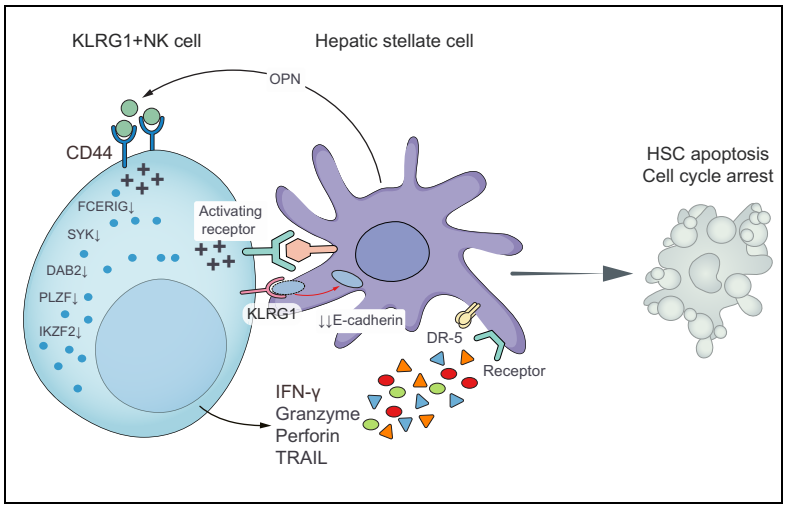
Research Article
Viral Hepatitis

JOURNAL
OF HEPATOLOGY

KLRG1+ natural killer cells exert a novel antifibrotic function in chronic hepatitis B

Graphical abstract



Highlights

- Patients with chronic HBV possess increased circulating and hepatic KLRG1+ NK cells.
- KLRG1+ NK cells possess a mature phenotype expressing elevated CD57 and DNAM-1, and reduced NKp46 and NKG2A.
- KLRG1+ NK cells are associated with reduced fibrosis stage in patients with chronic HBV.
- KLRG1+ NK cells induce hepatic stellate cell apoptosis in a TRAIL and CD44-osteopontin dependent manner.

Authors

Ratna S. Wijaya, Scott A. Read, Stephen Schibeci, ..., Jacob George, Mark W. Douglas, Golo Ahlenstiel

Correspondence

g.ahlenstiel@westernsydney.edu.au
(G. Ahlenstiel)

Lay summary

Individuals that are chronically infected with hepatitis B virus (HBV) possess an increased number of immune cells, called natural killer (NK) cells expressing the surface marker KLRG1 in the blood and liver. Here, we demonstrate that these specific NK cells are able to kill activated stellate cells in the liver. Because activated stellate cells contribute to liver scarring, *i.e.* fibrosis, and subsequent liver dysfunction in individuals with chronic HBV infection, KLRG1+ NK cells are a novel immune cell type that can limit liver scarring.

http://dx.doi.org/10.1016/j.jhep.2019.03.012
© 2019 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved. J. Hepatol. 2019, 71, 252–264

Research Article
Viral Hepatitis

JOURNAL OF HEPATOLOGY

KLRG1+ natural killer cells exert a novel antifibrotic function in chronic hepatitis B

Ratna S. Wijaya[1,2], Scott A. Read[1,3], Stephen Schibeci[1], Mohammed Eslam[1], Mahmoud K. Azardaryany[1], Korri El-Khobar[1], David van der Poorten[5], Rita Lin[5], Lawrence Yuen[5,6], Vincent Lam[5,6], Jacob George[1,5], Mark W. Douglas[1,5,7], Golo Ahlenstiel[1,3,4,*]

[1]*Storr Liver Centre, The Westmead Institute for Medical Research, The University of Sydney, Westmead, NSW 2145, Australia;* [2]*Faculty of Medicine, Pelita Harapan University, Tangerang, Indonesia;* [3]*Blacktown Clinical School, Western Sydney University, Blacktown, NSW 2148, Australia;* [4]*Blacktown Hospital, Blacktown, NSW 2148, Australia;* [5]*Westmead Hospital, University of Sydney, NSW, Australia;* [6]*Discipline of Surgery, University of Sydney, Australia;* [7]*Centre for Infectious Diseases and Microbiology, Marie Bashir Institute for Infectious Diseases and Biosecurity, University of Sydney at Westmead Hospital, Westmead NSW 2145, Australia*

Background & Aims: Natural killer (NK) cells are known to exert strong antiviral activity. Killer cell lectin-like receptor subfamily G member 1 (KLRG1) is expressed by terminally differentiated NK cells and KLRG1-expressing lymphocytes are known to expand following chronic viral infections. We aimed to elucidate the previously unknown role of KLRG1 in the pathogenesis of chronic hepatitis B (CHB).

Methods: KLRG1+ NK cells were taken from the blood and liver of healthy individuals and patients with CHB. The phenotype and function of these cells was assessed using flow cytometry and *in vitro* stimulation.

Results: Patients with CHB had a higher frequency of KLRG1+ NK cells compared to healthy controls (blood 13.4 *vs.* 2.3%, $p < 0.0001$ and liver 23.4 *vs.* 2.6%, $p < 0.01$). KLRG1+ NK cells were less responsive to K562 and cytokine stimulation, but demonstrated enhanced cytotoxicity (9.0 *vs.* 4.8%, $p < 0.05$) and IFN-γ release (8.0 *vs.* 1.5%, $p < 0.05$) via antibody dependent cellular cytotoxicity compared to their KLRG1− counterparts. KLRG1+ NK cells possessed a mature phenotype, demonstrating stronger cytolytic activity and IFN-γ secretion against hepatic stellate cells (HSCs) than KLRG1− NK cells. Moreover, KLRG1+ NK cells more effectively induced primary HSC apoptosis in a TRAIL-dependent manner. Increased KLRG1+ NK cell frequency in the liver and blood was associated with lower fibrosis stage (F0/F1) in patients with CHB. Finally, the expression of CD44, degranulation and IFN-γ production were all increased in KLRG1+ NK cells following stimulation with osteopontin, the CD44 ligand, suggesting that HSC-derived osteopontin may cause KLRG1+ NK cell activation.

Conclusions: KLRG1+ NK cells likely play an antifibrotic role during the natural course of CHB infection. Harnessing this antifibrotic function may provide a novel therapeutic approach to treat liver fibrosis in patients with CHB.

Lay summary: Individuals that are chronically infected with hepatitis B virus (HBV) possess an increased number of immune cells, called natural killer (NK) cells expressing the surface marker KLRG1 in the blood and liver. Here, we demonstrate that these specific NK cells are able to kill activated stellate cells in the liver. Because activated stellate cells contribute to liver scarring, *i.e.* fibrosis, and subsequent liver dysfunction in individuals with chronic HBV infection, KLRG1+ NK cells are a novel immune cell type that can limit liver scarring.

© 2019 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

Introduction

Natural killer (NK) cells are a primary effector population responsible for the innate immune response to viral infection, representing approximately 5–15% of blood and 40–60% of hepatic lymphocytes.[1,2] While NK cells play a role in controlling hepatitis B virus (HBV) infection,[3–9] they act as a double-edged sword, contributing to liver injury through sustained activation and tissue damage.[10–13]

In humans, NK cells are historically divided into 2 subsets according to the expression of CD56: $CD56^{dim}$ NK cells represent approximately 90% of circulating NK cells and demonstrate strong cytolytic capacity, whereas $CD56^{bright}$ NK cells constitute the remaining 10% of NK cells and are strong producers of cytokines.[14,15] With shorter telomeres and later appearance following haematopoietic stem cell transplantation,[16,17] $CD56^{dim}$ cells are considered further differentiated than $CD56^{bright}$ NK cells. They can nonetheless continue to differentiate, losing NKG2A and acquiring CD16, killer immunoglobulin-like receptors (KIRs), CD57, and killer cell lectin-like receptor G1 (KLRG1) surface markers that appear sequentially during NK cell maturation.[18–22]

KLRG1 is an inhibitory receptor of the C-type lectin-like family.[23] Approximately 20% of human CD4+ T cells and 40% of CD8+ T cells express KLRG1, most of which are effector or memory T cells.[24] KLRG1 expressing cells possess poor proliferative capacity indicative of senescence, but still maintain their ability to exert effector functions.[24–26] During persistent viral infections such as human cytomegalovirus (HCMV),[27,28] human

Keywords: Natural killer cells; Hepatitis B virus; HBV; Liver fibrosis.
Received 10 August 2018; received in revised form 6 March 2019; accepted 7 March 2019; available online 21 March 2019

* Corresponding author. Address: Blacktown Clinical School, Western Sydney University, 18 Blacktown Road, Blacktown, NSW 2148, Australia. Tel.: +61 2 9851 6073; fax: +61 2 9851 6050.
E-mail address: g.ahlenstiel@westernsydney.edu.au (G. Ahlenstiel).

immunodeficiency virus (HIV),[29] and Epstein-Barr virus infection,[26,30,31] the proportion of KLRG1+ T cells increases in peripheral blood. The interaction of KLRG1 with its ligand, E-cadherin, results in inhibition of cytotoxic activity, suggesting that KLRG1 may raise the activation threshold of T cells.[32–35] As a result, KLRG1 may limit the antiviral activity of T cells, thus contributing to viral persistence.[36] Unlike T cells, the role of KLRG1 on NK cells remains uncertain, particularly in chronic HBV infection.

In this study, we examined the quantity, phenotype and functional characteristics of KLRG1+ NK cells in healthy controls and patients with chronic HBV (CHB). Patients with CHB had a significantly higher frequency of mature KLRG1+ NK cells than healthy controls. These mature KLRG1+ NK cells rely on antibody dependent cellular cytotoxicity (ADCC) as a distinct effector mechanism. Moreover, peripheral blood KLRG1+ NK cells are negatively correlated with serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels and enriched in patients with CHB and no or mild fibrosis, suggesting a role of KLRG1+ NK cells in limiting liver injury and fibrosis. Consequently, KLRG1+ NK cells may play an important part in controlling the progression of liver disease during CHB infection.

Materials and methods

Patients and healthy donors

Blood samples were collected from 33 patients with CHB and from 10 age-matched healthy controls. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE Healthcare Life Sciences, UK) and cryopreserved for concurrent analysis. Ethics approval was obtained from the Western Sydney Local Health District and University of Sydney. All individuals signed informed consent to participate in the study (HREC2002/12/4.9(1564)). Patient characteristics are summarized in Table 1.

Immune cell purification

NK cells from healthy controls were magnetically isolated from PBMCs using the NK cell negative isolation kit according to the manufacturer's instructions (Miltenyi Biotec, Bergisch-Gladbach, Germany). NK cell purity was >95% for all experiments, assessed by flow cytometry.

Intrahepatic lymphocyte cell isolation was done by enzymatic digestion, based on described protocols.[37,38] Briefly, liver was minced into 1 mm^3 pieces, digested using RPMI 1640 medium containing 10% foetal calf serum (FCS), 0.1 mg/ml type IV collagenase, and 1 μg/ml DNase I, then incubated at 37 °C for 30 min with gentle agitation. Cells were passed through a 70 μm cell strainer and the cell suspension was assessed by flow cytometry. All flow/cell sorting panels are available in the supplementary materials and supplementary CTAT table.

Quantification of IFN-γ production

Cells were cultured at a density of $3x10^6$ cells/ml in complete medium (RPMI 1640 with L-glutamine medium containing 10% FCS, 10 mM/L HEPES pH 7.4, 100 IU/ml penicillin, 100 μg/ml streptomycin) at 37 °C and 5% CO_2 overnight. PBMCs were next stimulated with either (1) interleukin (IL)-12 (0.5 ng/ml; Sino Biological Inc., Beijing, China) and IL-15 (20 ng/ml; Sino Biological Inc.), (2) IL-12 (0.5 ng/ml) and IL-18 (100 ng/ml; Sino Biological Inc.), or (3) complete medium for 14 h. GolgiPlug (BD Biosciences) was added, and cells were incubated for 4 h. Cells were next labelled with surface antibodies (Supplementary materials and methods), then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and labelled with an IFN-γ antibody.

Degranulation and IFN-γ production upon target cell stimulation

CD107a expression was used to assess NK cell cytotoxicity following target cell co-culture. Cells patients with CHB (5×10^5 cells/ml) were incubated with K562 target cells at a ratio of 1:1 with an anti-CD107a antibody to monitor degranulation. To assess ADCC against Raji cells, 1 μg/ml of anti-CD20 (rituximab; BioVision, Inc.) was added during co-culture. After 1 h, GolgiStop and GolgiPlug were added, incubation was continued for an additional 5 h, and cells were labelled with NK phenotyping antibodies.

