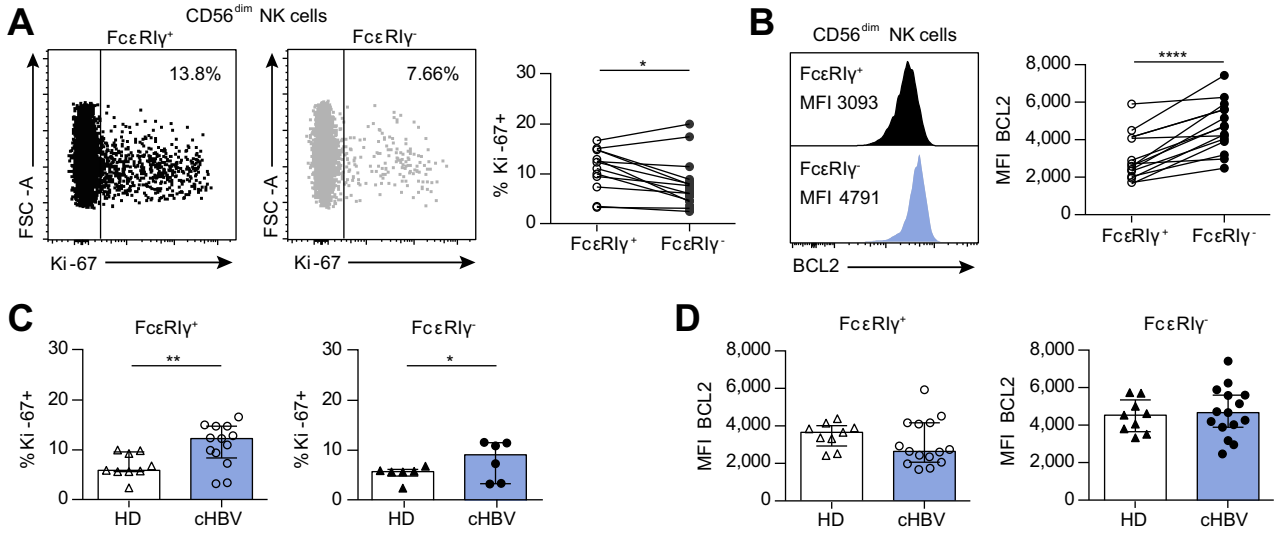

Fig. 4. Increased homeostatic proliferation of NK cells in cHBV infection. (A) Ki-67 and (B) BCL2 expression in FcεRIγ+ and FcεRIγ- CD56^{dim} NK cells of patients with cHBV/HCMV+. Comparison of (C) Ki-67 and (D) BCL2 expression in FcεRIγ+ and FcεRIγ- CD56^{dim} NK cells of patients with cHBV/HCMV+ and HD/HCMV+. Each dot represents an individual. Bars indicate the median with IQR. The following statistical analyses were performed: paired *t* test (A, B) and unpaired *t* test with Welch's correction (C, D). cHBV, chronic hepatitis B virus; cHCV, chronic hepatitis C virus; HCMV, human cytomegalovirus; HD, healthy donors; NK, natural killer.

measures of homeostatic proliferation and survival capacity, we analyzed Ki-67 and BCL2 expression, respectively, directly *ex vivo* in FcεRIγ- and FcεRIγ+ CD56^{dim} NK-cell subsets from patients with cHBV/HCMV+ and HD/HCMV+. In line with previous reports in HD/HCMV+,[15] Ki-67 expression (Fig. 4A) was significantly decreased and BCL2 expression (Fig. 4B) was significantly increased in memory-like FcεRIγ- compared to conventional FcεRIγ+ CD56^{dim} NK cells in patients with cHBV/HCMV+. Interestingly, expression of Ki-67 was significantly increased in CD56^{dim} NK cells obtained from patients with cHBV/HCMV+ compared to HD/HCMV+, irrespective of the FcεRIγ-based subset (Fig. 4C) whereas BCL2 levels were similar (Fig. 4D). In summary, memory-like FcεRIγ- CD56^{dim} NK cells seem to be more quiescent than FcεRIγ+ subsets despite a general increase in homeostatic proliferation in cHBV infection.

