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Abstract

$CD8^+$ T cells represent a sizable component of the liver inflammatory infiltrate in chronic hepatitis C and are thought to contribute to immune-mediated tissue injury. Since chronic stimulation may promote the expression by $CD8^+$ T cells of distinct human leukocyte antigen (HLA) Class I specific, Natural Killer cells receptors (NKRs) susceptible to both inhibit effector functions and promote cell survival, we examined the distribution and characteristics of $CD8^+$ T cells with such receptors in chronic hepatitis C patients. NKRs $CD8^+$ T cells were detectable in liver and peripheral blood from HCV-infected patients but were not major subsets. However, the frequency of $NKG2A^+CD8^+$ in liver and in a lesser extent in peripheral blood was positively correlated to histological activity in HCV-infected patients. No such correlation was found with KIR^+ T cells in liver in HCV-infected patients and with the both NKR CD8+ T cells in hepatitis B virus (HBV)- infected patients. Circulating $CD8^+$ T cells expressing killer cell immunoglobulin-like receptors (KIRs) exhibited phenotypic features of memory T cells but with exacerbated expression of the senescence marker CD57 in patients. $NKG2A^+CD8^+$ T cells were committed T cells that appeared less differentiated than $KIRs^+CD8^+$ T cells. In HCV-infected patients their content in perforin was low and similar to that observed in $NKG2A^-CD8^+$ T cells, a scenario not observed in healthy subjects and HBV-infected patients. Both NKG2A and KIRs could inhibit HCV-specific $CD8^+$ T cells response *ex vivo*. In conclusion, these results support the concept that accumulation in liver parenchyma of NKR^+CD8^+ T cells that have functional alterations could be responsible for liver lesions. They provide novel insights into the complexity of liver infiltrating $CD8^+$ T cells in chronic hepatitis C and reveal that distinct subsets of antigen-experienced $CD8^+$ T cells are differentially sensitive to the pervasive influence of HCV

Introduction

Hepatitis C is a liver-targeting infectious disease caused by a non cytopathic RNA virus (HCV). If HCV is not rapidly cleared by the immune system, the infection becomes chronic, causes inflammation, mononuclear cell infiltration, hepatocytes destruction, progression to fibrosis, can lead to cirrhosis and in some instance, to hepatocellular carcinoma (1, 2). While vigorous HCV specific T cell responses can allow for virus clearance, HCV persists in a majority of cases (3-6). Among liver lymphocytes of chronic hepatitis C (cHC) patients are many activated $CD8^+$ T cells (7). Such a population comprises very few T cells reactive to HCV because HCV-specific $CD8^+$ T cells are difficult to detect within the persistently infected liver (8-10). HCV-specific $CD8^+$ T cells differ from other virus-specific $CD8^+$ T cells by their incomplete differentiation status. For instance, many of them retain expression of CD27 and CD28 costimulatory molecules that are missing on fully differentiated cytomegalovirus (CMV)-specific $CD8^+$ T cells (11). Our knowledge of $CD8^+$ T cells in cHC has thus progressed. Nevertheless, some features remain poorly explored. In particular, some Natural Killer cells receptors (NKRs) that bind human leukocyte antigen class I (HLA-I) can be expressed by $CD8^+$ T cells and greatly influence their properties. Although the expression of some NKRs has been examined in some chronic viral infections, including Epstein-Barr virus (EBV), CMV and human immunodeficiency virus (HIV) infections (12-15) less is known with respect to NKRs expression by $CD8^+$ T cells in cHC.

Depending on the nature of the ligand binding domain, HLA-I reactive NKRs can be subdivided in two major groups. The first group corresponds to molecules whose ectodomain belongs to the C-type lectin domain family such as NKG2A that associates with CD94 to form inhibitory heterodimers. The second group corresponds to molecules whose ectodomain consists of immunoglobulin (Ig)-like domains. It includes KIRs (killer cell Ig-like receptors) such as CD158 molecules, and LIR1/ILT2 (leukocyte Ig-like receptor 1/Ig-like transcript 2). While CD94:NKG2 heterodimers react to the non classical HLA-I molecule HLA-E, KIR family members bind to polymorphic residues of classical HLA-I molecules. As to LIR1/ILT2, it reacts to a monomorphic portion of classical HLA-I with poor affinity and with marginal functional consequences relative to KIRs or NKG2A (16-20).

KIRs are absent on naïve $CD8^+$ T lymphocytes but they can be surface expressed on $CD8^+$ T cells harboring memory cell-associated phenotypic features. In most cases, they correspond to KIRs with high affinity for their HLA-I ligands and that can negatively regulate effector functions (inhibitory KIRs) (19). KIRs expression by $CD8^+$ T cells, reflecting prior TCR engagement, is stable and remains unaltered in culture (21, 22). With respect to the

inhibitory heterodimer CD94:NKG2A, its surface expression on $CD8^+$ T cells depends on TCR engagement and is observed on T cells with memory/effector phenotype (23). Various observations support the notion that HLA-I reactive inhibitory NKRs expressed by $CD8^+$ T cells can impair their effector functions (13, 14, 24-26). In addition, KIRs and NKG2A expression can impact on $CD8^+$ T cell persistence via modulation of their susceptibility to activation-induced cell death (AICD) and possibly, promote their accumulation (19).

In cHC patients, initiation and progression of liver damages, which are characterized by portal lymphoid infiltration and degenerative lobular lesions, are essentially driven by the host immune response (3). $CD8^+$ T cells are abundant within the diseased liver, both in the lobular and periportal areas (7, 27) and can be cytotoxic for HCV-infected as well as non-infected liver cells through various mechanisms (9, 28, 29). Given these characteristics and because HLA-I specific NKRs can be expressed by committed $CD8^+$ T cells, $NKRs^+CD8^+$ T cells could contribute to the progression toward chronicity both by impairing the function of HCV specific $CD8^+$ T cells and by promoting the accumulation of activated $CD8^+$ T cells at large within the liver. We therefore examined the expression of NKG2A (Z199 antibody) as well as of CD158a/h and CD158b/j KIRs members (EB6 and GL183 antibodies) by intrahepatic and circulating $CD8^{bright}$ T lymphocytes from patients with characterized HCV chronic infection compared to healthy controls and HBV-infected patients.

Patients and Methods

Patients.

Thirty four chronic HCV-infected patients seen in our clinical department for a liver biopsy from January to December 2005 were included in the study (**Table 1**). Each patient had serologic markers of HCV infection and presence of serum HCV RNA. Criteria for exclusion were age under 18 years, presence of serum HbsAg or anti-HIV antibodies, other causes of chronic liver disease, alcohol consumption higher than 30 g/day, and current or past immunomodulatory or antiviral treatment. Serum alanine aminotransferase (ALT) levels were determined at the time of the liver biopsy and were expressed as multiple of the upper limit of the normal. Anti-HCV antibodies were tested by third-generation ELISA (Ortho Diagnostic Systems, Raritan, NJ). Serum HCV-RNA titer was measured by RT-Q-PCR (Monitor 2.0 HCV, Roche Diagnostic Systems, Meylan, France) at the time of the liver biopsy. HCV genotype was determined by direct sequencing (Trugene HCV 5'NC; Visible Genetics Inc., Toronto, Canada). Patients underwent percutaneous liver biopsy (16-gauge hepafix needle) as part of their diagnostic evaluation. Blood samples from patients were collected at the same time of the biopsy in heparinized vacutainer tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by using Ficoll-hypaque density gradient separation. Two control groups were studied for PBMCs experiments : 13 chronically mono HBV-infected patients seen in the same clinical department, and 22 healthy volunteers obtained from our blood bank (Etablissement Français du Sang, La Tronche, France). The study was in agreement with the local ethics committee recommendations and all patients gave a written informed consent.