HBV antigen NK cell culture

Purified NK cells or PBMCs were cultured in RPMI 1640 medium containing 10% human serum from patients with CHB (HBV surface antigen [HBsAg] (+) and ALT level <60 IU/ml), patients with non-alcoholic fatty liver disease (NAFLD) (HBsAg (−) and ALT level <60 IU/ml), FCS, and 1 μg/ml recombinant HBV antigen (HBV core antigen [HBcAg, ab119471], HBV e antigen [HBeAg, ab91273], and HBsAg [ab73749], purchased from Abcam [Cambridge, MA, USA]) for 7 days. IL-15 (1 ng/ml) was added to support NK cell survival, with 50% of the medium being replenished every 3 days.

Table 1. Characteristics of healthy controls and patients with chronic hepatitis B, chronic hepatitis C, and NAFLD.

Clinical data	Healthy controls	Chronic hepatitis B	Chronic hepatitis C	NAFLD
Case	10	33	10	10
Age (years)	44.2 ± 9.6	46.2 ± 11.9	47.3 ± 11	46.4 ± 8.3
Gender (male/female)	5/5	21/12	5/5	5/5
HBeAg (+/−)	0/10	5/28	0/10	0/10
HBsAg (+/−)	0/10	33/0	0/10	0/10
ALT (U/L)	n.a.	40 ± 21.6	47 ± 23.9	59.5 ± 33.5
AST (U/L)	n.a.	25.7 ± 11.2	36.9 ± 38.7	27.8 ± 12.8
HBV DNA level (log_{10} IU/ml)	n.a.	3.2 ± 1.9	n.a.	n.a.
Fibrosis stage				
0–1	n.a.	27	7	5
2–3	n.a.	6	3	5

Data are showed as mean ± SD (standard deviation).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B early antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; n.a., not applicable; NAFLD, non-alcoholic fatty liver disease.

NK cell co-culture with hepatic stellate cells

The LX-2 immortalized human hepatic stellate cell (HSC) line was cultured at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle's Medium containing 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Progressive activation of LX-2 cells in culture was confirmed by the increase in mRNA expression of the *ACTA2* gene that encodes for α-smooth muscle actin (α-SMA) protein, quantified by quantitative real-time PCR (qPCR) during different time points (Fig. S1). NK cells (±IFN-α pre-activation) were co-cultured with LX-2 cells at the effector target ratio (E:T) of 10:1 for 6 h and IFN-γ and degranulation were measured as above.

Apoptosis assay

To differentiate NK cells from HSCs for flow analysis, LX-2 and primary human HSCs (hHSCs) (SciencCell, USA) were stained with 5 μM carboxyfluorescein succinimidyl ester (CFSE, Cayman Chemicals) for 10 min at room temperature and washed, before 5×10^4 cells/well were seeded in a 24-well plate. IFN-α stimulated CD3-CD56^{dim}CD16^{bright}KLRG1−and CD3-CD56^{dim}CD16^{bright}KLRG1+ NK cells were co-incubated with HSCs for 6 h at an E:T ratio of 1:1 ± the neutralizing tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) antibody (clone RIK-2; 10 μg/ml; Biolegend). LX-2 and primary hHSCs were harvested, and apoptosis studied by staining with propidium iodide and annexin V conjugated to allophycocyanin (BD Biosciences) according to the manufacturer's instruction. Target cells alone were used as controls.

RNA extraction and quantitative real time qPCR

NK cell RNA was extracted using the Favorgen total RNA mini kit and complementary DNA (cDNA) was synthesized according to the manufacturer's protocol (M-MLV reverse transcriptase, Promega). PCR probes and primers are available in supplementary materials and supplementary CTAT table.

Statistical analysis

Data were analysed with GraphPad Prism Version 7. The Mann-Whitney *U* test was used to compare variables between 2 independent groups, the Wilcoxon matched-pairs test was used for individual comparisons of paired groups, and Spearman rank correlation analysis was performed to examine correlations. In all analyses, a 2-tailed *p* value of less than 0.05 was considered statistically significant.

Results

KLRG1+ NK cells frequency is elevated in CHB patients

To examine the effect of CHB infection on circulating NK cells, blood NK cells were phenotyped based on KLRG1 expression by flow cytometry. NK cell frequencies were not significantly different between healthy controls (n = 10) and patients with CHB (n = 33) (Fig. 1A), however, a larger population of KLRG1+ NK cells were present in patients with CHB (13.4 *vs.* 2.3%, $p < 0.0001$, Fig. 1B). The phenotype of KLRG1+ NK cells was similar in healthy controls and patients with CHB (Fig. S2). Compared to CD56^{bright} NK cells, KLRG1 was highly expressed on CD56^{dim} NK cells (13.2 *vs.* 0.1%, $p < 0.0001$, Fig. 1B), supporting its role as a maturation marker. To confirm the larger percentage of KLRG1+ NK cells in patients with CHB compared to healthy controls, we utilized an unbiased analysis of non-linear dimensionality reduction approach via t-stochastic neighbour embedding (t-SNE). As presented in the 2D t-SNE plot in Fig. 1C, there was a markedly different distribution of NK cells within the CHB CD56^{dim} area when compared to healthy control samples. We further found that the KLRG1 marker was highly expressed in this area within CHB samples (Fig. 1C), confirming our findings using manual gating. Furthermore, a larger population of CD16+ cells was present in the KLRG1+ population (80.8 *vs.* 76%, $p < 0.01$, Fig. 1D) that additionally expressed higher levels of CD16 as represented by geometric mean fluorescence intensity (gMFI) (4,812.1 *vs.* 4,337.3, $p < 0.001$, Fig. 1D). A significant upregulation of the *FCGR3A* gene encoding CD16 (2.14 *vs.* 1.4-fold change; $p < 0.01$, Fig. 1D) on KLRG1+ was confirmed by qPCR.

We next investigated differences in maturation stage by studying CD57 and KIR, both of which reflect the mature phenotype of CD56^{dim}CD16^{bright} NK cells (Fig. 2A).[22] A greater frequency of CD57 positive cells (79.2 *vs.* 74.6%, $p < 0.05$, Fig. 2B) and gMFI (9,851 *vs.* 5,425.5, $p < 0.01$, Fig. 2B) were measured on KLRG1+ than KLRG1− NK cells, with minimal differences in KIR expression between subsets (Fig. 2B). We also investigated the expression of NK cell activating and inhibitory receptors (NKp46, DNAM-1, and NKG2A) on KLRG1+ and KLRG1− NK cells. Consistent with previous reports,[18,22] we found NKp46 and NKG2A were expressed at a lower frequency and density (gMFI) on the surface of KLRG1+ compared with KLRG1− NK cells (NKp46; 38.8% *vs.* 50.3%, $p < 0.0001$ and gMFI 337.6 *vs.* 355, $p < 0.05$, Fig. 2B; NKG2A; 2.3% *vs.* 7.2%, $p < 0.0001$ and gMFI 558.9 *vs.* 561.7, $p < 0.01$, Fig. 2B). The expression level of DNAM-1 (CD226) was significantly higher in frequency (77.6% *vs.* 74.2%, $p < 0.05$, Fig. 2B) and gMFI (1,454.5 *vs.* 1,240.4, $p < 0.01$, Fig. 2B) on KLRG1+ compared to KLRG1− NK cells. In contrast to chronic HCMV infection,[28,39–42] there was no difference in the frequency (18.2 *vs.* 21%, $p > 0.05$, Fig. 2B) and gMFI (916.1 *vs.* 980.9, $p > 0.05$, Fig. 2B) of NKG2C expression on NK cell populations. Representative flow analysis of KLRG1+ and KLRG1− NK cell surface markers are shown in Fig. S3. NK cell receptor expression was confirmed by qPCR, demonstrating a 1.8-fold increase in CD57 (*B3GAT1*) ($p < 0.05$), 3.6-fold reduction in NKp46 (*NCR1*) ($p < 0.05$), 2.1-fold reduction in NKG2A (*KLRC1*) ($p < 0.01$), and 2-fold increase in DNAM-1 (*CD226*) ($p < 0.01$, Fig. 2C) mRNA expression in KLRG1+ versus KLRG1− NK cells.

KLRG1+ NK cells possess superior antibody dependent cellular cytotoxicity

To assess NK cell function in relation to KLRG1 expression, IFN-γ production and degranulation were examined following cytokine treatment and target cell co-culture. While no differences in IFN-γ expression were observed following IL-12/15 stimulation, IL-12/18 stimulation resulted in fewer IFN-γ producing KLRG1+ NK cells compared to KLRG1− NK cells (9.5 *vs.* 14.9%, $p < 0.001$, Fig. 3A). Consistent with flow cytometry results, KLRG1+ NK cell *IFNG* expression was reduced following IL-12/18 treatment compared to KLRG1− NK cells (2.0 *vs.* 4.5-fold change; $p < 0.05$, Fig. 3A).

KLRG1+ NK cell activation in response to the K562 target cell co-culture was also diminished compared to KLRG1− cells. IFN-γ production was reduced in KLRG1+ NK cells (0.9 *vs.* 2.8%, $p < 0.05$, Fig. 3B), as was degranulation, measured using CD107a (12.2 *vs.* 14.4, $p < 0.01$, Fig. 3B). qPCR results confirmed these findings, demonstrating reduced KLRG1+ NK cell *IFNG* (0.7 *vs.* 1.6-fold change, $p < 0.05$, Fig. 3B) and *GZMB* (1.1 *vs.* 2.0-fold change, $p < 0.05$, Fig. 3B) expression after K562