Increased CD16-mediated degranulation of memory-like FcεRIγ- and conventional FcεRIγ+ CD56^{dim} NK cells in cHBV infection

In the next set of experiments, we explored the functional properties of memory-like FcεRIγ- compared to conventional FcεRIγ+ CD56^{dim} NK cells in cHBV infection. First, we assessed the degranulation capacity via the surrogate marker CD107a and the IFNγ production after crosslinking of CD16 by plate-bound monoclonal antibodies (Fig. 5A). CD16 mediates antibody-dependent cellular cytotoxicity of NK cells. We observed a CD16-induced increased degranulation and IFNγ production of FcεRIγ- compared to FcεRIγ+ CD56^{dim} NK cells in cHBV/HCMV+ infection (Fig. 5A). Of note, CD16-mediated degranulation and IFNγ production of bulk CD56^{dim} NK cells from patients with cHBV correlated with the frequencies of

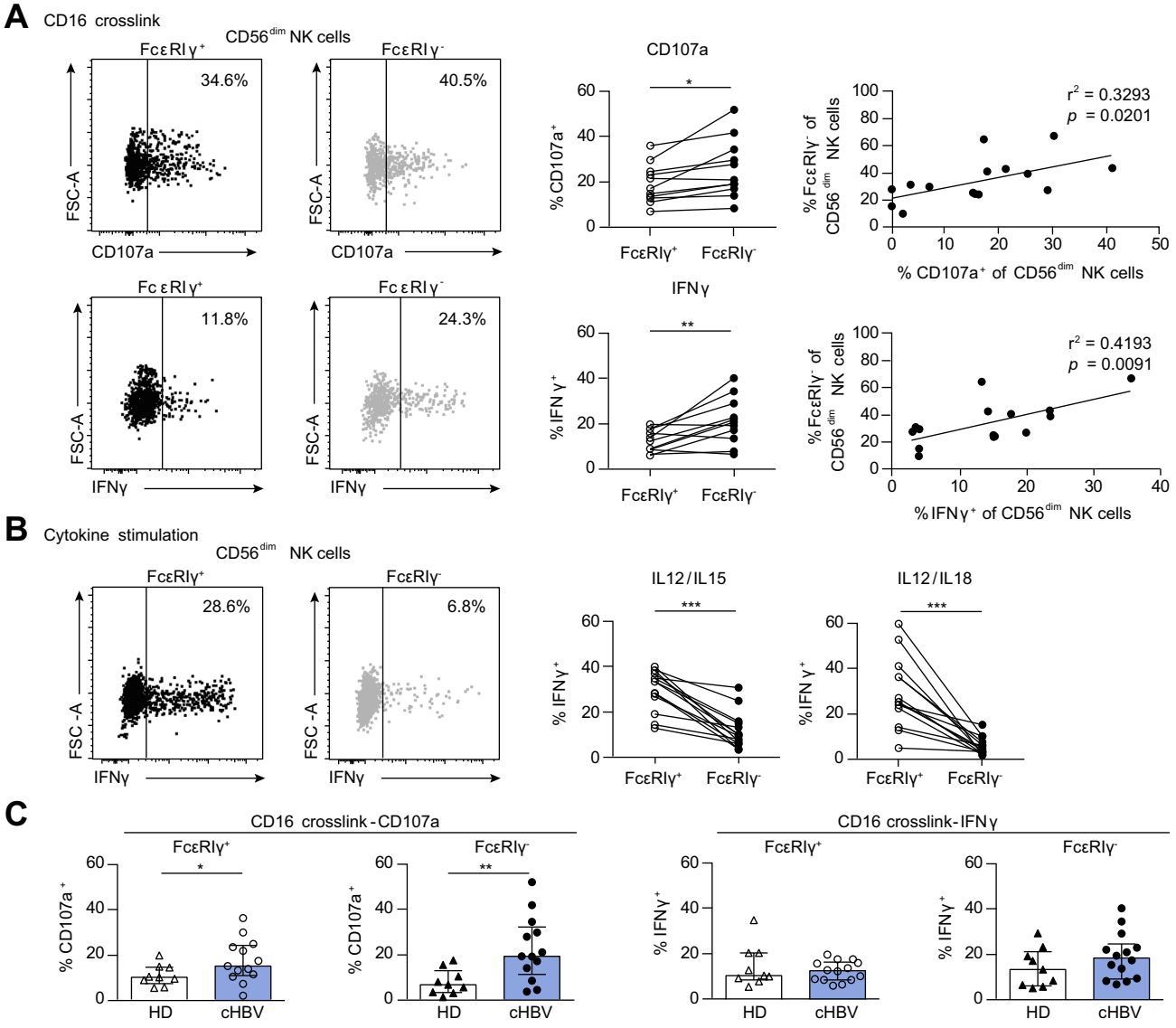

Fig. 5. Increased CD16-mediated NK-cell effector function in cHBV infection. (A) CD107a expression and IFNγ production of FcεRIγ+ and FcεRIγ- CD56^{dim} NK cells in patients with cHBV/HCMV+ after CD16 crosslinking for 5 h. (B) IFNγ production of FcεRIγ- and FcεRIγ+ CD56^{dim} NK cells in patients with cHBV/HCMV+ after stimulation with IL12/IL15 overnight. (C) Comparison of CD107a expression and IFNγ production after CD16 crosslinking for 5 h of FcεRIγ+ and FcεRIγ- CD56^{dim} NK cells in HD/HCMV+ and patients with cHBV/HCMV+. Each dot represents an individual. Bars indicate the median with IQR. The following statistical analyses were performed: paired *t* test (A), Wilcoxon test (B), unpaired *t* test with Welch's or Mann-Whitney (C). cHBV, chronic hepatitis B virus; cHCV, chronic hepatitis C virus; HCMV, human cytomegalovirus; HD, healthy donors; NK, natural killer.

memory-like FcεRIγ- CD56^{dim} NK cells (Fig. 5A). In response to cytokine stimulation with either IL12/IL15 or IL12/18 (Fig. 5B), however, IFNγ production was diminished in FcεRIγ- CD56^{dim} NK cells compared to FcεRIγ+ CD56^{dim} NK cells obtained from patients with cHBV/HCMV+. Next, we compared the functional properties of FcεRIγ-based CD56^{dim} NK-cell