Liver samples and Immunohistological analysis.

Liver biopsies were processed for histopathological examination by a single independent pathologist (NS) using the METAVIR scoring system. Each biopsy was cut in five micro meters thick cryostat sections, dried and fixed in formol. Immunostaining was performed using an indirect streptavidin biotin peroxydase method with an anti-CD8 antibody (C8/144B clone, Dako). For cell suspension analysis, fresh fragments collected in RPMI 1640 10 % of rhesus AB human serum were washed extensively to minimize blood cells contamination and disrupted mechanically into small pieces in complete media. Cell suspensions were prepared in complete media by using a glass mortar. Cells were washed and processed immediately for immunoanalysis.

Bulk expansion of intrahepatic lymphocytes.

Infiltrating T lymphocytes were isolated from fresh liver tissue and expanded as we described elsewhere (30). Briefly, cells were expanded polyclonally (unprimed, bulk-expanded) in the presence of irradiated (30 Gy) autologous PBMCs (10^5 cells/well), recombinant interleukin(IL)-2 (100 IU/ml) and phytohemaglutinin A (PHA) (2 μg/ml) in 96 well plates (10^5 cells/well) for 2 weeks in RPMI 1640 media containing 10% AB human serum, 1 mM glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 50 μg/ml penicillin/streptomycin (Gibco Life Sciences, Rockville, MD) at 37°C in 5% CO_2 atmosphere. Half of the media was replaced twice a week and cells were splitted at confluence. Expanding cells were maintained for 2-3 weeks without restimulation with addition of media and IL-2. Bulk-expanded lymphocytes were used directly or cryopreserved.

Antibodies and multimers

The antibodies used were anti-HLA-A2-Fluorescein Isothiocyanate (FITC) (clone BB7.2), anti-CD3-R-Phycoerythrin-Cyanine 5 (PE)Cy5 (UCHT1), anti-CD8α-FITC (SK1), anti-CD28-FITC (CD28.2), anti-CD27-FITC (M-T271), anti-CD45RA-FITC (HI100), anti-CD45RO-FITC (UCHL1), anti-CD57-FITC (NK-1), anti-CD85j/LIR1-PE /FITC (GHI/75) and anti-Perforin-FITC (δG9) from Becton-Dickinson Biosciences (Le Pont-de-Claix, France). Anti-CD8α-Allophycocyanin (APC) (B9.11), anti-CD158a,h purified or PE/APC-coupled (EB6), anti-CD158b1,b2,j purified or PE/APC-coupled (GL183), anti-NKG2A/CD159A purified or PE-coupled (Z199), anti-CD94-PE (HP3B1) were obtained from Beckman Coulter France (Villepinte, France). Anti-NKG2A-APC (131411) and purified anti-CD158b2/KIR2DL3 (180701) was from R&D Systems (Lille, France). Anti-CD62L-FITC (FMC46) was from Serotec (Oxford, UK). $F(ab')_2$ of goat anti-mouse (GAM) IgG-FITC was from Jackson ImmunoResearch (West Grove, PA). Soluble R-PE-labeled HCV peptide:HLA-A*0201 multimers containing the non-structural $(NS)3_{1073-1081}$, $NS3_{1406-1415}$ and $NS5_{2594-2602}$ peptides were obtained from Proimmune Limited (Oxford, UK). Staining was performed according to the manufacturer instructions.

Immunostaining and Flow Cytometry.

Fresh PBMCs/intrahepatic samples (0.2 to 1 x 10^6 cells) were incubated with a saturating amount of appropriate labeled antibodies in phosphate-buffered saline (PBS) (30 min, 4°C).

Cells were washed twice and analyzed immediately. For indirect staining, cells first were incubated with unconjugated primary mAb, followed by a $F(ab')_2$ fragment of goat GAM-FITC. For intracellular staining, cells were first surface stained, washed and subjected to fixation/permeabilisation (Cytofix/Cytoperm kit, BD PharMingen), prior to intracellular staining according to the manufacturer instructions. Multi-parameter flow cytometric analysis was performed on a FACSCalibur instrument with the CellQuest software (Becton Dickinson, Mountain View, CA). Nonviable cells were excluded by electronic gating or propidium iodide. Depending on the conditions, 20.000 to 200.000 gated events were acquired.

Functional assay.

For stimulation assay of T cells from HLA-A*0201$^+$ patients, cells used were total PBMCs or unprimed, bulk-expanded, iHLs plus autologous, T-depleted PBMCs. Cells were cultured for 16-18hrs with a mixture of HCV peptides (3 μg/ml) that bind to HLA-A2 in the presence of anti-KIRs (EB6 + GL183), anti-NKG2A (Z199) or an isotype-matched (IgG1), irrelevant antibodies (5 μg/ml). Cultures were set up in duplicates in a final volume of 200 μl of complete media. The peptides used were : Core35-44, 90-98, 131-140, 156-165 et 177-185 et NS31073-1081, 1131-1139, 1169-1177, 1284-1298, 1406-1415, 1443-1451 et 1585-1593. Supernatants were harvested for detection of TNF-α by ELISA (OptEIA ELISA kit, BD Biosciences).

Statistical analysis

Comparisons between group of patients and controls were performed using the nonparametric Mann-Whitney U-test and Kruskall-Wallis tests. Within-group comparisons were evaluated using the Wilcoxon signed rank's test. Bivariate correlations were done by computing a Spearman's correlation coefficient. All comparisons were 2-tailed. When multiple comparisons were done on a given experiment the p value was corrected by multiplying by the number of comparisons. P values less than 0.05 were considered significant. The statistical analysis was conducted using the SPSS 9.0 software (SPSS Inc., Chicago, IL).

Results

$CD8^{bright}$ T lymphocytes enrichment in damaged liver from cHC patients.

We previously observed a positive correlation between liver lesions severity and the frequency of intrahepatic $CD3^+$ lymphocytes in cHC patients (30). We also found an accumulation of CD8β transcripts in liver biopsies with high histological activity index relative to samples with milder index (31). We therefore examined here the frequency of $CD3^+CD8^{bright}$ lymphocytes (thereafter referred to as $CD8^+$ T cells) in liver biopsies from cHC patients presenting with various degrees of histological lesions. The flow cytometry analysis showed that an elevated fraction of $CD3^+CD8^+$ intrahepatic lymphocytes was associated with a more pronounced histological activity ($p < 0.04$) (**Fig. 1**). This observation was corroborated by an *in situ* analysis by immunostaining showing a large number of $CD8^+$ T cells surrounding areas of lobular and periportal necrosis **(Fig. 2)**. The latter result is in line with a recent immunochemistry study that has shown a higher $CD8^+$ T cell infiltrate in liver from patients with Metavir activity superior to 1 compared to those who had mild activity (32). Taken together, these results strongly support the concept that in cHC patients, the progression from mild to marked histological lesions is associated with an increase in the size of the liver $CD8^+$ T cell pool.