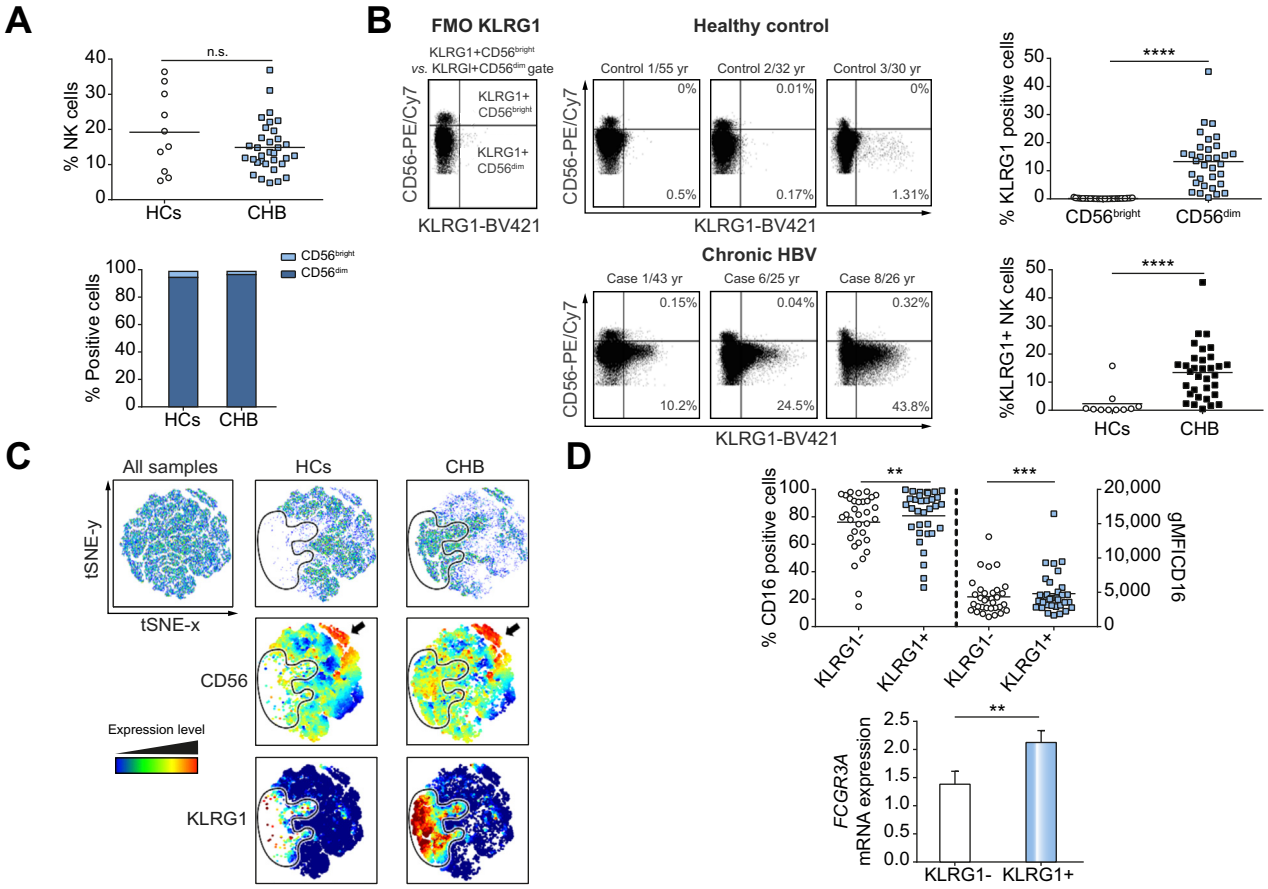


Fig. 1. Increased KLRG1 expression on CD56^{dim} NK cells in patients with CHB. (A) PBMCs obtained from 10 HCs and 33 patients with CHB were investigated for the total frequency, CD56^{bright}, and CD56^{dim} NK cells. (B) NK cells were gated based on CD3-CD56+ and assessed for the expression KLRG1. Distribution of KLRG1 expression between CD56^{bright} and CD56^{dim} subset in patients with CHB (left graph). Frequency of total KLRG1+ NK cell (KLRG1+ CD56^{bright} and CD56^{dim}) in HCs and patients with CHB (right graph). (C). Dimensionality reduction using t-SNE. Flow cytometry data from HCs and CHB (gated on single cells, live, CD19-, and CD3-CD56+) were concatenated, transformed and plotted in 2D t-SNE plots using R software. Encircled areas indicate the NK cell subset enriched in patients with CHB compared to HCs. Indicated markers are colour mapped from blue (low expression) to red (high expression). Arrows indicate the CD56^{bright} NK cells (D) CD16 expression on the **CD56^{dim}KLRG1− and CD56^{dim}KLRG1+ NK cell population. Mann-Whitney *U* tests were performed for comparisons of 2 independent groups and Wilcoxon's tests were performed for comparisons of paired groups. $^{**}p$ <0.01, $^{***}p$ <0.001, $^{****}p$ <0.0001. CHB, chronic hepatitis B virus infection; HCs, healthy controls; NK, natural killer; PBMCs, peripheral blood mononuclear cells; t-SNE, t-stochastic neighbour embedding.

challenge. As in patients with CHB, KLRG1+ NK cells from healthy controls showed reduced effector activities following stimulation with cytokines and target cells (Fig. S4). These data suggest that KLRG1+ NK cells have reduced functionality towards standard stimuli, akin to reduced cytokine sensitivity that has been documented in highly differentiated NK cells.[22,43]

KLRG1+ NK cell activation can additionally occur through CD16 recognition of antibody bound antigen, called the ADCC pathway.[44] To evaluate the capacity for ADCC by KLRG1+ NK cells, PBMCs from patients with CHB were co-cultured with CD20-expressing Raji cells ± the anti-CD20 monoclonal antibody rituximab. In contrast to our previous findings, KLRG1+ NK cells showed increased IFN-γ production (8.0 *vs.* 1.5%, p <0.05, Fig. 3C) and degranulation (9.0 *vs.* 4.8%, p <0.05, Fig. 3C) following stimulation with anti-CD20+ Raji cells compared to KLRG1− NK cells. KLRG1+ NK cells additionally demonstrated a 1.8-fold increase in *IFNG* (p <0.05) and a 2.9-fold increase in *GZMB* expression (p <0.05, Fig. 3C) compared to KLRG1− NK cells.

KLRG1 is upregulated on FcεRIγ-negative NK cells

Considering that effective ADCC is a hallmark of FcεRIγ-negative NK cells (g-NK cells or gamma-negative NK cells), we examined KLRG1 expression in this context. We gated on CD56^{dim} NK cells to identify FcεRIγ-negative and FcεRIγ-positive NK cells[45–48] and analysed KLRG1 expression in these subsets (Fig. 4A). Our results showed the expression of KLRG1 was significantly increased on FcεRIγ-negative NK cells compared to FcεRIγ-positive NK cells (16.6 *vs.* 11.9%, p <0.01, Fig. 4A). To verify whether KLRG1+ NK cells belong to the group FcεRIγ-negative NK cells in general, we performed qPCR on sorted KLRG1+ and KLRG1− NK cells for genes that are known to be related to FcεRIγ-negative NK cells. Indeed, KLRG1+ NK cells had significantly lower expression of *FCER1G* mRNA encoding for FcεRIγ (1.6 *vs.* 3.7-fold change, p <0.01, Fig. 4B). In addition, the KLRG1+ NK cells showed decreased expression of genes typically decreased in FcεRIγ-negative NK cells,[48] for example signalling molecules *SYK* (0.4 *vs.* 1.34, p <0.01, Fig. 4B), *DAB2* (1 *vs.* 2.1, p <0.05, Fig. 4B) and transcriptional regulators *PLZF* (0.8 *vs.* 1.6, p <0.05, Fig. 4B) and *IKZF2* (1.5 *vs.* 1.9, p <0.05, Fig. 4B).

Given the strong association between HCMV infection and the presence of FcεRIγ-negative NK cells,[46] we further investigated whether the KLRG1+ NK cells persist in patients with CHB based on HCMV exposure. We tested IgG HCMV antibody titres and found no significant difference in KLRG1 expression

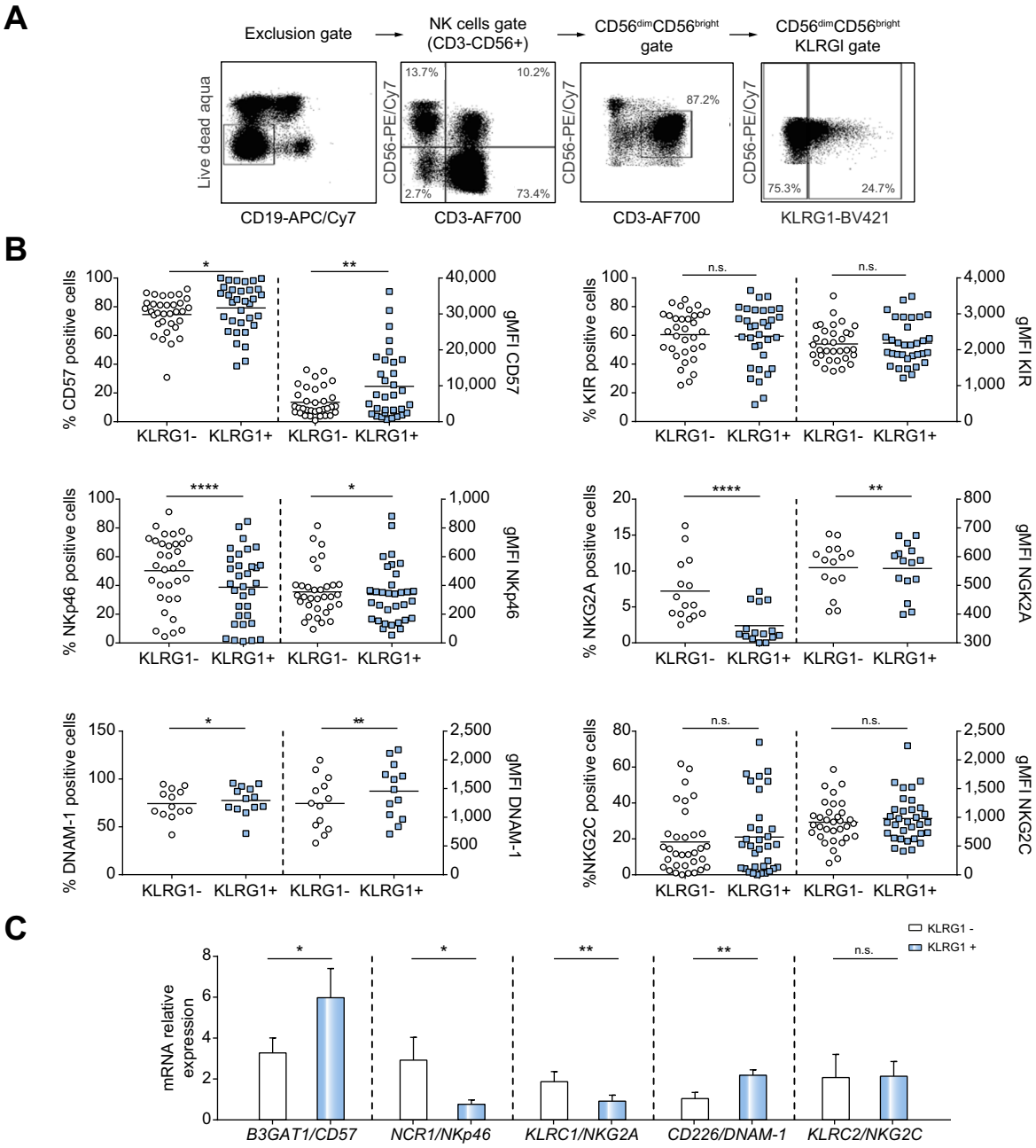


Fig. 2. CD56^{dim}CD16^{bright}KLRG1+ NK cells are phenotypically more mature than CD56^{dim}CD16^{bright}KLRG1− NK cells. Flow cytometry was performed on total PBMCs from patients with CHB. NK cells (CD3-CD56+) were assessed for expression of CD16 and CD56. CD56^{dim}CD16^{bright} NK cells were gated based on KLRG1 expression in order to assess the expression of NK cell surface markers. (A) The flow gating strategy on single live cells was shown for 1 representative donor. (B) The proportion and gMFI expression of CD57, KIR, NKp46, NKG2A, DNAM-1, and NKG2C between KLRG1− and KLRG1+ CD56^{dim}CD16^{bright} NK cells (C) The mRNA level of surface receptor genes; *B3GAT1, NCR1, KLRC1, CD226,* and *KLRC2* in KLRG1− and KLRG1+ NK cells. Wilcoxon's tests were performed for individual comparisons of paired groups, *p <0.05, **p <0.01, ****p <0.0001. CHB, chronic hepatitis B virus infection; gMFI, geometric mean fluorescence intensity; HCs, healthy controls; NK, natural killer; PBMCs, peripheral blood mononuclear cells.

based on HCMV serology status either in healthy controls or patients with CHB (Fig. S6). This indicates that increased frequency of KLRG1+ NK cells was likely due to HBV infection, and not CMV exposure.

Chronic hepatitis B infection stimulates the expression of NK cell KLRG1

To examine whether the expression of KLRG1 is induced by persistent antigen stimulation, we cultured NK cells from healthy controls for 7 days with either (1) CHB serum (HBsAg (+) and ALT level <60 IU/ml), (2) NAFLD serum (HBsAg (−) and ALT level <60 IU/ml), or (3) FCS as a control. CHB serum had no effect on CD56^{dim} NK cell CD16, CD57, or KIR, but stimulated KLRG1 surface expression (CHB *vs.* FCS; 4.1 *vs.* 0.9%, p <0.01 and gMFI: 2,042.5 *vs.* 1,685, p <0.05, CHB *vs.* NALFD; 4.1 *vs.* 0.5%, p <0.01 and gMFI: 2,042.5 *vs.* 1,602.3, p <0.05, Fig. 5A). Indeed, CHB serum stimulated an activated NK cell phenotype, increasing the frequency of CD69 positive cells compared to FCS controls (9.2 *vs.* 1.1%, p <0.01 and gMFI: 1,508.8 *vs.* 1,123.3, p <0.05, Fig. 5A). Representative flow dot plots are shown in Fig. S6.