subsets in cHBV/HCMV+ infection and HD/HCMV+. IFNγ production was conserved in FcεRIγ- and FcεRIγ+ subsets from patients with cHBV/HCMV+ compared to HD/HCMV+ irrespective of stimulation via CD16 (Fig. 5C) or cytokines (Fig. S4). However, CD16-induced degranulation was significantly increased in FcεRIγ- and FcεRIγ+ CD56^{dim} NK cells from patients with cHBV/HCMV+ compared to HD/HCMV+. Hence, CD16-mediated CD56^{dim} NK-cell effector function is increased in cHBV infection.

Different metabolic properties of memory-like and conventional CD56^{dim} NK cells are conserved in cHBV infection

Since functional properties of NK cells are associated with alterations in metabolic pathways,[24] we next tested whether we can also detect metabolic changes comparing memory-like and conventional CD56^{dim} NK-cell subsets from patients with cHBV/HCMV+ and HD/HCMV+. First, we assessed glucose uptake that is increased in cells with active glycolysis. Since intracellular staining was not compatible with protocols assessing metabolic properties, we defined memory-like NK cells by the cell surface markers NKG2C, CD57 or CD2 instead of using FcεRIγ expression (Fig. S5A). Glucose uptake was reduced in memory-like NK cells compared to conventional NK cells in patients with cHBV/HCMV+ (Fig. 6A, Fig. S5B). A prerequisite for mitochondrial oxidative phosphorylation (OXPHOS) is an intact mitochondrial membrane and polarized mitochondria. We therefore analyzed fluctuations in mitochondrial membrane potential via Mito-ID (Fig. 6B, Fig. S5C). A higher fraction of memory-like compared to conventional NK cells obtained from patients with cHBV/HCMV+ displayed polarized and thus functional mitochondria, whereas mitochondrial mass was similar (Fig. S6A). As shown, the metabolic properties of memory-like and conventional CD56^{dim} NK-cell subsets were similar in patients with cHBV/HCMV+ and HD/HCMV+ (Fig. 6C,D; Fig. S5D,E; Fig. S6B). Thus, while memory-like and conventional CD56^{dim} NK cells differ in their metabolic characteristics, cHBV infection does not alter glucose uptake and OXPHOS under steady-state conditions.