Ex vivo detection of $CD8^+$ T cells expressing HLA-I specific NKRs

Because $NKRs^+CD8^+$ T cells are antigen-experienced cells whose presence might be associated to chronic stimulation (12, 33), we investigated whether substantial fractions of intrahepatic $CD8^+$ T cells from cHC patients could express CD158a/h and CD158b/j KIR as well as NKG2A and CD94 receptors. The data in **Fig. 3** indicate that both PBMCs and IHL from cHC patients do comprise $CD8^+$ T cells with surface expression of NKG2A, CD94 or CD158a/h-CD158b/j KIR molecules. These populations were not major subsets among intrahepatic T cells, with frequencies in liver ranging on average from 4% for CD158a to 12% for CD94 **(Table 2)**. Interestingly, the percentage of $NKRs^+CD8^+$ T visualized in liver was significantly correlated to that observed in blood. The strongest correlations were observed for NKG2A ($r=0.72, p<0.006$) and CD94 ($r=0.82$, $p<0.001$) **(Fig. 4)**. For each NKR^+CD8^+ T cell subset there was a trend toward an enrichment in liver compared to blood that was significant only for CD158b (11 *vs* 4%, $p<0.01$). Finally, we compared circulating $NKRs^+CD8^+$ T cells frequencies observed in cHC patients to both cHB patients and healthy controls and we observed no significant differences between the three groups **(Table 2).**

Increased liver injury correlates with high frequency of NKG2A$^+$CD8$^+$ T lymphocytes.
Since KIRs and NKG2A expression by CD8$^+$ T cells reflect prior TCR-mediated activation in vivo (19, 33), we searched for possible correlation between the frequency of NKRs$^+$CD8$^+$ T cells and disease severity. We found a significant correlation between histological activity and frequency of intrahepatic NKG2A$^+$CD8$^+$ T cells (r = 0.645, p<0.02), and in a lesser extent with circulating peripheral NKG2A$^+$CD8$^+$ T cells (r=0.41, p<0.05). By contrast, the frequency of liver CD94$^+$CD8$^+$ T cells seemed to negatively correlate with lesion severity but this trend failed to reach statistical significance (r = -0.543, p = 0.055). No correlation was found with the fibrosis stage. Correlations between the frequency of KIR$^+$CD8$^+$ T cells and biochemical and virological characteristics were not significant. The correlation between circulating NKG2A$^+$CD8$^+$ T cells and histological activity was not observed in the cHB group (r=0.04, p=0.82). Thus, the salient feature of this analysis is that in cHC, but not in cHB, patients, an increase percentage of NKG2A$^+$CD8$^+$ T cells subset is associated to an enhanced histological activity.

NKG2A or KIRs expression by intrahepatic HCV-specific CD8$^+$ T cells in cHC patients.
To determine whether NKR expression could be detected among HCV-specific liver CD8$^+$ T cells, we performed costaining with HCV-peptide/HLA-class I multimers reagents. Although the frequency of CD8$^+$ T cells specific for a given peptide/HLA-A2 complex may be higher in the liver than in the blood (34, 35), it usually remains very low. For this reason, we used a cocktail of three multimer reagents made of HLA-A*0201 molecules complexed to the $NS3_{1073\text{-}1081}$, $NS3_{1406\text{-}1415}$ and $NS5_{2594\text{-}2602}$ HCV epitopes. **Fig. 5A** shows that it is possible to detect very low fractions of HCV-specific CD8$^+$ T cells expressing NKG2A or KIRs receptors among unprimed, bulk-expanded, intrahepatic lymphocytes. Both NKG2A$^+$ and KIRs$^+$ HCV-specific CD8$^+$ T cell frequencies were lower than that of HCV-specific cells expressing LIR1/ILT2 molecules. We included the LIR1/ILT2 control since in other infectious diseases these molecules can be expressed by higher fractions of virus-specific CD8$^+$ T cells than KIRs (19). Although a limited number of epitopes were studied here, the data indicate that liver CD8$^+$ T cells from cHC patients can comprise fractions of NKRs$^+$ virus-specific cells. Since KIRs expression is a feature of antigen-experienced, terminally differentiated, CD8$^+$ T cells (12), the data indicates that some HCV specific CD8$^+$ T cells, albeit at low frequency, do undergo extensive differentiation during cHC.

NKG2A and KIRs modulate the reactivity of HCV-specific CD8$^+$ T cells from cHC patients.

To determine whether NKG2A and KIRs expression is susceptible to impact on the reactivity of HCV specific CD8$^+$ T cells, we stimulated PBMCs from HLA-A2$^+$ patients with various HCV peptides, that bind to HLA-A2, in the presence or absence of anti- NKG2A or -KIRs Abs and measured TNF-α secretion in the supernatant. We observed that neutralizing KIRs engagement, and in a minor extend NKG2A engagement, did enhance the response of virus-specific CD8$^+$ T cells to specific stimulation *in vitro* (**Fig. 5B**). Similar results were obtained when unprimed, bulk-expanded, intrahepatic lymphocytes were used in the assay. Thus, NKRs expressed by HCV-specific CD8$^+$ T cells from patients are functional and are capable of interfering with their specific response *in vitro*.

Phenotypic features of NKRs$^+$CD8$^+$ T cells

To study the differentiation status of NKRs$^+$CD8$^+$ T cells, we examined their phenotype. Due to the limited CD8$^+$ T cell number that can be recovered from biopsies, we used PBMCs (**Table III**). In all groups, most KIRs(CD158a/h plus CD158b/j)$^+$CD8$^+$ T cells expressed CD45RA and only few expressed CD45RO. Concerning the replicative senescence marker CD57, we observed that, while the frequency of positive cells was comparable between KIRs$^+$CD8$^+$ T cells from patients and controls, there was a marked increase in the CD57 expression level on CD57$^+$KIRs$^+$CD8$^+$ T cells from cHC patients as revealed by the mean fluorescence intensity (MFI)(**Fig. 6A, B**). This effect was not observed in the case of cHB patients. KIR$^+$CD8$^+$ T cells from all cohorts comprised a minority of cells positive for CD27 and CD28 relative to their KIR$^-$ counterparts with however, a trend toward a further reduced CD28$^+$ cell frequency in HCV$^+$ patients (**Fig. 6C**), that was also detected for cHB patients. With respect to NKG2A$^+$CD8$^+$ T cells, the high proportions of CD45RO$^+$, CD27$^+$ and CD28$^+$ cells seen in controls were virtually unchanged in cHC patients. As to CD57, the proportion of positive cells among NKG2A$^+$CD8$^+$ T cells was lower than among KIR$^+$CD8$^+$ T cells and there was no change in expression level (not shown). Overall, the analysis of CD8$^+$ T cells expressing either KIRs or NKG2A indicates that both subsets display characteristics of effector/memory CD8$^+$ T cells. NKG2A$^+$CD8$^+$ T cells appeared less differentiated than KIRs$^+$CD8$^+$ T cells. Finally, the phenotype of distinct NKRs$^+$CD8$^+$ subsets can show subtle distinctions between cHC patients and controls.

Scarcity of perforin$^+$ cells among blood NKG2A$^+$CD8$^+$ T cells from cHC patients.

Because perforin is central to granule exocytosis-dependent cytotoxicity, the lytic potential of NKRs$^+$CD8$^+$ T cells was estimated by studying the perforin cellular content. Intracellular stainings in controls showed that blood KIRs$^+$CD8$^+$ and NKG2A$^+$CD8$^+$ T cells were both enriched in perforin$^+$ cells relative to NKR$^-$CD8$^+$ T cells (**Fig. 7A**). However, while this enrichment was fairly stable among KIRs$^+$CD8$^+$ T cells, the proportion of perforin$^+$ cells among NKG2A$^+$CD8$^+$ T lymphocytes was reduced in cHC patients (**Fig. 7B**). This difference is reminiscent of the reduced frequency of perforin$^+$CD8$^+$ T cells previously noticed in HCV$^+$ patients (36) and was not noticed in the case of HBV$^+$ patients (**Fig. 7B**). Thus, during cHC, it might exist a differential modulation of perforin expression among subsets of CD8$^+$ T cells expressing distinct NKRs.