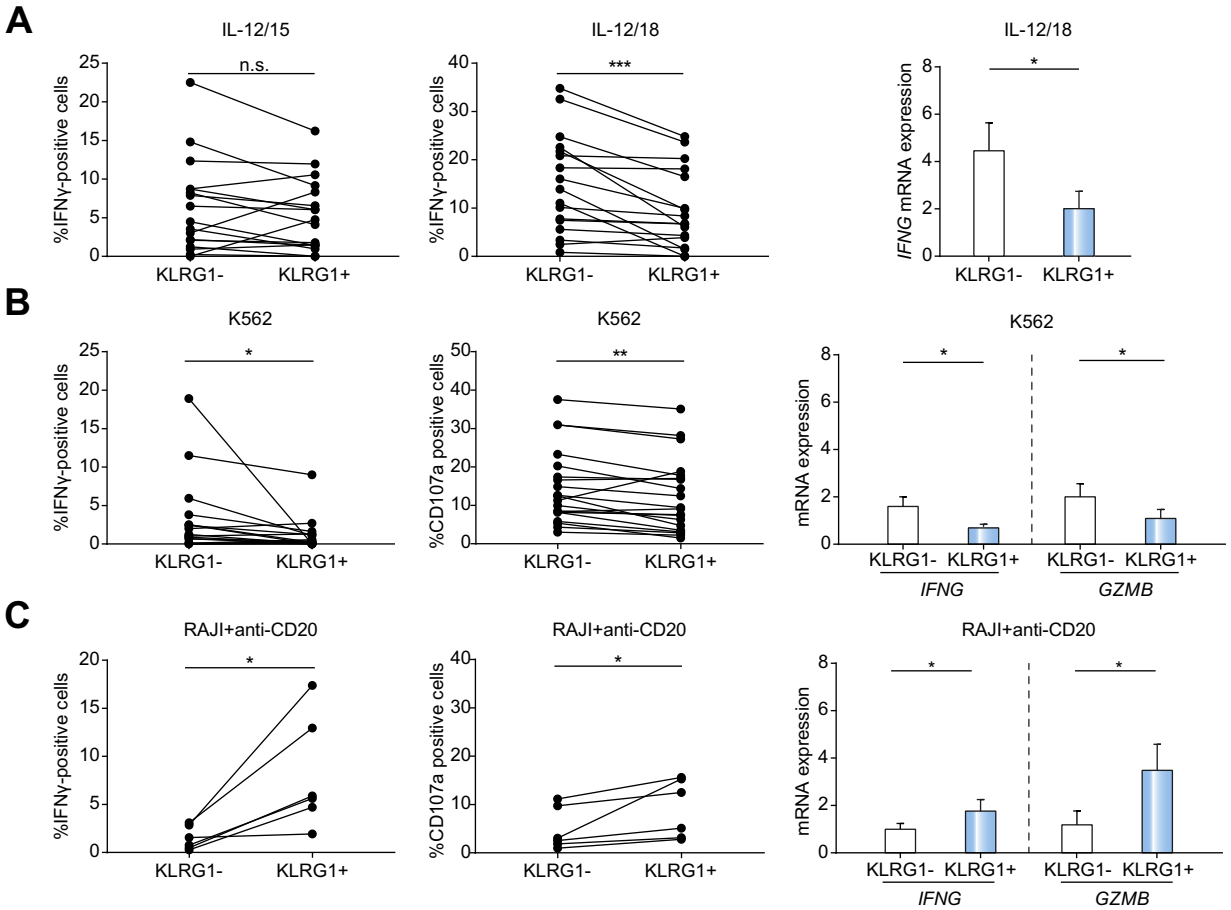


Fig. 3. KLRG1 mediates differences in NK cell IFN-γ secretion and CD107a expression in response to cytokine treatment and co-culture. For all experiments, IFN-γ production and CD107a expression were determined by flow cytometry, gating on CD56^{dim}CD16^{bright}KLRG1− and CD56^{dim}CD16^{bright}KLRG1+ NK cells. (A) IFN-γ production of NK cells after stimulation with IL-12/15 or IL-12/18 for 14 h and qPCR of NK cells following stimulation with IL-12/18. Following target cell stimulation; (B) K562 and (C) Raji+ anti-CD20 mAb, degranulation and IFN-γ expression were measured by flow cytometry and qPCR. Wilcoxon's tests were performed for individual comparisons of paired groups, *p <0.05, **p <0.01, ***p <0.001. mAb, monoclonal antibody; NK, natural killer; qPCR, quantitative real-time PCR.

Furthermore, there was no significant difference in KLRG1+ NK cells after culture with HBV serum from HBeAg positive or negative patients (Fig. 5B). Additionally, the percentage of KLRG1+ NK cells within patients with CHB in the presence or absence of HBeAg was similar (Fig. 5C) and no correlation was found between the percentage of KLRG1+ NK cells and HBsAg concentration (Fig. 5C). However, a significant increase in KLRG1 expression was observed in PBMCs stimulated with purified HBsAg (p <0.05) or serum from a patient with acute HBV for 7 days, compared to PBMCs cultured with FCS or NAFLD serum (Fig. 5D), suggesting that antigen stimulation is required for induction of KLRG1 expression. A similar pattern of KLRG1 expression on CD8+ T cells has been observed during persistent antigen stimulation in chronic viral infection.[30,31]

Peripheral KLRG1+ NK cells are activated and correlate negatively with liver injury serum markers

CD69 expression levels were measured to determine the activation state of CD56^{dim}CD16^{bright} NK cells in relation to KLRG1 expression: The frequency of the activation marker CD69 was significantly higher on KLRG1+ NK cells than KLRG1− NK cells in patients with CHB (14.6 *vs.* 6.6%, p <0.0001 and gMFI: 1,385.6 *vs.* 1,154.2, p <0.0001, Fig. 6A). This was confirmed by qPCR, showing an increase in *CD69* mRNA expression on KLRG1+ NK cells (3.4 *vs.* 1.7-fold change; p <0.05, Fig. 6A).

Peripheral KLRG1+ NK cells in patients with CHB showed a negative correlation with levels of ALT (r: −0.538, p = 0.0012, Fig. 6B) and AST (r: −0.542, p = 0.0011, Fig. 6B). Additionally, we found peripheral and intrahepatic KLRG1+ NK cells are enriched in patients with CHB and no or mild fibrosis stage (F0-F1) compared to moderate fibrosis stages (F2-F3), as assessed by liver biopsy or transient elastography (Fibroscan) (peripheral: 14.8 *vs.* 7%, p <0.05; intrahepatic: 38.8 *vs.* 8%, p <0.05, Fig. 6C)

Hepatic KLRG1+ NK cells are enriched in the livers of chronic hepatitis B patients

To better understand the relationship between KLRG1+ NK cells in peripheral blood and liver, we performed phenotyping of hepatic NK cells from liver resections from 13 patients undergoing surgery for colorectal metastases or hepatocellular carcinoma (HCC): 8 patients were HBsAg (+), anti-HBc (+), anti-HBs (−) and 5 patients were HBsAg (−), anti-HBc (−), anti-HBs (−). We observed NK cells were slightly enriched in liver compared to peripheral blood both in healthy controls and patients with CHB (Fig. 7A). KLRG1+ NK cells were significantly enriched

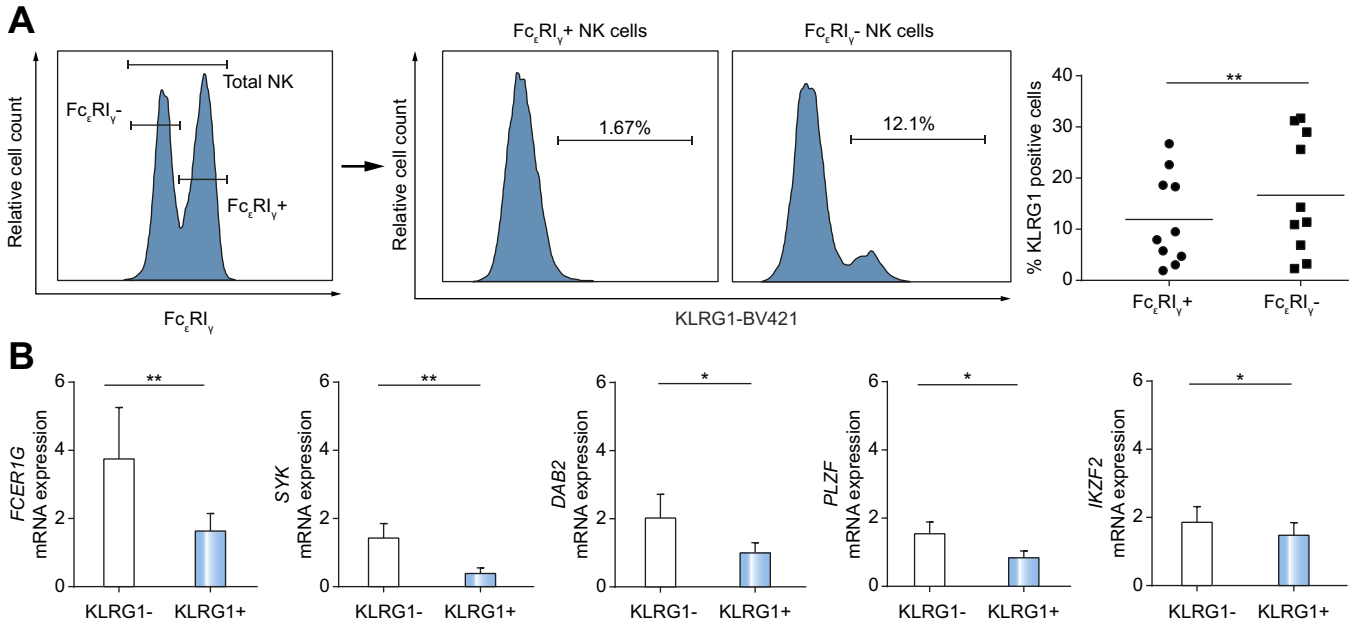


Fig. 4. Identification KLRG1 expression on FcεRIγ-negative NK cells in patients with CHB. (A) PBMCs of patients with CHB were analysed using flow cytometry, gating on CD56^{dim}CD3-CD19- NK cells. Histograms demonstrating FcεRIγ expression among NK cells, and KLRG1 expression within FcεRIγ positive and negative groups. (B) Relative mRNA expression of *FCER1G*, *SYK*, *DAB2*, *PLZF*, and *IKZF2* on KLRG1− and KLRG1+ NK cells. Wilcoxon's tests were performed for individual comparisons of paired groups and Mann-Whitney *U* tests were performed for comparisons of 2 independent groups, **p* <0.05, ***p* <0.01. CHB, chronic hepatitis B virus infection; NK, natural killer; PBMCs, peripheral blood mononuclear cells;

in liver of patients with CHB compared to non-CHB controls (23.4 *vs.* 2.6%, *p* <0.01, Fig. 7A).

These findings prompted us to further characterize whether KLRG1+ NK cells were liver-resident NK cells. Because the expression of CD69 is high on liver-resident NK cells[49,50] and correlates with other liver-resident NK cells markers such as CXCR6 and CD49a,[49,51–53] we investigated KLRG1 expression based on CD69 positivity on intrahepatic NK cells subsets. Using t-SNE plots, we identified distinct CD69+ and CD69− NK cell populations (Fig. 7B), and further, that CXCR6 and CD49a markers were highly expressed on CD69+ NK cells (Fig. 7C). These findings are in line with previous publications[53,54] that identified CD69 as a tissue retention marker.

Additionally, KLRG1 was highly expressed in CD69− compared to CD69+ NK cells, as measured by gMFI (723.8 *vs.* 566.8, *p* <0.05, Fig. 7C). Mature NK cell characteristics such as lower CD56 expression and higher CD16 and CD57 expression were found in the CD69− NK cell subset (Fig. 7C). Altogether, these findings suggest that intrahepatic KLRG1+ NK cells were not liver-resident NK cells, but more closely resemble the peripheral KLRG1+ NK cell phenotype.

Finally, to investigate the activation state of intrahepatic KLRG1+ NK cells, we compared the expression of NKp44 activating receptor within CD69− and CD69+ NK cell subsets. Unlike the other natural cytotoxicity receptors, NKp30 and NKp46, which are constitutively expressed, NKp44 is only expressed after NK cell activation.[55,56] The expression of NKp44 was significantly higher on KLRG1+ NK cells compared to KLRG1− NK cells, as measured by gMFI (228.6 *vs.* 126.3, *p* <0.05, Fig. 7C).