Memory-like CD56^{dim} NK cells can be subdivided in FcεRIγ/Helios-based subsets with a stable epigenetic signature in cHBV infection

Memory cells of the adaptive immune system are comprised of different subsets with distinct phenotypic and functional characteristics. We therefore wondered whether memory-like CD56^{dim} NK cells are also heterogeneous. For this, we performed FcεRIγ/Helios co-expression analyses of CD56^{dim} NK cells obtained from patients with cHBV/HCMV+ since downregulation of both molecules has been linked to HCMV-associated memory-like subsets.[12,13] We observed the presence of 4 FcεRIγ/Helios-based CD56^{dim} NK-cell subsets: subset I, FcεRIγ+Helios+; subset II, FcεRIγ+Helios-; subset III, FcεRIγ-Helios-; subset IV, FcεRIγ-Helios+. The FcεRIγ+Helios+ conventional subset represented the highest proportion of CD56^{dim} NK cells,

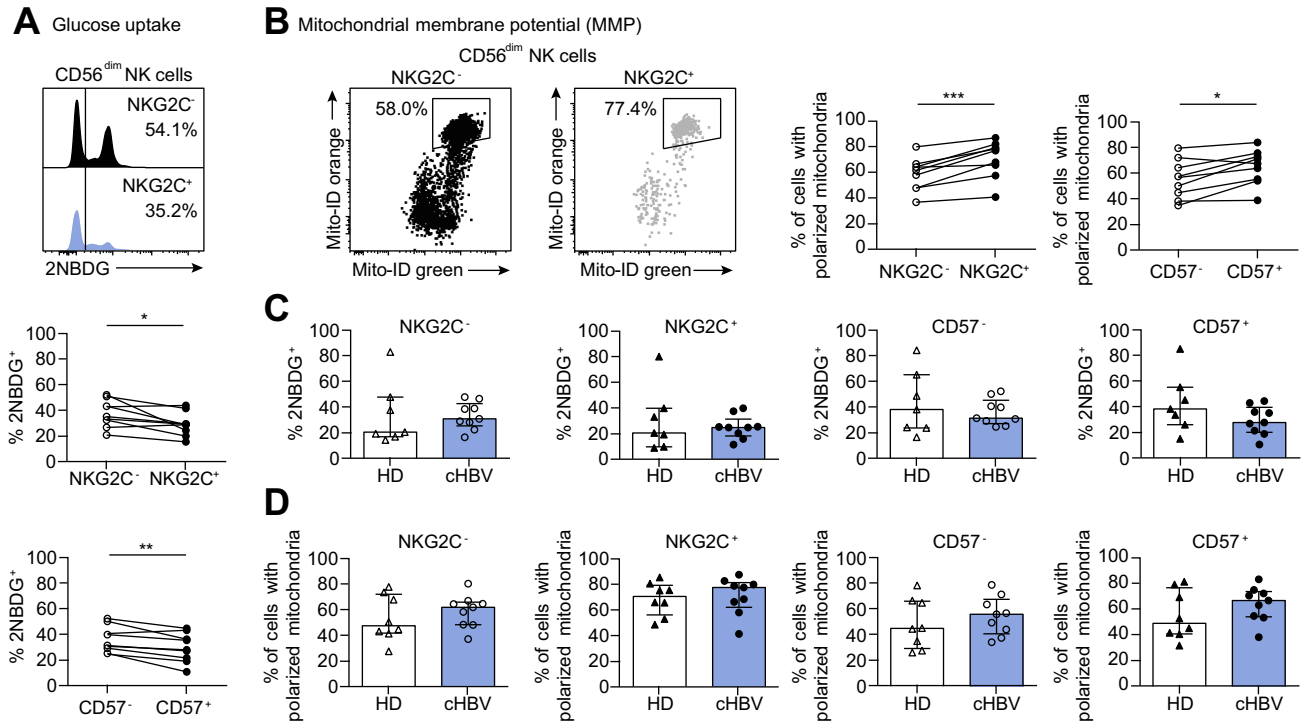

Fig. 6. Metabolic profile of memory-like NK cells reflects a resting state in cHBV infection. Uptake of (A) 2NBDG and (B) percentage of polarized mitochondria of NKG2C- and NKG2C+ (left panel) and CD57- and CD57+ (right panel) CD56^{dim} NK cells of patients with cHBV/HCMV+. Comparison of (C) 2NBDG uptake and (D) percentage of polarized mitochondria of NKG2C- and NKG2C+ (left panel) and CD57- and CD57+ (right panel) CD56^{dim} NK cells in HD/HCMV+ and patients with cHBV/HCMV+. Each dot represents an individual. Bars indicate the median with IQR. The following statistical analyses were performed: paired *t* test (A, B) Mann-Whitney (C), unpaired *t* test with Welch's correction (D). cHBV, chronic hepatitis B virus; cHCV, chronic hepatitis C virus; HCMV, human cytomegalovirus; HD, healthy donors; NK, natural killer.