Discussion

Conventional $CD8^+$ T lymphocytes are largely suspected to be involved in the pathogenesis of chronic hepatitis C (7, 27, 30-32, 37, 38). The first finding of our study supports this concept by unraveling a significant correlation between frequencies of intra-hepatic $CD8^{bright}$ T cells and histological activity and by showing *in situ* CD8+ immunostainings in close contact to lobular and periportal hepatocyte necrosis. It has been reported in various pathologies that KIRs and NKG2A receptors could be expressed on antigen-experienced $CD8^+$ T cells under conditions of chronic stimulation/inflammation and impact on their functions and/or persistence (12, 33). We have then examined $CD8^{bright}$ T cells expressing these markers in liver and blood of cHC patients in terms of frequencies, functionalities and association to liver pathogenesis. While there was no overt accumulation of $KIRs^+CD8^+$ or $NKG2A^+CD8^+$ T cells in patients, our results indicate that within the liver, the frequency of $NKG2A^+CD8^+$, but not $KIRs^+CD8^+$ T cells correlates with lesion severity. Possibly, the increased frequency of $NKG2A^+CD8^+$ T cells we found in liver samples with marked lesion severity could relate to the fact that the liver can retain committed $CD8^+$ T cells (39-41), a phenomenon that could be reinforced during chronic inflammation. In theory, liver $NKG2A^+CD8^+$ T cells could contribute to lesions because some of them were $perforin^+$ and there were blasting cells with a high forward light scatter (not shown). In addition, the inhibitory effect of NKG2A is not necessarily as efficient as that triggered by inhibitory KIRs at interfering with $CD8^+$ T cell lytic activity (19, 20, 25, 42). We have indeed shown that KIRs, rather than NKG2A, were effectively capable of modulating the reactivity of specific anti-HCV $CD8^+$ T cells. It is worth to note, the scarcity of HCV specific $CD8^+$ T cells we observed in liver is consistent with the estimated frequency of intra-hepatic CTLs usually reported (8-10). Therefore, the substantial deleterious role for liver of HCV reactive $NKG2A^+CD8^+$ T cells may result not only from their direct action, but would rely also on bystander phenomena. On the other hand, liver $NKG2A^+CD8^+$ T cells could be neutralized because NKG2A can, in some systems, efficiently control $CD8^+$ T cell functions (26, 43). It is known that such an inhibition greatly depends on HLA-E availability (19, 20) and in the case of hepatitis C, liver HLA-E expression appears enhanced (44) making $NKG2A^+CD8^+$ liver T cells very likely to have their cytotoxic potential constrained *in situ*. In addition, since repeated TCR engagements is necessary for high NKG2A expression (45), and because the interaction with HLA-E does not modulate NKG2A expression (46), our observation that NKG2A MFI was slightly lower on liver $NKG2A^+CD8^+$ T cells from patients than on their circulating counterparts (not shown) suggests that intrahepatic $NKG2A^+CD8^+$ T cells are not

overtly reactive *in situ*. Finally, an elevated HLA-E expression is likely to provide $NKG2A^+CD8^+$ liver T cells with anti-apoptotic signals since CD94 expression is high on $NKG2A^+CD8^+$ T cells (47-48) and high CD94 expression associates with an enhanced resistance to cell death (49). Thus, the increased frequency of $NKG2A^+CD8^+$ T cells in biopsies with marked lesions could reflect a selective survival advantage. It is possible that this association indeed reflects a combination of several factors including the retention of activated/memory $CD8^+$ T cells, an HLA-E-mediated resistance to cell death and perhaps, some level of activation/expansion. Albeit technically difficult, further experiments are hence needed to fully understand the properties of liver $NKG2A^+CD8^+$ T cells during cHC. With respect to blood cells, we detected the increased frequency of $NKG2A^+CD8^+$ T cells reported by Nattermann et al.(50) in some (nearly 20%), but not all, cHC patients. The reasons for this difference could relate to genetic factors, risk factors, and/or to differences in the virus genotype as well as in duration of infection. Taken together, the decrease of cytotoxic functions of NKG2A+ CD8+ T at single cell level could be compensated by increase survival favoring a long lasting accumulation of this population which eventually induces hepatocyte lesions.

Phenotypically, $KIRs^+CD8^+$ T cells from patients were committed cells with a memory-like phenotype resembling that of terminal effector cytotoxic $CD8^+$ T cells (19). $NKG2A^+CD8^+$ T cells were also antigen-experienced cells since there were enriched in $CD45RO^+$ cells but they were less differentiated than $KIRs^+CD8^+$ T cells because many were $CD27^+CD28^+$ cells. TCR engagement is required for NKG2A surface expression by activated $CD8^+$ T cells during infection (23). Therefore, $NKG2A^+CD8^+$ T cells from patients must have responded to TCR-mediated stimulations *in vivo*. Given that the frequency of $NKG2A^+CD8^+perforin^+$ T cells seemed reduced in patients, the data suggest that the differentiation of $NKG2A^+CD8^+$ T cells might be altered in persistent HCV infection. While the fractions of $NKG2A^+CD8^+$ and $KIRs^+CD8^+$ T cells that expressed CD57 were quite comparable between patients and controls, the CD57 expression was enhanced on $KIRs^+CD8^+$ T cells from HCV but not HBV patients. Since CD57 expression by $CD8^+$ T cells reflects a history of intense mitosis and a status of proliferative inability (51), we conclude that the majority $KIRs^+CD8^+$ T cells from cHC patients are truly terminally differentiated cells that have undergone more extensive *in vivo* division than their counterparts from controls.

Through yet unknown mechanism(s), HCV infection can influence the phenotype of antigen-specific $CD8^+$ T cells. Thus, CMV-specific $CD8^+$ T cells from HCV^+ patients are less differentiated than their counterparts from controls. This phenomenon is referred to as

pervasive influence (36). In this line, we found phenotypic distinctions between $NKRs^+CD8^+$ T cells from cHC patients and both healthy individuals and cHB patients. For example, most $KIRs^+CD8^+$ T cells from cHC patients showed signs of enhanced replicative senescence, and $perforin^+$ cells tended to be less frequent among $NKG2A^+CD8^+$ T cells. This suggests therefore that HCV pervasive influence can affect various subsets of committed $CD8^+$ T cells and that distinct such subsets can be differentially susceptible to this influence.

In summary, $CD8^+$ T lymphocytes that express either KIRs or NKG2A are T cell subsets which have undergone TCR-mediated activation *in vivo* in cHC patients. Their expression on HCV-specific $CD8^+$ T cells is rare but associated, especially for KIRs, to a clear impairment of HCV-specific reactivity. Within the inflamed liver, $CD8^+$ T cells expressing KIRs or NKG2A are variable among patients in terms of frequencies and display phenotypic and functional alterations which are not observed in controls or cHB patients. We hypothesize these changes are the consequence of the known pervasive influence of HCV that differentially affects $CD8^+$ subsets. Finally, $NKG2A^+$ $CD8^+$ T cells, whose functional impairment seems to be compensated by a survival advantage favoring accumulation in liver, appear to be a major player in HCV pathogenesis.

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Footnotes:

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List of abbreviations
HLA: human leukocyte antigen
NKRs: Natural Killer receptors
KIR: killer cell immunoglobulin-like receptors
HCV : Hepatitis C virus
HCB: Hepatitis B virus
cHC: chronic Hepatitis C
cHB: chronic Hepatitis B
IHL : Intra Hepatic Lymphocytes
CMV: cytomegalovirus
EBV: Epstein-Barr virus
HIV: human immunodeficiency virus

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Figures and Legends.

Figure 1. Increased frequency of CD8$^+$ T lymphocytes in more severely injured liver during chronic Hepatitis C.
Significant association between the fraction of intrahepatic CD3$^+$CD8^{bright} assessed flow cytometry and METAVIR activity (A1: mild; A2 : moderate, A3 severe) ($p < 0.03$, $n = 17$).