Antifibrotic activity of KLRG1+ NK cells

To assess whether KLRG1+ NK cells possess antifibrotic activity, we examined their ability to kill activated stellate cells. IFN-α was used to stimulate NK cells, as it has been extensively used to treat chronic HBV and limit the progression of liver fibrosis.[57,58] NK cells were sorted based on KLRG1 expression and treated ± 1,000 U/ml IFN-α for 14 h. Following co-culture with LX-2 cells for 6 h, KLRG1+ NK cell IFN-γ production was significantly increased compared to KLRG1− cells in the absence (3 *vs.* 0.3%, *p* <0.05, Fig. 8A) or presence (6.1 *vs.* 1%, *p* <0.01, Fig. 8A) of IFN-α stimulation. Consistent with IFN-γ production, KLRG1+ NK cells demonstrated stronger cytotoxicity than KLRG1− NK cells against LX-2 cells after IFN-α stimulation (26.9 *vs.* 13.3%, *p* <0.01, Fig. 8A). To validate these findings, NK cells were sorted based on KLRG1 expression and treated ± -IFN-α. *IFNG* and *GZMB* mRNA expression was significantly increased on KLRG1+ compared to KLRG1− NK cells following LX-2 co-culture in the absence (*IFNG*: 3.1 *vs.* 1.2; *GZMB:* 3.2 *vs.* 2.2, *p* <0.05, Fig. 8B) or presence (*IFNG*: 4.4 *vs.* 2.3; *GZMB:* 4.2 *vs.* 1.9, *p* <0.05, Fig. 8B) of IFN-α treatment.

Apart from granzyme/perforin-dependent and IFN-γ-mediated mechanisms, NK cells can limit fibrosis progression via TRAIL-, FasL-, or NKG2D- interactions with activated HSC receptors.[59] To determine whether KLRG1+ NK cells can induce LX-2 cell death via these mechanisms, we performed phenotypic analysis of TRAIL, FasL, and NKG2D expression on CD56^{dim}CD16^{bright}KLRG1+ and KLRG1− NK cells in the presence, or absence, of IFN-α. TRAIL expression alone was significantly upregulated on KLRG1+ NK cells compared to KLRG1− NK cells (13.5 *vs.* 9.2%, *p* <0.01, Fig. 8C) following IFN-α stimulation. Consistently, a significant increase in TRAIL (*TNFSF10)* mRNA in KLRG1+ NK cells was measured following IFN-α treatment (1.9-fold increase, *p* <0.05, Fig. 8C). TRAIL receptor expression on LX-2 cells was assessed by qPCR for 7 days after plating, demonstrating maximal expression on day 7, consistent with increased activation status (Fig. S1). Consistent with IFN-γ and cytotoxicity assays, KLRG1+ NK cells were more effective at inducing apoptosis of LX-2 cells (Annexin V+) compared to

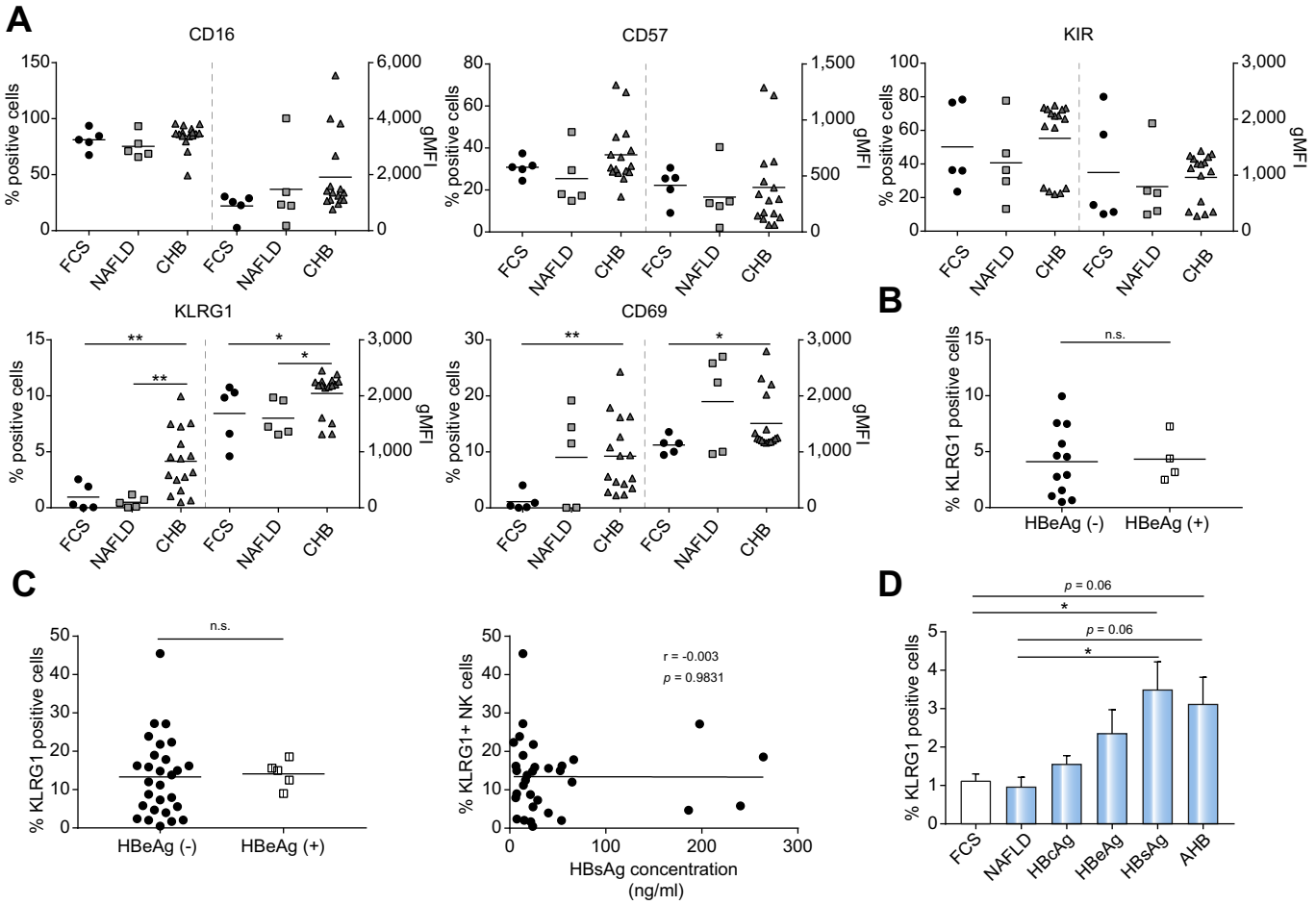


Fig. 5. Culture of NK cells or PBMCs from healthy controls with NAFLD/HBV serum or hepatitis B antigens. (A) The percentage and gMFI of CD16, CD57, KIR, KLRG1, and CD69 NK cells after culture for 7 days in media containing FCS as a control, NAFLD patient serum or chronic HBV patient serum. (B) The percentage of KLRG1+ cells in the absence or presence HBeAg in culture serum. (C) The frequency of KLRG1+ NK cells in patients with CHB related to the positivity of HBeAg and the concentration of HBsAg. (D) The percentage of KLRG1+ NK cells after culture of PBMCs from HCs in the presence of FCS, NALFD serum, HBV antigens, or acute HBV serum. Mann-Whitney *U* tests were performed for comparisons of 2 independent groups, Wilcoxon's tests were performed to assess differences in KLRG1+ NK cells after culture of PBMCs from HCs, and Spearman rank correlation analysis was used to evaluate correlations, *p <0.05, **p <0.01. FCS, foetal calf serum; gMFI, geometric mean fluorescence intensity; HBeAg, HBV e antigen; HBsAg, HBV surface antigen; HBV, hepatitis B virus; HCs, healthy controls; NAFLD, non-alcoholic fatty liver disease; NK, natural killer; PBMCs, peripheral blood mononuclear cells.

KLRG1-NK cells (10.6 *vs.* 0.4%, p <0.01, Fig. 8D). The apoptotic role of TRAIL was confirmed in blocking experiments, where pre-incubation with a neutralizing TRAIL antibody significantly decreased LX-2 apoptosis in KLRG1+ NK cells (10.6 *vs.* 2.2%, p <0.01, Fig. 8D), but not in KLRG1− NK cells.

To confirm the antifibrotic function of KLRG1+ NK cells with activated HSCs, we used primary hHSCs that more closely resemble *in vivo* activated HSCs. A progressive increase of hHSC TRAIL-R2 (DR5) mRNA expression was observed in parallel with progressive hHSC activation after plating (Fig. S1). Similar to their ability to kill LX-2 cells, KLRG1+ NK cells killed primary hHSCs more effectively than KLRG1− NK cells (16.1 *vs.* 10.8%, p <0.01, Fig. 8D), and blocking TRAIL with a neutralizing antibody significantly reduced the TRAIL-mediated apoptosis by KLRG1+ NK cells (16.1 *vs.* 10.7%, p <0.01, Fig. 8D), but not by KLRG1− NK cells. Taken together, this shows a mechanism by which KLRG1+ NK cells may limit liver fibrosis by killing activated HSCs.

HSC-derived osteopontin (OPN) has recently been associated with the promotion of HSC activation, collagen deposition and fibrogenesis.[60–64] Along with HSC activation, increased concentrations of OPN were detected in the supernatant of LX-2 cultures (Fig. S1). To determine whether KLRG1+ NK cells potentially interact with OPN, we examined the surface expression of OPN receptors CD44 and VLA-4 on NK cells, and found that CD44 only was highly expressed on KLRG1+ NK cells compared to KLRG1− NK cells (19,398.1 *vs.* 12,784.2, p <0.01, Fig. 8E). To confirm whether the antifibrotic effect of KLRG1+ NK cells is mediated by osteopontin, NK cells were incubated with 1 μg/ml of recombinant OPN after which NK cell activation status was measured: IFN-γ secretion and degranulation were significantly increased in KLRG1+ NK cells compared to KLRG1− NK cells (IFN-γ; 3.6 *vs.* 1.2, p <0.01; CD107a; 37.5 *vs.* 31.9%, p <0.01, Fig. 8F) supporting an antifibrotic effect. These findings suggest that activation of KLRG1+ NK cells can be mediated by OPN, which is produced by activated HSCs, and thus reduce liver fibrosis.

Discussion

HBV infection remains a major public health concern, with more than 257 million people chronically infected and at high risk of

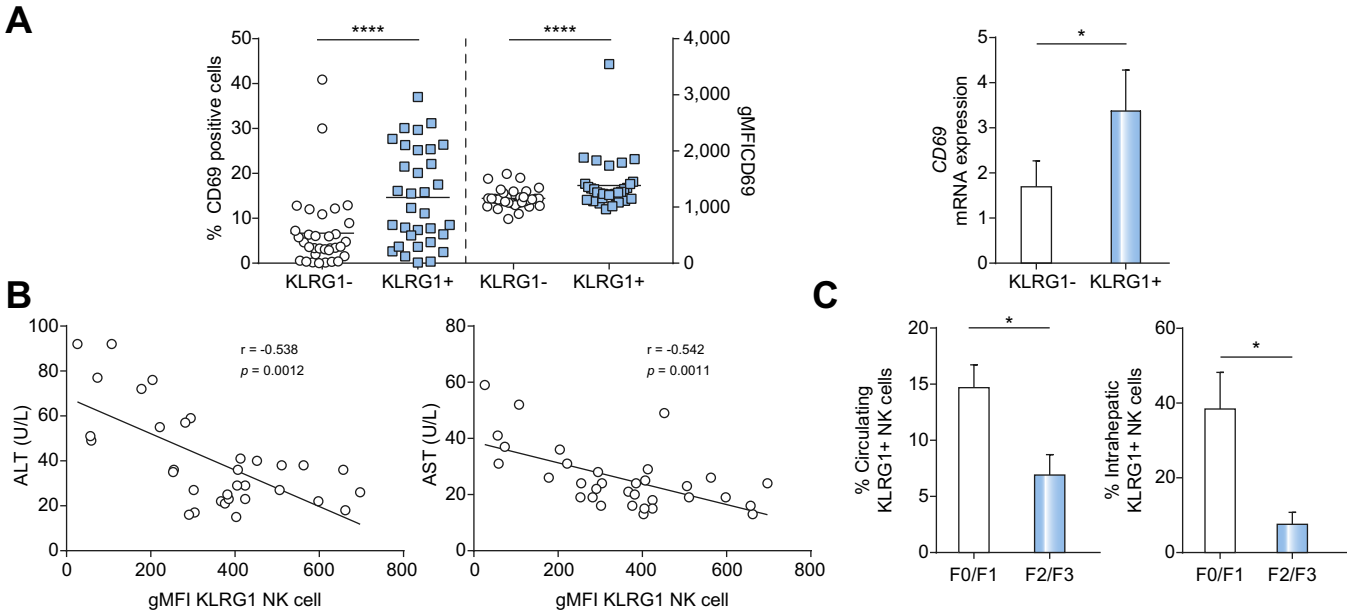


Fig. 6. The activated peripheral KLRG1+ NK cells function in patients with CHB. (A) Flow cytometry and qPCR analysis of CD69 expression on peripheral KLRG1+ NK cells. (B) Correlations between peripheral KLRG1+ NK cells with ALT and AST serum levels (U/L). (C) The proportion of circulating and intrahepatic KLRG1+ NK cells according to fibrosis stages. Mann-Whitney *U* tests were performed for comparisons of 2 independent groups, the Wilcoxon's tests were performed for comparisons of paired groups, and Spearman rank correlation was used to evaluate correlations, $^{*}p < 0.05$, $^{****}p < 0.0001$. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHB, chronic hepatitis B virus infection; NK, natural killer; qPCR, quantitative real-time PCR.