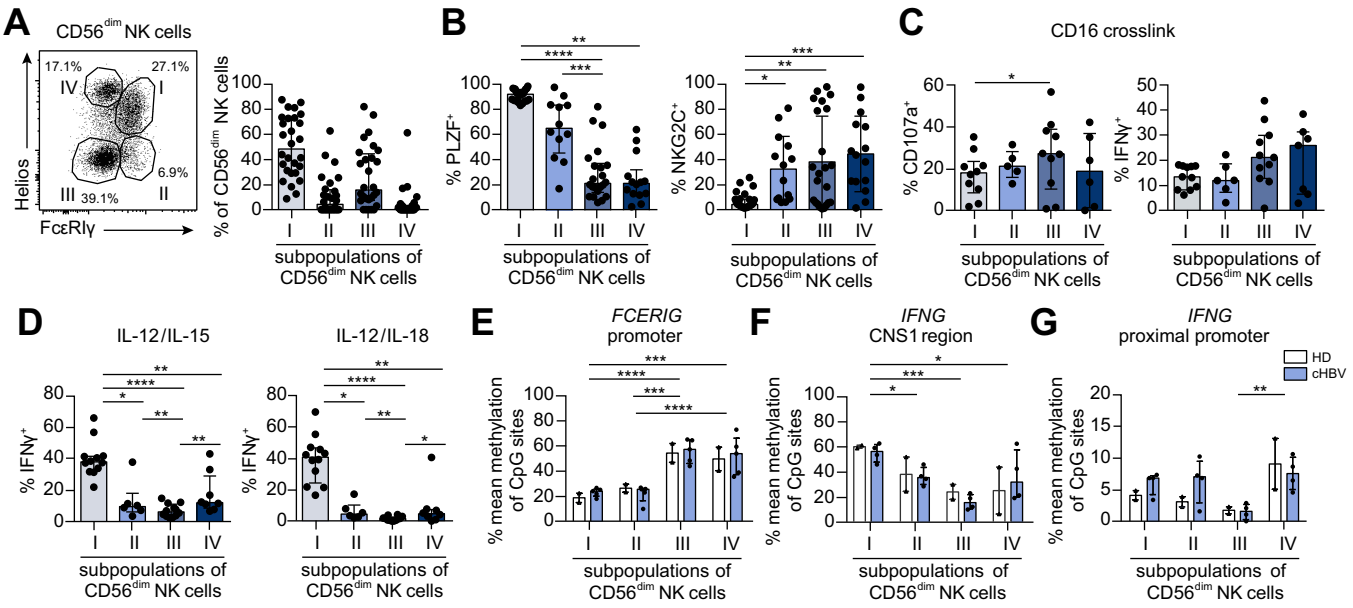

Fig. 7. Stable epigenetic signature of FcεRIγ/Helios-based CD56^{dim} NK-cell subsets. (A) FcεRIγ/Helios co-expression on CD56^{dim} NK cells of patients with cHBV/HCMV+. (B) *Ex vivo* PLZF and NKG2C expression, (C) CD107a expression and IFNγ production upon CD16 crosslinking for 5 h and (D) IFNγ secretion after IL12/15 or IL12/18 stimulation overnight of FcεRIγ/Helios-based CD56^{dim} NK-cell subsets obtained from patients with cHBV/HCMV+. (E) Mean methylation of CpG sites of *FCER1G* promoter, (F) *IFNG* CNS1 region and (G) *IFNG* proximal promoter in FcεRIγ/Helios-based CD56^{dim} NK-cell subsets of patients with cHBV/HCMV+ (n = 4) and HD/HCMV+ (n = 2). The following statistical analyses were performed: Friedman test (B), RM one-way ANOVA (C, D), two-way ANOVA (E-G). cHBV, chronic hepatitis B virus; cHCV, chronic hepatitis C virus; HCMV, human cytomegalovirus; HD, healthy donors; NK, natural killer.

followed by FcεRIγ-Helios- and FcεRIγ+Helios- subsets. FcεRIγ-Helios+ CD56^{dim} NK cells represented the least frequent subset in patients with cHBV. All FcεRIγ/Helios-based NK-cell subsets were also present in HD (Fig. S6A), suggesting a conserved subset diversification of memory-like CD56^{dim} NK cells in the context of cHBV infection. As shown, PLZF was significantly downregulated in FcεRIγ- subsets III and IV compared to the conventional FcεRIγ+Helios+ NK-cell subset I (Fig. 7B). In addition, NKG2C was upregulated in subsets II (FcεRIγ+Helios-), III (FcεRIγ-Helios-) and IV (FcεRIγ-Helios+) compared to conventional NK cells (Fig. 7B, right). Furthermore, CD16-induced degranulation