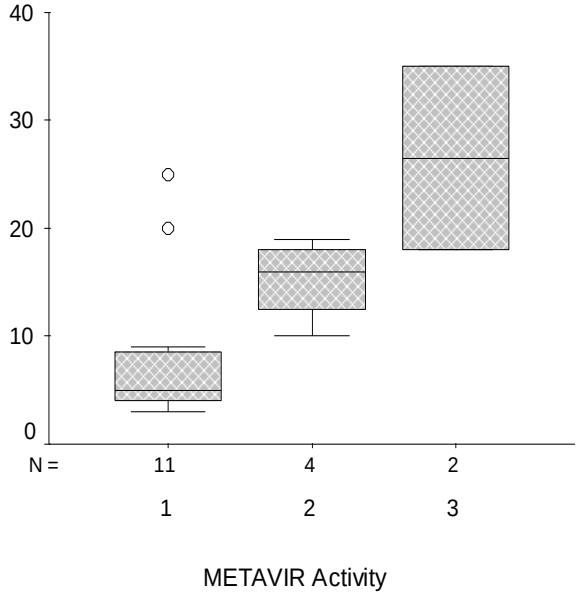


Figure 2 Immunohistology of CD8 expressing cells in liver of chronic viral hepatitis C patients.
The distribution of CD8 expressing cells in liver biopsies from patients, with low activity (A1) in panel A and B, and high activity (A3) in panel C and D, is detected by staining with anti-CD8 antibody. Few CD8 positive cells are present in fibrous septa (S) and portal tracts (PT), some of them in contact with rare foci of parcellar necrosis (arrow). Numerous CD8 positive T lymphocytes are seen in fibrous septa and portal tracts and also in lobules, in close contact with foci of lobular necrosis (asterisk) and in large areas of parcellar necrosis. Magnification is x 10 for panel A and C, and x 20 for panel B and D.

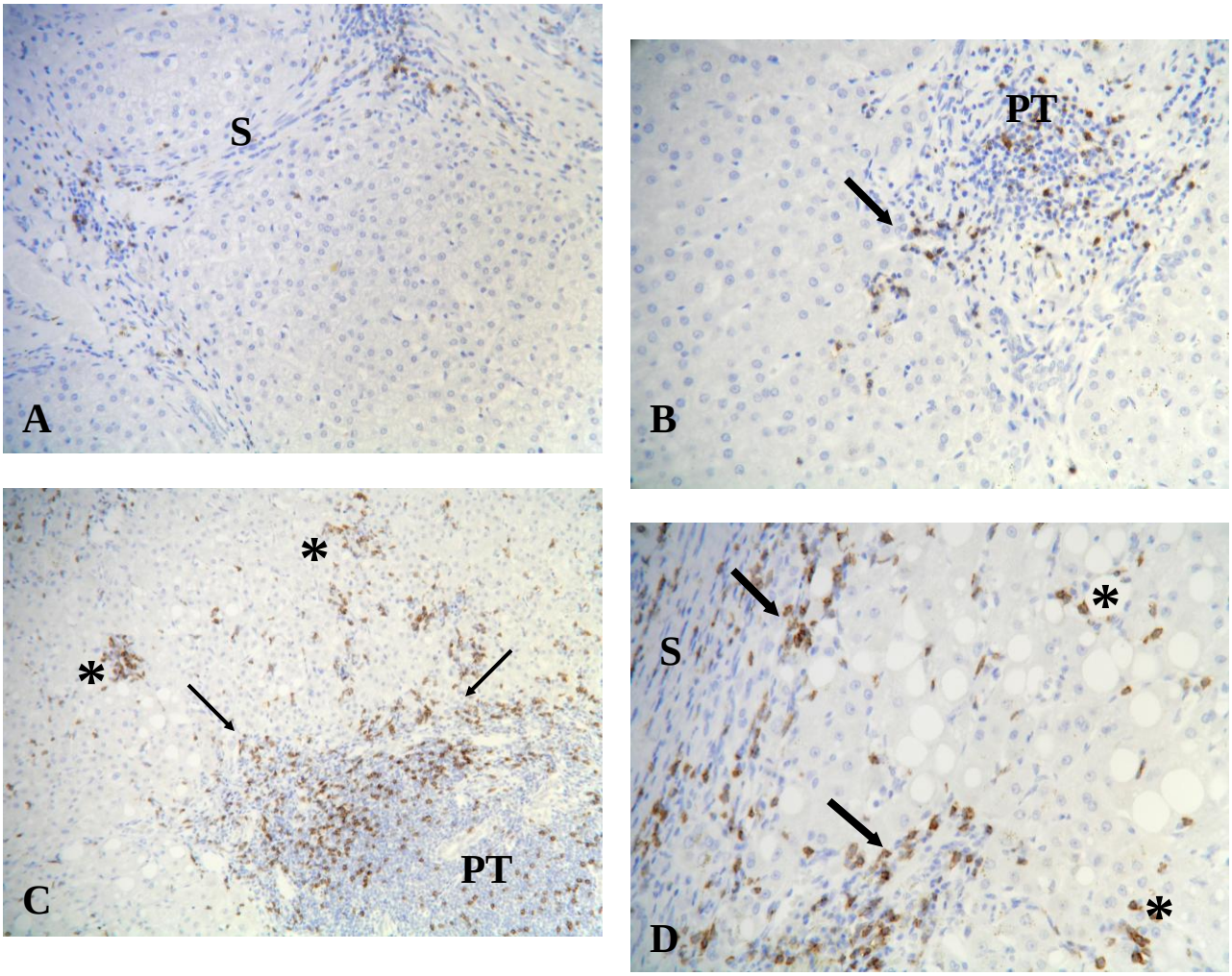


Figure 3. Presence of CD8$^+$ T cells with surface expression of HLA class I specific NKRs in peripheral blood and liver tissue from cHC patients.
The surface expression of CD158a/h, CD158b/j, NKG2A and CD94 was examined on peripheral blood cells (PBMCs) (**A**) and on intrahepatic lymphocytes (iHLs) (**B**) in patients with chronic hepatitis C by using four-color immunostaining as outlined in Methods. Representative flow cytometry data were plotted after electronic gating on CD3$^+$CD8^{bright} lymphocytes (R2 in top panels). y axis: cell count.