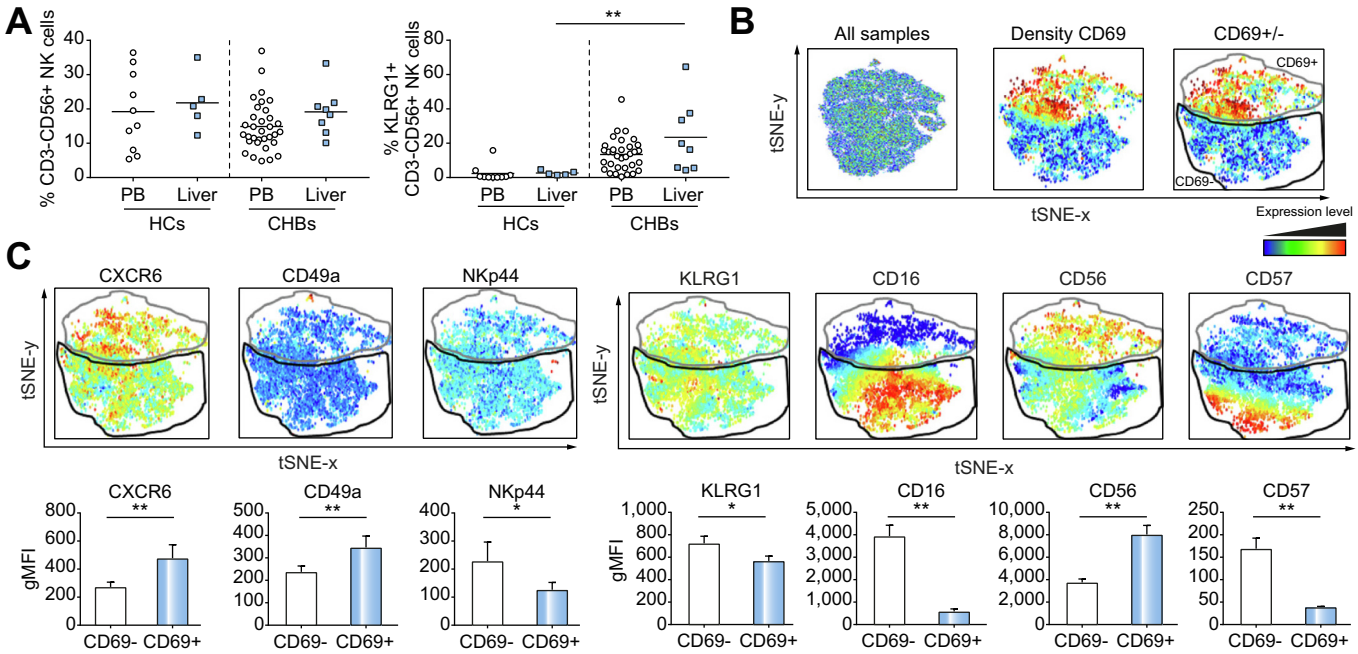


Fig. 7. KLRG1+ NK cells enriched in liver patients with CHB. Hepatic lymphocytes were harvested from 8 patients with CHB who underwent liver resections. (A) Frequency of peripheral blood (PB) and intrahepatic (liver) NK and KLRG1+ NK cells in HCs and patients with CHB. (B) Gated CD3-CD56+ NK cells from 8 patients with CHB were concatenated and represented in t-SNE plots. The CD69+ and CD69− NK cell subsets were gated based on the expression intensity, identified by the colour coding, red being higher expressed and blue being lower expressed. (C) Gated CD69+ and CD69− NK cell subsets were analysed for differences in CXCR6, CD49a, NKp44, KLRG1, CD16, CD56, and CD57 surface markers. The colour gradient indicates the expression intensity of each marker signal. Mann-Whitney *U* tests were performed for comparisons of 2 independent groups and Wilcoxon's tests were performed for comparisons of paired groups, $^{*}p < 0.05$, $^{**}p < 0.01$. CHB, chronic hepatitis B virus infection; HCs, healthy controls; NK, natural killer; PBMCs, peripheral blood mononuclear cells; t-SNE, t-stochastic neighbour embedding.

developing liver failure, cirrhosis, and HCC.[65] The mechanism of HBV chronicity remains ill-defined but involves a complex interaction between viral immune suppression and the host immune response. Because NK cells play an important role in the early stages of host defence against viral infection, an impaired NK cell response may contribute to viral persistence

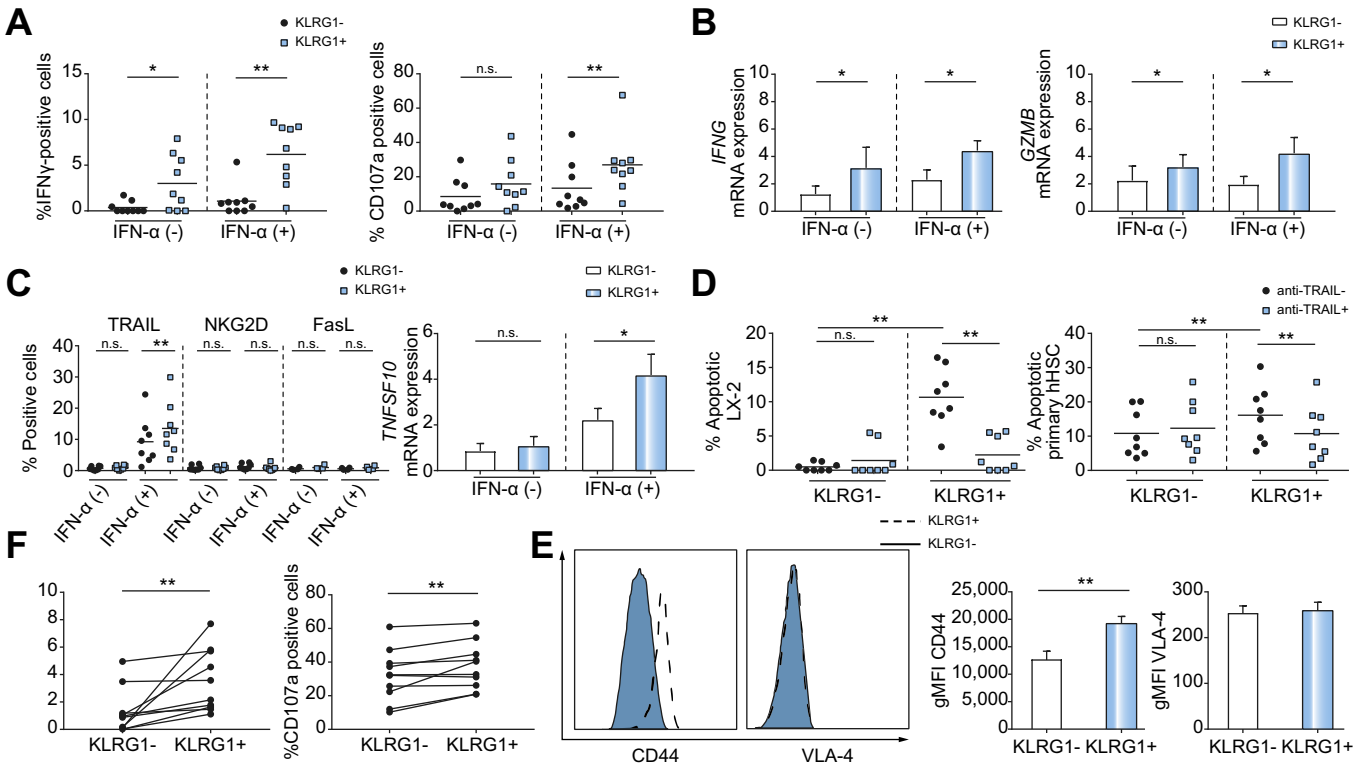


Fig. 8. Antifibrotic activities of KLRG1+ NK cells. (A) The proportion of IFN-γ and CD107a positive cells after culturing purified NK cells with LX-2 for 6 h in the presence or absence IFN-α stimulation. (B) qPCR analysis of *IFNG* and *GZMB* expression following LX-2 target cell stimulation. (C) TRAIL, NKG2D and FasL expression and *TNFSF10* mRNA expression level on KLRG1− and KLRG1+ NK cell in the presence or absence IFN-α stimulation. (D) The percentage of apoptotic cell after co-incubation of LX-2 or primary hHSCs with NK cells in the presence or absence TRAIL-neutralization antibody. (E) Representative histogram demonstrating the CD44 and VLA-4 expression on KLRG1− and KLRG1+ NK cells. Bar diagram showing the expression (gMFI) of CD44 and VLA-4. (F) The proportion of IFN-γ and CD107a positive cells following culture with 1 μg/ml osteopontin. Mann-Whitney *U* tests were performed for comparisons of 2 independent groups and Wilcoxon's tests were performed for comparisons of paired groups. Data are showed as mean ± SEM, $^{*}p$ <0.05, $^{**}p$ <0.01. gMFI, geometric mean fluorescence intensity; hHSCs, human hepatic stellate cells; NK, natural killer; qPCR, quantitative real-time PCR.

in HBV infection.[4,66–67] Unfortunately, the NK cell response that drives viral clearance also contributes to liver injury and subsequent fibrosis in CHB.[10,12,68–70] Nonetheless, emerging evidence suggests that NK cell populations in the liver can have antifibrotic activities by killing activated HSCs, or via the production of the antifibrogenic cytokine, IFN-γ.[57,59,71–78]

Recent studies in mice and humans have demonstrated the presence of significant heterogeneity among NK cells based on maturation stage and functional capability,[19,79–81] making it unclear which NK cell population possesses antifibrotic potential. We have demonstrated that KLRG1+ NK cells can potentially inhibit liver fibrosis by targeting activated stellate cells in a TRAIL-dependent manner. Furthermore, we have established a link between KLRG1+ NK cells in the regulation of liver fibrosis, as well as the protection against liver injury, a common precursor of liver fibrosis. Therefore, the production of KLRG1+ NK cells in patients with CHB may offer advantageous clinical effects for treating liver fibrosis.

The effect of KLRG1 expression on NK cells and their biological role in chronic HBV infection remains unknown. We have shown a significant increase in KLRG1+ NK cells in patients with CHB compared to healthy donors. KLRG1 expression is elevated on CD56^{dim} NK cells and is co-expressed with CD16 and CD57 surface markers, indicative of NK cell maturity. This mature state relates to the reduced IFN-γ and cytotoxic responses to cytokine stimulation[22,43] and target cell co-culture that we observed,[82–86] as well as to the elevated ADCC immune responses by recognition of antibody-coated target cells (Fig. 3).[46,84,87]. Moreover, we found that KLRG1 was highly expressed on FcεRIγ-NK cells that expand during HCMV or chronic HBV/HCMV co-infection and represent memory-like NK cells.[46,88] While KLRG1+ NK cells share similar expression of maturation markers (↑CD57), signalling molecules (↓Syk, ↓DAB2), and transcription factors (↓PLZF, ↓Helios) with FcεRIγ-NK cells, we found that KLRG1 is upregulated on NK cells of patients with CHB regardless of HCMV serology status (Fig. S5). In addition, we found no correlation between KLRG1 + NK cells with NK cells subsets that expand during HCMV infection (KIR+CD57+NKG2C+)[89] (Fig. S5). These findings suggest that the presence of KLRG1+ NK cells in patients with CHB is not affected by the presence of HCMV infection.