was increased in FcεRIγ-Helios- subset III, compared to conventional FcεRIγ+Helios+ NK cells (Fig. 7C). IFNγ production of subsets II-IV compared to conventional FcεRIγ +Helios+ NK cells of subset I was substantially decreased in response to cytokine stimulation (Fig. 7D) pointing towards memory-like functional features generally biased towards CD16-mediated effector functions.

To further confirm the memory-like profile of FcεRIγ/Helios-based CD56^{dim} NK-cell subsets II-IV, we determined methylation patterns of the *FCER1G* and *IFNG* promoter, reported to be distinct in memory-like *vs.* conventional NK cells.[12,13,25] For this, we FACS-sorted FcεRIγ/Helios-based NK-cell subsets derived from 4 patients with cHBV/HCMV+ and 2 HD/HCMV+ and subsequently performed bisulfite sequencing of genomic DNA. We detected hypermethylation of the *FCER1G* promoter in CD56^{dim} NK-cell subsets lacking FcεRIγ expression (subset III and IV; Fig. 7E). Furthermore, CpG hypomethylation of the *IFNG* CNS1 region in FcεRIγ/Helios-based NK-cell subsets II, III and IV (Fig. 7F), and of the proximal *IFNG* promoter in subset III (Fig. 7G), not only associated epigenetic marks, reported for memory-like NK cells, to CD56^{dim} NK-cell subsets with decreased FcεRIγ expression, but also linked diminished Helios expression to epigenetic regulation associated with memory-like NK-cell differentiation in patients with cHBV/HCMV+ and HD/HCMV+ (Fig. 7F, G). In summary, co-expression of FcεRIγ and Helios diversifies phenotypically, functionally and epigenetically distinct memory-like CD56^{dim} NK-cell subsets with a stable signature in cHBV infection.