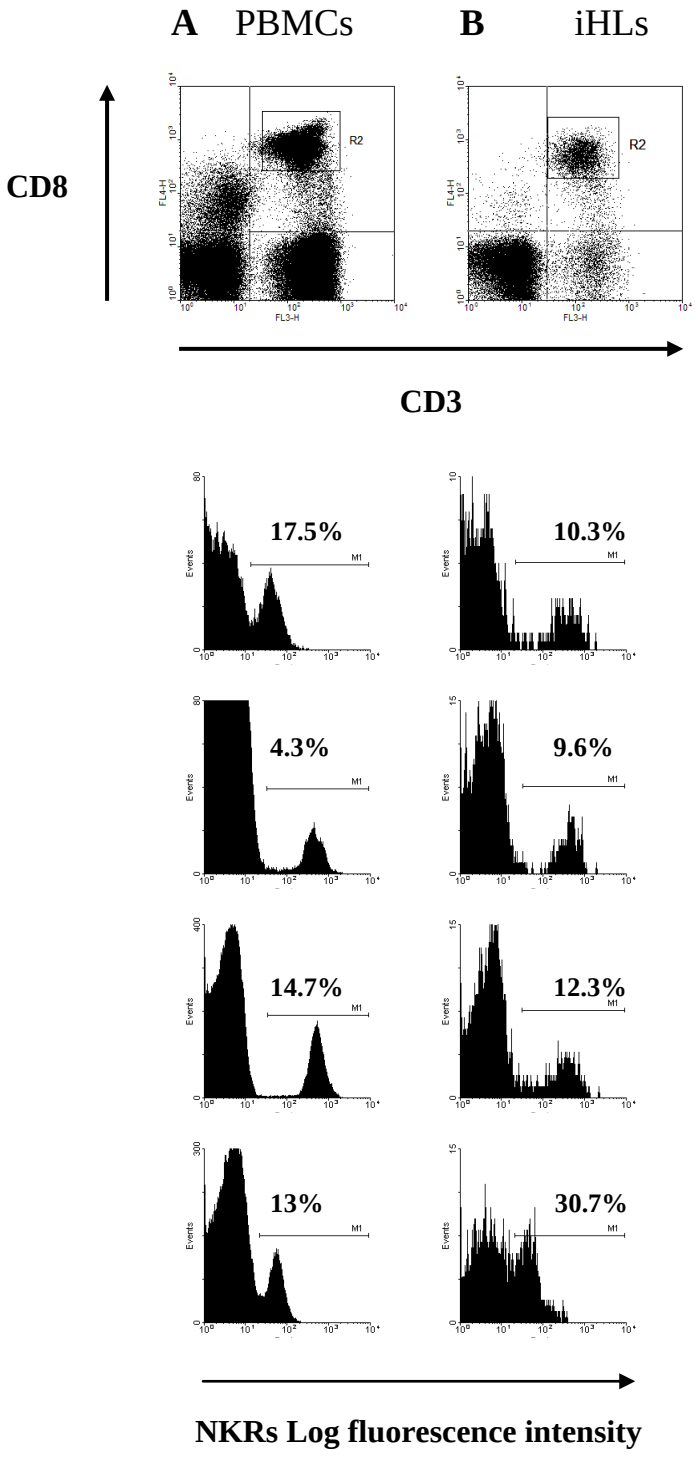
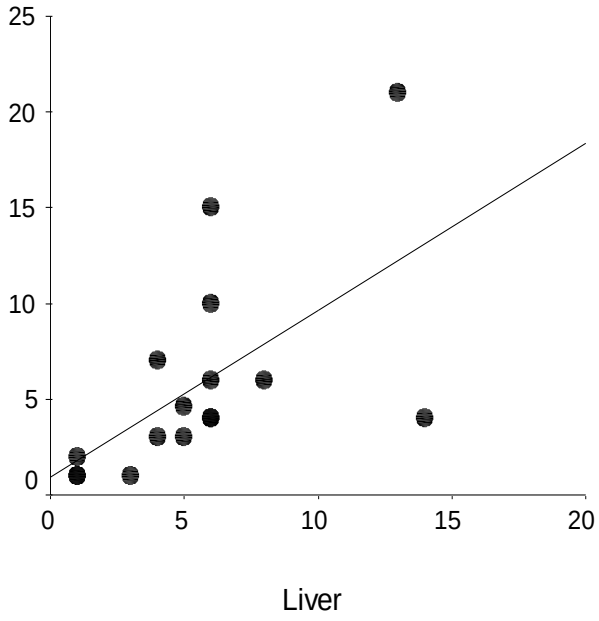


Figure 4 : Correlation between frequencies of NKG2A+ (A) and CD94+ (B) CD8+ T cells in PBMCs and liver

Frequencies (expressed as percentage) assessed in 16 patients whose IHL and PBMCs collected at the same time were available. Results are significant for NKG2A ($r=0.72, p<0.006$) and CD94 ($r=0.82, p<0.001$).

A (NKG2A)



B (CD94)

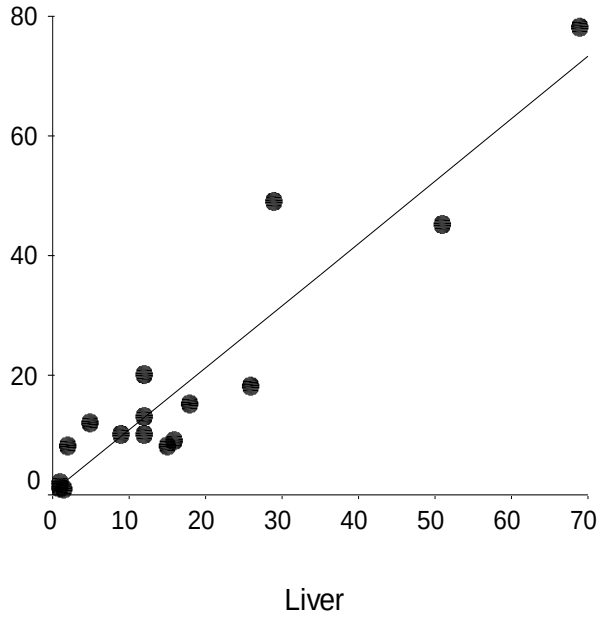
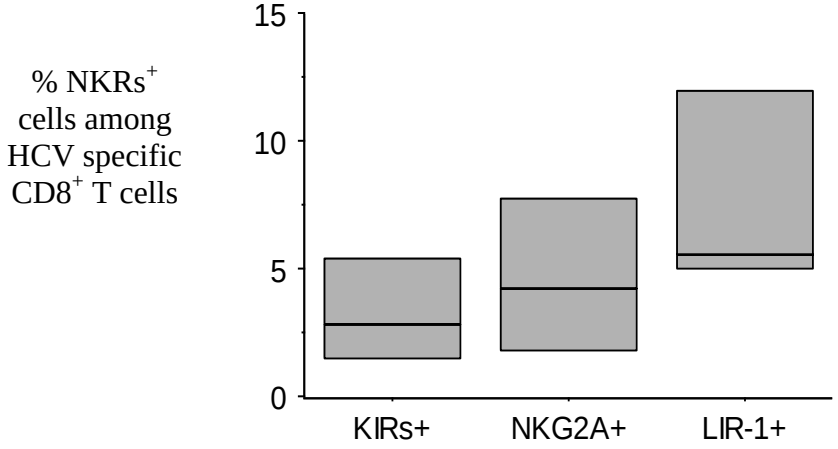


Figure 5. Expression of functional KIRs and NKG2A receptors by HCV-specific CD8$^+$ T cells from HLA-A*0201$^+$ cHC patients.

(A) HCV-specific CD8$^+$ T cells present among bulk-expanded, iHLs were examined for expression of KIRs or NKG2A. KIRs (CD158a,h,b,j) and NKG2A expression were plotted after a combination of electronic gating defining CD3$^+$CD8$^+$ T cells and HCV peptide:HLA-A*0201 Multimer$^+$ cells. The peptides bound to HLA-A*0201 were $NS3_{1073-1081}$, $NS3_{1406-1415}$ and $NS5_{2594-2602}$. The data are from four independent experiments where HCV-specific cells were detected among expanded iHLs.

(B) Modulation of HCV-specific CD8$^+$ T cell reactivity by KIRs and NKG2A. PBMCs or bulk-expanded, iHLs were stimulated with multiple HCV peptides that bind to HLA-A2 (see methods) in the presence of anti-KIRs Abs (EB6 + GL183), anti-NKG2A Ab (Z199) or isotype-matched control (Is.C., IgG1) Ab (5 μg/ml). Supernatants were harvested prior to detection of TNF-α by ELISA. The data are representative of three independent experiments.