In HBV infection, NK cell cytolytic activity is positively correlated with liver injury, as measured by ALT or AST levels and liver histological activity index.[10,12] We found a negative correlation between the gMFI of KLRG1 on NK cells and serum ALT/AST levels suggesting an opposite effect. The low cytotoxicity of KLRG1+ NK cells may be mediated by the inhibitory function of KLRG1 that prevents hepatocyte lysis by binding to its biological ligand E-cadherin on hepatocytes.[90] Additionally, it may be mediated by the low expression of NKp46 activating receptor, which plays a significant role in regulating NK cell cytotoxicity during HBV infection. Indeed, the abundance of NKp46+ NK cells

correlates with NK cytotoxicity in several viral infections[71,91,92] and blocking NKp46 reduces NK cytotoxicity against HepG2 cell lines.[91,93,94]

Our finding that KLRG1+ NK cells can stimulate HSC apoptosis indicates that they may represent a unique NK cell population with antifibrotic potential. These findings are in line with published works showing inhibition of HSC proliferation, activation and induction of HSC apoptosis via NK cell IFN-γ production and cytotoxicity.[71–73] Although KLRG1+ NK cells lack NKp46 receptor expression which has previously been shown to play a role to in liver fibrosis,[71] the loss of E-cadherin, the ligand for KLRG1, on activated HSCs (Fig. S1) could mediate the activation of KLRG1+ NK cells via the reduction of inhibitory signals in the regulation of NK cell activity. In addition, IFN-α enhanced NK cell activation and attenuation of liver fibrosis progression have been reported in patients with HCV[95,96] and HBV,[57,58] prompting our examination of its effects (Fig. 8). Our results demonstrate that IFN-α stimulates TRAIL-mediated HSC killing by KLRG1+ NK cells *in* vitro, indicating that IFN-α may not only be beneficial for inhibiting viral replication in patients with HBV, but also in limiting liver fibrosis progression by activating KLRG1+ NK cells. Finally, we found that KLRG1+ NK cells can be activated by OPN, which is produced by activated HSCs, suggesting a novel antifibrotic role for KLRG1+ NK cells.

The stimulus required to induce the production of KLRG1+ NK cells remains uncertain; however, we have demonstrated that HBV antigen is likely required. By culturing NK cells from healthy donors with serum from patients with CHB and NAFLD with similar ALT (<60 IU/ml) or recombinant HBV antigen, we found that KLRG1 expression is induced in the presence of HBV antigen alone. These data suggest that an antigenic stimulus, as is present in the HBV vaccine or during active infection, is capable of stimulating KLRG1+ NK cell maturation.

In conclusion, we have shown that KLRG1+ NK cells persist in the blood and liver of patients with CHB, where they display a mature phenotype. Their inability to produce IFN-γ after cytokine stimulation and limited cytotoxic activity are consistent with their differentiated status. KLRG1+ NK cells are however more responsive to ADCC stimulus and display a greater capacity to induce HSC apoptosis, perhaps explaining their enrichment in patients with no or mild fibrosis. A better understanding of the factors that influence KLRG1 expression, NK cell maturation, and the antifibrotic activity of KLRG1+ NK cells may provide insight into novel therapeutic strategies to limit fibrosis in CHB.

Financial support

This project was supported by the Robert W. Storr Bequest to the Sydney Medical Foundation, University of Sydney; a National Health and Medical Research Council of Australia (NHMRC) Program Grant No. 1053206; Australia Awards Scholarships. Golo Ahlenstiel is supported by a Sylvia and Charles Viertel Charitable Foundation Investigatorship (VTL2015C022).

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.

Authors' contributions

Designing research studies (RW, SR, GA), conducting experiments (RW, SR), acquiring/analysing data (RW, SR, SS, MA, KE, GA), providing tissues and reagents and interpreting results (ME, DP, RL, LY, VL, MD, JG, GA), and writing the manuscript (SR, RW, GA).

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.03.012.

References

Author names in bold designate shared co-first authorship

[1] Schuch A, Hoh A. Thimme R: the role of natural killer cells and CD8+ T cells in hepatitis B virus infection. Front Immunol 2014;5:1–8.
[2] Mondelli MU, Varchetta S, Oliviero B. Natural killer cells in viral hepatitis: facts and controversies. Eur J Clin Invest 2010;40:851–863.
[3] Fisicaro P, Valdatta C, Boni C, Massari M, Mori C, Zerbini A, et al. Early kinetics of innate and adaptive immune responses during hepatitis B virus infection. Gut 2009;58:974–982.
[4] **Zhao J**, **Li Y**, Jin L, Zhang S, Fan R, Sun Y, et al. Natural killer cells are characterized by the concomitantly increased interferon-gamma and cytotoxicity in acute resolved hepatitis B patients. PLoS ONE 2012;7 e49135.
[5] **Li J**, **Han Y**, Jin K, Wan Y, Wang S, Liu B, et al. Dynamic changes of cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, and natural killer T (NKT) cells in patients with acute hepatitis B infection. Virol J 2011;8:199.
[6] Tong S, Liu G, Li M, Li X, Liu Q, Peng H, et al. Natural killer cell activation contributes to hepatitis B viral control in a mouse model. Sci Rep 2017;7:314.
[7] Zheng M, Sun R, Wei H, Tian Z. NK cells help induce anti-hepatitis B virus CD8+ T cell immunity in mice. J Immunol 2016;196:4122–4131.
[8] Tang H, Li C, Wang L, Zhang H, Fan Z. Granzyme H of cytotoxic lymphocytes is required for clearance of the hepatitis B virus through cleavage of the hepatitis B virus X protein. J Immunol 2012;188:824–831.
[9] Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. Science 1999;284:825–829.
[10] **Zhang Z**, **Zhang S**, **Zou Z**, Shi J, Zhao J, Fan R, et al. Hypercytolytic activity of hepatic natural killer cells correlates with liver injury in chronic hepatitis B patients. Hepatology 2011;53:73–85.
[11] Dunn C, Brunetto M, Reynolds G, Christophides T, Kennedy PT, Lampertico P, et al. Cytokines induced during chronic hepatitis B virus infection promote a pathway for NK cell-mediated liver damage. J Exp Med 2007;204:667–680.
[12] Zheng Q, Zhu YY, Chen J, Ye YB, Li JY, Liu YR, et al. Activated natural killer cells accelerate liver damage in patients with chronic hepatitis B virus infection. Clin Exp Immunol 2015;180:499–508.
[13] Peppa D, Gill US, Reynolds G, Easom NJ, Pallett LJ, Schurich A, et al. Up-regulation of a death receptor renders antiviral T cells susceptible to NK cell-mediated deletion. J Exp Med 2013;210:99–114.
[14] Fan YY, Yang BY, Wu CY. Phenotypically and functionally distinct subsets of natural killer cells in human PBMCs. Cell Biol Int 2008;32:188–197.
[15] Fu B, Tian Z, Wei H. Subsets of human natural killer cells and their regulatory effects. Immunology 2014;141:483–489.
[16] Juelke K, Killig M, Luetke-Eversloh M, Parente E, Gruen J, Morandi B, et al. CD62L expression identifies a unique subset of polyfunctional CD56dim NK cells. Blood 2010;116:1299–1307.
[17] Romagnani C, Juelke K, Falco M, Morandi B, D'Agostino A, Costa R, et al. CD56brightCD16- killer Ig-like receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation. J Immunol 2007;178:4947–4955.
[18] Bjorkstrom NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, et al. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. Blood 2010;116:3853–3864.
[19] Moretta L. Dissecting CD56dim human NK cells. Blood 2010;116:3689–3691.