Discussion

In patients with cHBV, HCMV has a high prevalence.[20] HCMV establishes lifelong infection with alternating phases of latency and reactivation of productive infection. The lifelong persistence of HCMV leads to a dynamic interplay of the virus and the immune response resulting in imprinted changes of the immune system.[26,27] Moreover, the liver is a major site of HCMV replication.[28,29] HBV can establish a chronic and therefore actively persisting infection that is also associated with alterations in the immune response.[30,31] This study now revealed that the NK-cell repertoire of patients with cHBV/HCMV+ is mutually affected by HCMV and cHBV coinfection, indicating co-adaptation to both persisting infections.

We observed that HCMV-associated expansion of memory-like FcεRIγ- CD56^{dim} NK cells in cHBV was increased compared to HD. This increase was evident in both prevalence and frequency, probably resulting from facilitated induction and pronounced expansion of memory-like FcεRIγ- CD56^{dim} NK cells in cHBV/HCMV+ infection. The mechanisms responsible for this pronounced expansion of memory-like FcεRIγ- NK-cell subsets in cHBV/HCMV+ infection are unclear. For example, enforced repetitive HCMV reactivation in the context of cHBV infection may lead to boosted FcεRIγ- CD56^{dim} NK-cell expansion, especially since HCMV reactivation has been shown to induce memory-like NK-cell expansion in hematopoetic stem cell transplantations.[32,33] Yet, clinical HCMV reactivation was not diagnosed in the patients with cHBV/HCMV+, cHCV/HCMV+ or

HD/HCMV+ analyzed in this study. However, sub-clinical or tissue-specific HCMV reactivations cannot be excluded. In addition, it has been reported that FcεRIγ- CD56^{dim} NK cells obtained from HD/HCMV+ strongly proliferated in response to anti-HCMV IgG targeted HCMV-infected cells[12,13] and memory-like NK cells in patients infected with HIV correlated with HCMV serum antibody levels.[34,35] Hence, antibodies targeting HCMV- and/or HBV-derived epitopes may also play a role in the enhanced expansion of FcεRIγ- NK-cell subsets in patients with cHBV/HCMV+. Other reasons for enhanced memory-like FcεRIγ- CD56^{dim} NK-cell expansion may include genetic factors, *e.g.* combined KIR/MHC class I repertoire, inflammatory cytokines and selection of distinct HCMV strains.[36–40] Indeed, HCMV-associated NK-cell expansions, as well as chronic hepatitis, have previously been associated with self-reactive KIR expression.[21,38] Furthermore, the cytokine IL12 has been shown to represent a key player in memory-like NK-cell expansion in a cell culture model,[41] and elevated levels of IL12 were detectable in sera of patients with cHBV.[42]

An HCMV-associated expansion of NKG2C+ NK cells in chronic viral hepatitis, including cHBV and cHCV infection, has previously been reported.[21,22] In this study, we showed that a large fraction of FcεRIγ- CD56^{dim} NK cells express NKG2C and that frequencies of NKG2C+ and FcεRIγ- CD56^{dim} NK cells correlate with cHBV/HCMV+ infection. Thus, NKG2C+ and FcεRIγ- CD56^{dim} NK-cell expansions most likely represent overlapping observations. Yet, it has been recently shown that HCMV-associated expansion of NKG2C+ NK cells is not affected by concomitant cHBV infection.[22] As depicted by our phenotypic analyses, this most probably reflects the fact that NKG2C+ and FcεRIγ- CD56^{dim} NK cells do not completely represent the same populations. However, further analyses, *e.g.* by single cell RNA sequencing, are necessary to integrate heterogeneous phenotypes of expanded memory-like NK-cell populations and to reveal their relationship. In line with this, we demonstrated that HCMV-associated memory-like NK cells are indeed not homogeneous. FcεRIγ/Helios co-expression defines 3 phenotypically, functionally and epigenetically distinct memory-like CD56^{dim} NK-cell subsets contrasting with conventional FcεRIγ+ Helios+ CD56^{dim} NK cells. NKG2C, for example, was upregulated on all memory-like subsets, also on memory-like FcεRIγ+ Helios- CD56^{dim} NK cells revealing a memory-like CD56^{dim} NK-cell subset that co-expresses FcεRIγ and NKG2C. Of note, CD56^{dim} NK cells that lack FcεRIγ expression still constituted the majority of memory-like NK cells.