(A)



(B)

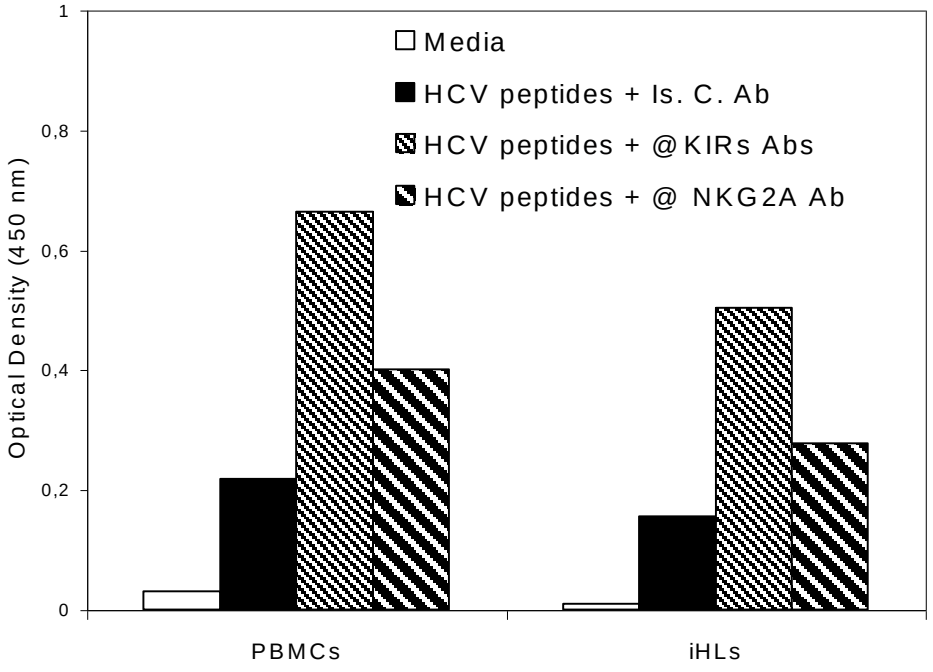
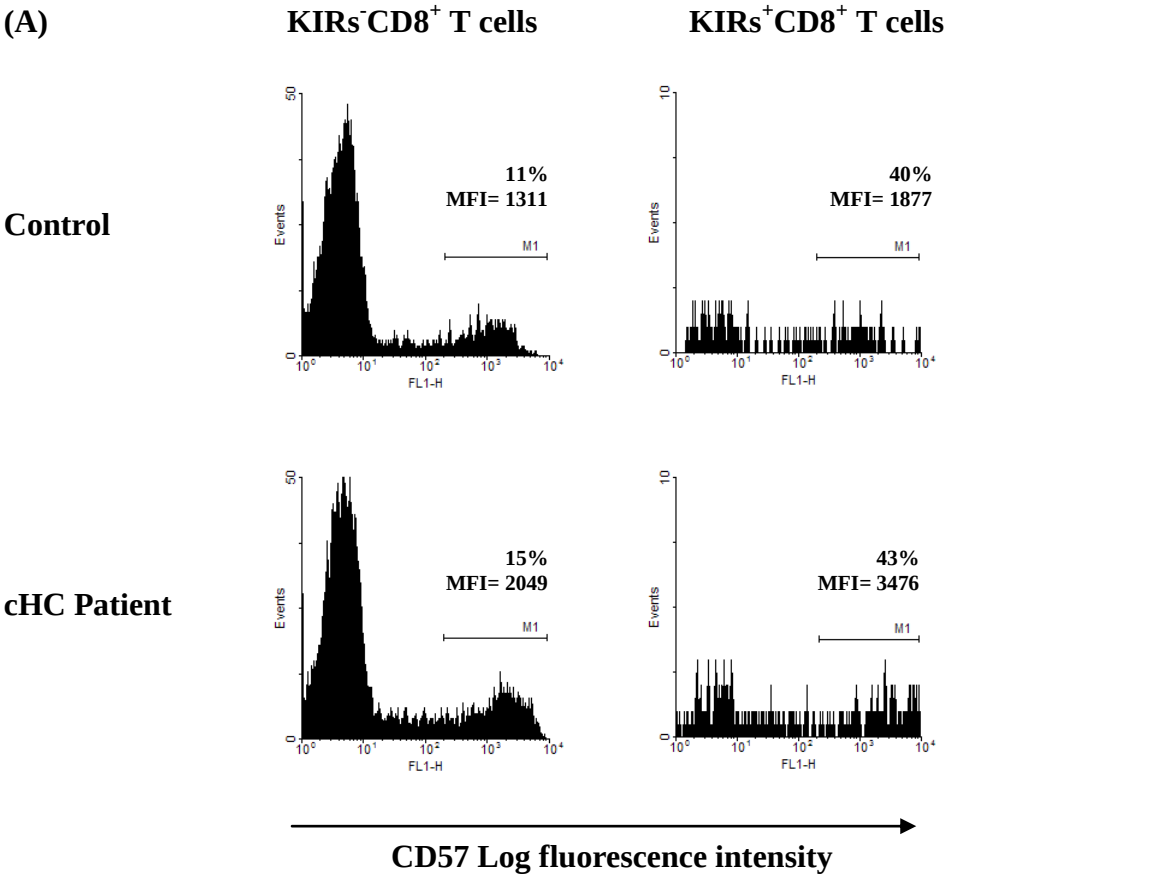


Figure 6. Phenotypic traits of KIRs$^+$CD8$^+$ T cells in chronically HCV-infected patients. **(A)** Representative profiles for CD57 expression by KIRs$^-$ and KIRs$^+$CD8$^+$ blood T cells from a control and a cHC patient. The fraction of CD57$^+$ cells is shown for both KIRs$^-$ and KIRs$^+$ cells after gating on CD3$^+$CD8$^+$ cells. The mean fluorescence intensity (MFI) is indicated. The antibodies used comprised a mixture of anti-CD158a,h and anti-CD158b,j mAbs. **(B)** The expression level of CD57 was increased in cHC patients (n = 5) relative to both controls (n = 4) (p = 0.014) and HBV$^+$ patients (n = 13) (p = 0.001). The data correspond to the ratio of CD57 MFI KIRs$^+$CD8$^+$ T cell to CD57 MFI KIRs$^-$CD8$^+$ T cells. **(C)** The frequency of blood KIR$^+$CD8$^+$ T cells expressing CD28 tended to be decreased in cHC patients (n = 7) relative to controls (n = 5) (p = 0.061) and was decreased in HVB$^+$ patients (n = 13) (p = 0.012). The KIR antibodies used comprised a mix of anti-CD158a,h and anti-CD158b,j mAbs.