[20] Henson SM, Akbar AN. KLRG1–more than a marker for T cell senescence. Age (Dordr) 2009;31:285–291.
[21] Huntington ND, Tabarias H, Fairfax K, Brady J, Hayakawa Y, Degli-Esposti MA, et al. NK cell maturation and peripheral homeostasis is associated with KLRG1 up-regulation. J Immunol 2007;178:4764–4770.
[22] Lopez-Verges S, Milush JM, Pandey S, York VA, Arakawa-Hoyt J, Pircher H, et al. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. Blood 2010;116:3865–3874.
[23] Tessmer MS, Fugere C, Stevenaert F, Naidenko OV, Chong HJ, Leclercq G, et al. KLRG1 binds cadherins and preferentially associates with SHIP-1. Int Immunol 2007;19:391–400.
[24] Voehringer D, Koschella M, Pircher H. Lack of proliferative capacity of human effector and memory T cells expressing killer cell lectinlike receptor G1 (KLRG1). Blood 2002;100:3698–3702.
[25] Robbins SH, Terrizzi SC, Sydora BC, Mikayama T, Brossay L. Differential regulation of killer cell lectin-like receptor G1 expression on T cells. J Immunol 2003;170:5876–5885.
[26] Voehringer D, Blaser C, Brawand P, Raulet DH, Hanke T, Pircher H. Viral infections induce abundant numbers of senescent CD8 T cells. J Immunol 2001;167:4838–4843.
[27] Della Chiesa M, Falco M, Bertaina A, Muccio L, Alicata C, Frassoni F, et al. Human cytomegalovirus infection promotes rapid maturation of NK cells expressing activating killer Ig-like receptor in patients transplanted with NKG2C-/- umbilical cord blood. J Immunol 2014;192:1471–1479.
[28] Foley B, Cooley S, Verneris MR, Pitt M, Curtsinger J, Luo X, et al. Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C+ natural killer cells with potent function. Blood 2012;119:2665–2674.
[29] Hong HS, Eberhard JM, Keudel P, Bollmann BA, Ballmaier M, Bhatnagar N, et al. HIV infection is associated with a preferential decline in less-differentiated CD56dim CD16+ NK cells. J Virol 2010;84:1183–1188.
[30] Thimme R, Appay V, Koschella M, Panther E, Roth E, Hislop AD, et al. Increased expression of the NK cell receptor KLRG1 by virus-specific CD8 T cells during persistent antigen stimulation. J Virol 2005;79:12112–12116.
[31] Ibegbu CC, Xu YX, Harris W, Maggio D, Miller JD, Kourtis AP. Expression of killer cell lectin-like receptor G1 on antigen-specific human CD8+ T lymphocytes during active, latent, and resolved infection and its relation with CD57. J Immunol 2005;174:6088–6094.
[32] Ito M, Maruyama T, Saito N, Koganei S, Yamamoto K, Matsumoto N. Killer cell lectin-like receptor G1 binds three members of the classical cadherin family to inhibit NK cell cytotoxicity. J Exp Med 2006;203:289–295.
[33] Grundemann C, Bauer M, Schweier O, von Oppen N, Lassing U, Saudan P, et al. Cutting edge: identification of E-cadherin as a ligand for the murine killer cell lectin-like receptor G1. J Immunol 2006;176:1311–1315.
[34] Li Y, Hofmann M, Wang Q, Teng L, Chlewicki LK, Pircher H, et al. Structure of natural killer cell receptor KLRG1 bound to E-cadherin reveals basis for MHC-independent missing self recognition. Immunity 2009;31:35–46.
[35] Colonna M. Cytolytic responses: cadherins put out the fire. J Exp Med 2006;203:261–264.
[36] Rosshart S, Hofmann M, Schweier O, Pfaff AK, Yoshimoto K, Takeuchi T, et al. Interaction of KLRG1 with E-cadherin: new functional and structural insights. Eur J Immunol 2008;38:3354–3364.
[37] Kawarabayashi N, Seki S, Hatsuse K, Ohkawa T, Koike Y, Aihara T, et al. Decrease of CD56(+)T cells and natural killer cells in cirrhotic livers with hepatitis C may be involved in their susceptibility to hepatocellular carcinoma. Hepatology 2000;32:962–969.
[38] Morsy MA, Norman PJ, Mitry R, Rela M, Heaton ND, Vaughan RW. Isolation, purification and flow cytometric analysis of human intrahepatic lymphocytes using an improved technique. Lab Invest 2005;85:285–296.
[39] Beziat V, Dalgard O, Asselah T, Halfon P, Bedossa P, Boudifa A, et al. CMV drives clonal expansion of NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients. Eur J Immunol 2012;42:447–457.
[40] Della Chiesa M, Muccio L, Moretta A. CMV induces rapid NK cell maturation in HSCT recipients. Immunol Lett 2013;155:11–13.
[41] Foley B, Cooley S, Verneris MR, Curtsinger J, Luo X, Waller EK, et al. Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand in vivo in response to recipient CMV antigen. J Immunol 2012;189:5082–5088.
[42] Malmberg KJ, Beziat V, Ljunggren HG. Spotlight on NKG2C and the human NK-cell response to CMV infection. Eur J Immunol 2012;42:3141–3145.
[43] Luetke-Eversloh M, Cicek BB, Siracusa F, Thom JT, Hamann A, Frischbutter S, et al. NK cells gain higher IFN-gamma competence during terminal differentiation. Eur J Immunol 2014;44:2074–2084.
[44] Nimmerjahn F, Ravetch JV. Fcgamma receptors as regulators of immune responses. Nat Rev Immunol 2008;8:34–47.
[45] **Hwang I**, **Zhang T**, **Scott JM**, Kim AR, Lee T, Kakarla T, et al. Identification of human NK cells that are deficient for signaling adaptor FcRgamma and specialized for antibody-dependent immune functions. Int Immunol 2012;24:793–802.
[46] Zhang T, Scott JM, Hwang I, Kim S. Cutting edge: antibody-dependent memory-like NK cells distinguished by FcRgamma deficiency. J Immunol 2013;190:1402–1406.
[47] **Schlums H**, **Cichocki F**, Tesi B, Theorell J, Beziat V, Holmes TD, et al. Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function. Immunity 2015;42:443–456.
[48] **Lee J**, **Zhang T**, Hwang I, Kim A, Nitschke L, Kim M, et al. Epigenetic modification and antibody-dependent expansion of memory-like NK cells in human cytomegalovirus-infected individuals. Immunity 2015;42:431–442.
[49] Stegmann KA, Robertson F, Hansi N, Gill U, Pallant C, Christophides T, et al. CXCR6 marks a novel subset of T-bet(lo)Eomes(hi) natural killer cells residing in human liver. Sci Rep 2016;6:26157.
[50] Fan X, Rudensky AY. Hallmarks of tissue-resident lymphocytes. Cell 2016;164:1198–1211.
[51] **Martrus G**, **Kautz T**, Lunemann S, Richert L, Glau L, Salzberger W, et al. Proliferative capacity exhibited by human liver-resident CD49a+CD25+ NK cells. PLoS ONE 2017;12 e0182532.
[52] Cuff AO, Robertson FP, Stegmann KA, Pallett LJ, Maini MK, Davidson BR, et al. Eomeshi NK cells in human liver are long-lived and do not recirculate but can be replenished from the circulation. J Immunol 2016;197:4283–4291.
[53] Lugthart G, Melsen JE, Vervat C, van Ostaijen-Ten Dam MM, Corver WE, Roelen DL, et al. Human lymphoid tissues harbor a distinct CD69+CXCR6 + NK cell population. J Immunol 2016;197:78–84.
[54] Shiow LR, Rosen DB, Brdickova N, Xu Y, An J, Lanier LL, et al. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. Nature 2006;440:540–544.
[55] Vitale M, Bottino C, Sivori S, Sanseverino L, Castriconi R, Marcenaro E, et al. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. J Exp Med 1998;187:2065–2072.
[56] Nel I, Lucar O, Petitdemange C, Beziat V, Lapalus M, Bedossa P, et al. Accumulation of intrahepatic TNF-alpha-producing NKp44+ NK cells correlates with liver fibrosis and viral load in chronic HCV infection. Medicine (Baltimore) 2016;95 e3678.
[57] Yang YF, Zhao W, Zhong YD, Xia HM, Shen L, Zhang N. Interferon therapy in chronic hepatitis B reduces progression to cirrhosis and hepatocellular carcinoma: a meta-analysis. J Viral Hepat 2009;16:265–271.
[58] Poynard T, Massard J, Rudler M, Varaud A, Lebray P, Moussalli J, et al. Impact of interferon-alpha treatment on liver fibrosis in patients with chronic hepatitis B: an overview of published trials. Gastroenterol Clin Biol 2009;33:916–922.
[59] Glassner A, Eisenhardt M, Kramer B, Korner C, Coenen M, Sauerbruch T, et al. NK cells from HCV-infected patients effectively induce apoptosis of activated primary human hepatic stellate cells in a TRAIL-, FasL- and NKG2D-dependent manner. Lab Invest 2012;92:967–977.
[60] Xiao X, Gang Y, Gu Y, Zhao L, Chu J, Zhou J, et al. Osteopontin contributes to TGF-beta1 mediated hepatic stellate cell activation. Dig Dis Sci 2012;57:2883–2891.
[61] **Chen Y**, **Ou Y**, Dong J, Yang G, Zeng Z, Liu Y, et al. Osteopontin promotes collagen I synthesis in hepatic stellate cells by miRNA-129-5p inhibition. Exp Cell Res 2018;362:343–348.
[62] **Urtasun R**, **Lopategi A**, George J, Leung TM, Lu Y, Wang X, et al. Osteopontin, an oxidant stress sensitive cytokine, up-regulates collagen-I via integrin alpha(V)beta(3) engagement and PI3K/pAkt/NFkappaB signaling. Hepatology 2012;55:594–608.
[63] Lee SH, Seo GS, Park YN, Yoo TM, Sohn DH. Effects and regulation of osteopontin in rat hepatic stellate cells. Biochem Pharmacol 2004;68:2367–2378.
[64] Leung TM, Wang X, Kitamura N, Fiel MI, Nieto N. Osteopontin delays resolution of liver fibrosis. Lab Invest 2013;93:1082–1089.
[65] Stasi C, Silvestri C, Voller F. Emerging trends in epidemiology of hepatitis B virus infection. J Clin Transl Hepatol 2017;5:272–276.

[66] Zhang QF, Shao JY, Yin WW, Xia Y, Chen L, Wang X, et al. Altered immune profiles of natural killer cells in chronic hepatitis b patients: a systematic review and meta-analysis. PLoS ONE 2016;11 e0160171.
[67] Sun C, Sun H, Zhang C, Tian Z. NK cell receptor imbalance and NK cell dysfunction in HBV infection and hepatocellular carcinoma. Cell Mol Immunol 2015;12:292–302.
[68] **Ghosh S**, **Nandi M**, Pal S, Mukhopadhyay D, Chakraborty BC, Khatun M, et al. Natural killer cells contribute to hepatic injury and help in viral persistence during progression of hepatitis B e-antigen-negative chronic hepatitis B virus infection. Clin Microbiol Infect 2016;22, 733 e739–733 e719.
[69] **Zou Y**, **Chen T**, Han M, Wang H, Yan W, Song G, et al. Increased killing of liver NK cells by Fas/Fas ligand and NKG2D/NKG2D ligand contributes to hepatocyte necrosis in virus-induced liver failure. J Immunol 2010;184:466–475.
[70] Maini MK, Peppa D. NK cells: a double-edged sword in chronic hepatitis B virus infection. Front Immunol 2013;4:57.
[71] **Kramer B**, **Korner C**, Kebschull M, Glassner A, Eisenhardt M, Nischalke HD, et al. Natural killer p46High expression defines a natural killer cell subset that is potentially involved in control of hepatitis C virus replication and modulation of liver fibrosis. Hepatology 2012;56:1201–1213.
[72] Jeong WI, Park O, Gao B. Abrogation of the antifibrotic effects of natural killer cells/interferon-gamma contributes to alcohol acceleration of liver fibrosis. Gastroenterology 2008;134:248–258.
[73] Jeong WI, Park O, Radaeva S, Gao B. STAT1 inhibits liver fibrosis in mice by inhibiting stellate cell proliferation and stimulating NK cell cytotoxicity. Hepatology 2006;44:1441–1451.
[74] Radaeva S, Wang L, Radaev S, Jeong WI, Park O, Gao B. Retinoic acid signaling sensitizes hepatic stellate cells to NK cell killing via upregulation of NK cell activating ligand RAE1. Am J Physiol Gastrointest Liver Physiol 2007;293:G809–816.
[75] Radaeva S, Sun R, Jaruga B, Nguyen VT, Tian Z, Gao B. Natural killer cells ameliorate liver fibrosis by killing activated stellate cells in NKG2D-dependent and tumor necrosis factor-related apoptosis-inducing ligand-dependent manners. Gastroenterology 2006;130:435–452.
[76] Gur C, Doron S, Kfir-Erenfeld S, Horwitz E, Abu-Tair L, Safadi R, et al. NKp46-mediated killing of human and mouse hepatic stellate cells attenuates liver fibrosis. Gut 2012;61:885–893.
[77] Bansal R, Prakash J, De Ruiter M, Poelstra K. Interferon gamma peptidomimetic targeted to hepatic stellate cells ameliorates acute and chronic liver fibrosis in vivo. J Control Release 2014;179:18–24.
[78] **Melhem A**, **Muhanna N**, Bishara A, Alvarez CE, Ilan Y, Bishara T, et al. Anti-fibrotic activity of NK cells in experimental liver injury through killing of activated HSC. J Hepatol 2006;45:60–71.
[79] Luetke-Eversloh M, Killig M, Romagnani C. Signatures of human NK cell development and terminal differentiation. Front Immunol 2013;4:499.
[80] Montaldo E, Vacca P, Moretta L, Mingari MC. Development of human natural killer cells and other innate lymphoid cells. Semin Immunol 2014;26:107–113.
[81] Yu J, Freud AG, Caligiuri MA. Location and cellular stages of natural killer cell development. Trends Immunol 2013;34:573–582.
[82] Guma M, Angulo A, Vilches C, Gomez-Lozano N, Malats N, Lopez-Botet M. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. Blood 2004;104:3664–3671.
[83] Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol 2001;19:197–223.
[84] Hammer Q, Romagnani C. About Training and memory: NK-cell adaptation to viral infections. Adv Immunol 2017;133:171–207.
[85] Bae DS, Hwang YK, Lee JK. Importance of NKG2D-NKG2D ligands interaction for cytolytic activity of natural killer cell. Cell Immunol 2012;276:122–127.
[86] Sivori S, Pende D, Bottino C, Marcenaro E, Pessino A, Biassoni R, et al. NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. Eur J Immunol 1999;29:1656–1666.
[87] Costa-Garcia M, Vera A, Moraru M, Vilches C, Lopez-Botet M, Muntasell A. Antibody-mediated response of NKG2Cbright NK cells against human cytomegalovirus. J Immunol 2015;194:2715–2724.
[88] **Schuch A**, **Zecher BF**, Muller PA, Correia MP, Daul F, Rennert C, et al. NK-cell responses are biased towards CD16-mediated effector functions in chronic hepatitis B virus infection. J Hepatol 2019;70:351–360.
[89] Hendricks DW, Balfour Jr HH, Dunmire SK, Schmeling DO, Hogquist KA, Lanier LL. Cutting edge: NKG2C(hi)CD57+ NK cells respond specifically to acute infection with cytomegalovirus and not Epstein-Barr virus. J Immunol 2014;192:4492–4496.
[90] Gonzalez-Sanchez E, Vaquero J, Fouassier L, Chignard N. E-cadherin, guardian of liver physiology. Clin Res Hepatol Gastroenterol 2015;39:3–6.
[91] Pembroke T, Christian A, Jones E, Hills RK, Wang EC, Gallimore AM, et al. The paradox of NKp46+ natural killer cells: drivers of severe hepatitis C virus-induced pathology but in-vivo resistance to interferon alpha treatment. Gut 2014;63:515–524.
[92] Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, Bushkin Y, et al. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature 2001;409:1055–1060.
[93] Li W, Jiang Y, Wang X, Jin J, Qi Y, Chi X, et al. Natural killer p46 controls hepatitis B virus replication and modulates liver inflammation. PLoS ONE 2015;10 e0135874.
[94] Yoshioka T, Tatsumi T, Miyagi T, Mukai K, Nishio K, Nishio A, et al. Frequency and role of NKp46 and NKG2A in hepatitis B virus infection. PLoS ONE 2017;12 e0174103.
[95] Shiratori Y, Imazeki F, Moriyama M, Yano M, Arakawa Y, Yokosuka O, et al. Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. Ann Intern Med 2000;132:517–524.
[96] Guerret S, Desmouliere A, Chossegros P, Costa AM, Badid C, Trepo C, et al. Long-term administration of interferon-alpha in non-responder patients with chronic hepatitis C: follow-up of liver fibrosis over 5 years. J Viral Hepat 1999;6:125–133.