HCMV-associated memory-like FcεRIγ- CD56^{dim} NK cells exhibit a distinct molecular signature compared with conventional FcεRIγ+ CD56^{dim} NK cells sharing similarities with CD8+ T cells.[12,13] Here, we demonstrated that this phenotypic, functional and epigenetic profile, as well as the transcriptional regulation of memory-like FcεRIγ- NK-cell subsets is mostly conserved in patients with cHBV/HCMV+ despite ongoing chronic infection. This is in stark contrast to CD8+ T cells that exhibit different molecular signatures in chronic infections.[43] Furthermore, our data show that memory-like NK cells exhibit different metabolic properties to conventional NK cells, including reduced steady-state glucose uptake and increased capacity for OXPHOS characteristic for long-living/resting cells. This is in line with a recent study by Cichocki *et al.* showing that memory-like/adaptive NK cells exhibit increased OXPHOS linked to the upregulation of the transcriptional regulator ARID5B.[44] Importantly, in this study, we observed that the steady-state metabolic profile, specifically glucose uptake and mitochondrial membrane potential, of memory-like and conventional NK cells was similar in patients with cHBV/HCMV+ and HD/HCMV+, confirming the high stability of HCMV-associated CD56^{dim} NK-cell subsets in chronic infection. With respect to metabolism, memory-like NK cells also behave differently to CD8+ T cells that exhibit an altered metabolism in chronic infection.[45] Hence, despite molecular, especially epigenetic similarities of memory-like NK cells and CD8+ T cells, these cells are clearly different with respect to their adaptation in chronic infection.

Based on the largely conserved phenotype of both FcεRIγ- and FcεRIγ+ CD56^{dim} NK-cell subsets in patients with cHBV/HCMV+, the relative diversification of these 2 distinct subsets is closely linked to the overall profile of CD56^{dim} NK cells in cHBV infection. Hence due to the additionally high prevalence of HCMV in patients with cHBV, HCMV-associated FcεRIγ- NK cells consequently represent major determinants of CD56^{dim} NK-cell alterations observed in the context of cHBV infection. This is even consolidated by the enhanced expansion of FcεRIγ- CD56^{dim} NK cells in patients with cHBV/HCMV+ compared to HD/HCMV+. Of note, FcεRIγ- CD56^{dim} NK-cell expansion was similar in patients with cHCV/HCMV+ and HD/HCMV + and it has recently been reported that HCMV infection does not determine NK-cell alterations in cHCV infection,[46] contrasting with our aforementioned findings in cHBV infection. However, we also detected a few properties of CD56^{dim} NK cells that differ in patients with cHBV/HCMV+ compared to HD/HCMV+, irrespective of FcεRIγ expression. In particular, we observed a higher homeostatic proliferation and increased CD16-mediated degranulation of CD56^{dim} NK cells, demonstrating cHBV-specific effects on the overall NK-cell repertoire including HCMV-associated NK-cell populations. The underlying molecular mechanisms of these cHBV-specific NK-cell alterations remain to be clarified. Yet, it has been previously reported that IL10 and TGFB1 can contribute to changes in NK-cell functionality in patients with cHBV.[6]

In summary, this study shows that based on mutual effects of HCMV infection and HBV chronicity, the NK-cell repertoire in patients with cHBV/HCMV+ is biased towards CD16-mediated effector functions, thereby supporting the antibody-dependent immune response. Specifically, a general increase of CD16-mediated degranulation and enhanced expansion of FcεRIγ- CD56^{dim} NK cells with higher CD16-mediated effector capacity are evident. Hence, this study underpins the knowledge that co-infection, especially with HCMV, can shape the immune repertoire and consequently affects the immune response in cHBV infection. Therefore, coinfection must be considered in the design and application of new immunotherapeutic approaches for HBV cure that involve NK cells, *e.g.* TLR7 agonists[47] or antibody-mediated checkpoint blockade.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

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Supplementary data

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