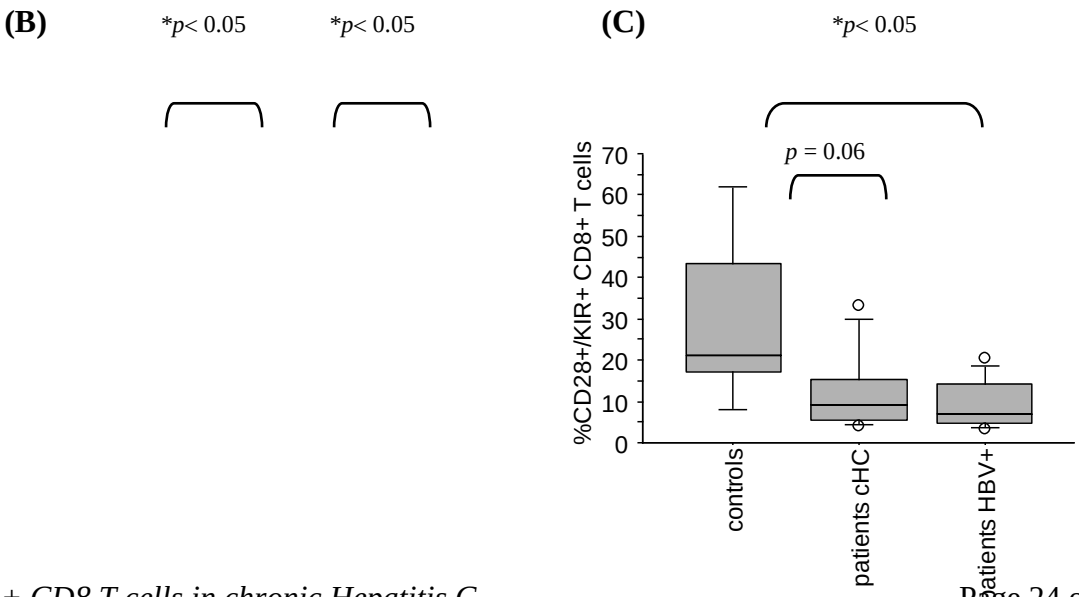
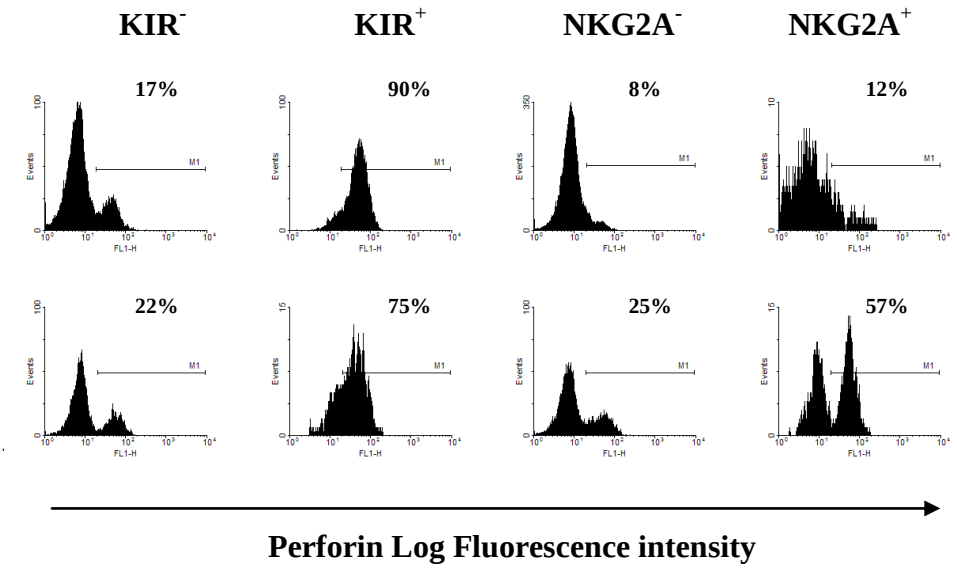


Figure 7. The perforin content of blood $CD8^+$ T cells with surface expression of NKRs in cHC patients.

(A) Representative profiles for perforin content of $NKRs^-CD8^+$ versus $NKRs^+CD8^+$ circulating T cell populations for a control and a cHC patient. The fraction of perforin positive cells is indicated for both $NKRs^-$ and $NKRs^+$ cells after gating on $CD3^+CD8^{bright}$ lymphocytes. The perforin content was examined by intracellular immunostaining as outlined in Methods. A mixture of anti-CD158a,h and anti-CD158b,j mAbs was used to assess KIRs surface expression. **(B)** The proportion of perforin positive cells among $NKG2A^+CD8^+$ T cells tended to be reduced in cHC patients (n = 5) relative to controls (n = 5) (p = 0.06) and cHB (n=13) (p=0.11).

(A)



(B)

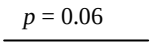


Table 1: Demographic and clinical characteristics of patients included in the study.

	HCV^+ (n = 34)	HBV^+ (n = 13)
Female *Sex**	15 (30 %)	7 (53,8 %)
*Age (years)***	46 (28 - 75)	39 (21 - 58)
*Source of infection**		
Intravenous drug abuse	13 (38 %)	0
Transfusion	10 (29 %)	0
Materno-foetal	0	3 (23%)
Other modes	4 (12 %)	5 (38%)
Unknown	7 (21 %)	5 (38%)
*Duration of infection (years)***	21 (2-42)	-
*Plasma Viral load (log IU/ml) **	6.10 (4.47 - 6.95)	3,75 (2,37 – 6,91)
*Viral genotype**		
1a	4 (12%)	
1b	13 (38 %)	
2	5 (15 %)	
3	7 (14 %)	
4	4 (12 %)	
ND	2 (6 %)	
*ALT (IU/ml)***	57 (22 - 226)	45 (20 - 132)
Liver Histology*		
Metavir activity score		
1	16 (47 %)	6 (46%)
2	13 (38 %)	5 (38%)
3	5 (15%)	2 (15%)
Metavir fibrosis score		
0	2 (6 %)	2 (15%)
1	13 (38 %)	5 (38%)
2	10 (29 %)	3 (23%)
3	3 (9 %)	2 (15%)
4	5 (15 %)	1 (8%)

* Number of cases/total (percentage %)
** Median (range)
IU/ml : international units par milliliter
ALT : alanine aminotransferase
Note that for technical reasons (number of cells available per sample), the entire set of experiments described could not be performed for every single patients.

Table 2: Frequencies of NKRs$^+$CD8$^+$ T cells in liver and blood

	CD158a$^+$	CD158b$^+$	NKG2A$^+$	CD94$^+$
HCV IHLs (n=17)	4 (1-9)	10 (2-28)	5 (1-14)	12 (1-69)
HCV Blood (n=28)	2 (1-18)	4 (1-18)	4 (1-21)	10 (1-78)
HBV Blood (n=13)	2 (1-20)	3 (1-16)	4 (2-21)	5 (2-21)
Controls (n=22)	2 (1-40)	4 (1-26)	3 (1-70)	7 (1-72)

Results expressed as percentages (median and ranges)

TABLE 3: Phenotypic features of peripheral blood CD8$^+$ T cells subsets expressing HLA-class I specific NK receptors during chronic Hepatitis C.

	within CD3+CD8+KIR+		within CD3+CD8+NKG2A+	
	Controls	Patients cHC	Controls	Patients cHC
%CD45RA+	85 (65-97)	76 (59-97)	52 (32-75)	67 (26-99)
%CD45RA^{high}	68 (58-92)	58 (45-92)	30 (18-37)	25 (18-91)
%CD45RO+	23 (2-28)	11 (7-50)	81 (71-83)	76 (27-90)
%CD27+	52 (19-69)	28 (5-66)	86 (75-91)	84 (8-97)
%CD28+	21 (8-62)	9 (4-33)	74 (52-88)	75 (23-91)
%CD62L+	56 (7-75)	19 (7-45)	36 (23-65)	16 (7-45)
%CD62L^{high}	24 (3-56)	12 (2-25)	22 (5-37)	4 (2-10)
%CD57+	64 (38-62)	56 (35-95)	28 (11-52)	29 (15-86)

	within CD3+CD8+KIR-		within CD3+CD8+NKG2A-	
	Controls	Patients cHC	Controls	Patients cHC
%CD45RA+	72 (47-85)	55 (42-68)	76 (47-84)	68 (55-92)
%CD45RA^{high}	64 (36-71)	46 (23-57)	68 (37-72)	59 (37-83)
%CD45RO+	21 (16-34)	44 (37-53)	28 (16-31)	37 (15-48)
%CD27+	90 (72-96)	81 (37-92)	92 (69-97)	79 (42-94)
%CD28+	79 (52-96)	71 (57-79)	86 (58-93)	79 (37-91)
%CD62L+	40 (26- 73)	32 (27-41)	61 (41-77)	31 (10-40)
%CD62L^{high}	11 (6-58)	11 (7-24)	51 (8-62)	9 (2-27)
%CD57+	18 (4-20)	16 (5-56)	22 (6-25)	25 (8-49)

The values represent the percentage (median (range)) of positive cells among CD8^{bright} T cells expressing or lacking KIRs or NKG2A receptors. Differences in frequency were not statistically significant.