

# NK cells from HCV-infected patients effectively induce apoptosis of activated primary human hepatic stellate cells in a TRAIL-, FasL- and NKG2D-dependent manner

Andreas Glässner, Marianne Eisenhardt, Benjamin Krämer, Christian Körner, Martin Coenen, Tilman Sauerbruch, Ulrich Spengler and Jacob Nattermann

In mouse models it has been shown that natural killer (NK) cells can attenuate liver fibrosis via killing of activated hepatic stellate cells (HSCs) in a NKG2D- and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-dependent manner. However, only little data exist regarding interactions of human NK cells with HSCs and their potential role in hepatitis C virus (HCV)-associated fibrogenesis. Therefore, purified NK cells from untreated HCV RNA(+) patients ( $n = 33$ ), interferon- $\alpha$  (IFN- $\alpha$ )-treated patients ( $n = 17$ ) and healthy controls ( $n = 18$ ) were cocultured with activated primary HSCs, and were tested for degranulation (CD107a expression) and secretion of IFN- $\gamma$  and TNF- $\alpha$ , respectively. Induction of HSC apoptosis was analyzed using an active caspase-3 assay. We found that following cocultivation with HSCs a significant increase in CD107a expression could be observed in both NK cells from HCV(+) patients and healthy controls, whereas only negligible secretion of IFN- $\gamma$  and TNF- $\alpha$  could be detected. More importantly, NK cells from untreated HCV RNA(+) patients were significantly more effective in induction of HSC apoptosis ( $17.8 \pm 9.2\%$ ) than NK cells from healthy controls ( $6.2 \pm 2.1\%$ ;  $P < 0.0001$ ). Additionally, we observed an inverse correlation of liver fibrosis stage and the ability of NK cells to induce HSC apoptosis. Induction of HSC apoptosis was contact dependent and could partly be blocked by antibodies specific for TRAIL, NKG2D and FasL, respectively. It is noteworthy that NK cells from IFN- $\alpha$ -treated HCV(+) patients displayed the highest capability to kill HSCs ( $27.6 \pm 10.5\%$ ). Accordingly, pre-stimulation of NK cells with recombinant IFN- $\alpha$  significantly increased the ability of NK cells to induce cell death in primary HSCs and was dependent on upregulated expression of TRAIL. Here we demonstrate that NK cells from HCV-infected patients are highly efficient in inducing apoptosis of activated HSCs. Thus, NK cells may have an important anti-fibrotic role in chronic hepatitis C.

*Laboratory Investigation* (2012) **92**, 967–977; doi:10.1038/labinvest.2012.54; published online 26 March 2012

**KEYWORDS:** apoptosis; hepatitis C virus; hepatic stellate cells; natural killer cell; NKG2D; TRAIL

A specific feature of the hepatitis C virus (HCV) is the capacity to establish chronic infection leading to progressive liver cell damage and fibrosis/cirrhosis.<sup>1,2</sup> Together with alcohol abuse and non-alcoholic steatohepatitis HCV-infection is considered a major cause of chronic liver disease resulting in liver fibrosis.<sup>1,3,4</sup>

Both parenchymal and non-parenchymal cells have been shown to contribute to liver fibrogenesis.<sup>2</sup> Hepatic stellate cells (HSCs) are believed to have a key role in the pathogenesis of liver fibrosis.<sup>1,3,4</sup> In healthy livers, HSCs are generally quiescent but become activated and subsequently differentiate into myofibroblastic cells that are characterized

by a loss of retinol and increased collagen expression during progressive liver injury.<sup>3–5</sup>

As HCV has been shown to replicate non-cytopathically,<sup>6</sup> immune responses have been suggested to have a central role in HCV-associated liver damage. Cytotoxic T lymphocytes (CTL) destroy HCV-infected hepatocytes via the perforin/granzyme pathway and release inflammatory cytokines such as interferon- $\gamma$  (IFN- $\gamma$ ) and TNF $\alpha$ .<sup>1,2</sup> Release of these soluble effector molecules is important to control viral infection, but may also destroy uninfected liver cells and can recruit HCV non-specific lymphocytes into the liver. These non-specific inflammatory cells can aggravate

Department of Internal Medicine I, University of Bonn, Bonn, Germany

Correspondence: Professor J Nattermann, MD, Department of Internal Medicine I, University of Bonn, Sigmund-Freud-Street 25, 53105 Bonn, Germany.

E-mail: jacob.nattermann@ukb.uni-bonn.de

Received 25 July 2011; revised 23 December 2011; accepted 23 January 2012

CTL-induced tissue damage and ultimately activate fibrogenesis.

On the other hand, there is increasing evidence that natural killer (NK) cells, a major component of intra-hepatic lymphocytes, can mediate anti-fibrotic effects.<sup>7–14</sup> In mouse models it was shown that NK cells can directly kill activated HSCs via a NKG2D-mediated mechanism, and are able to induce apoptosis of HSCs.<sup>7–14</sup> As activated HSCs are considered to critically contribute to the establishment of hepatic fibrosis via excessive production of collagen, killing of HSCs by immune cells may represent an important anti-fibrotic effect. Accordingly, in mouse studies dysregulated NK cell function has been shown to be associated with accelerated progression of liver fibrosis.<sup>8,10,12</sup>

However, it remained unclear whether human NK cells also may have a role in hepatic fibrogenesis and how HCV infection may affect interactions of NK cells with human HSCs.

## MATERIALS AND METHODS

### Patients

A total of 56 HCV-infected patients were enrolled into this study, including 33 treatment-naïve HCV RNA(+) patients with chronic infection, 17 HCV-infected patients under treatment with pegylated IFN- $\alpha$ /ribavirin and 6 HCV RNA(-) individuals who had obtained a sustained virological response under therapy.

Grading and staging of liver biopsies were performed according to the METAVIR score as part of the routine diagnostic work-up.

As a control 18 healthy donors with no history of liver disease were studied. For further information on patients characteristics see Table 1.

Informed consent was obtained from all patients. The study had been approved by the local ethics committee of the University of Bonn.

### Liver Specimens

Liver specimens were obtained from explanted livers of HCV(+) patients ( $n=3$ ). Fresh liver samples were washed twice in fresh medium and shaken gently to avoid blood contamination. After mechanical disruption, the fragments were homogenized on a cell strainer (BD Labware). To obtain intra-hepatic lymphocytes from the resulting cell suspension, density gradient centrifugation with percoll (PAA) was performed. The lymphocytes were washed and resuspended in RPMI 1640 medium. Intra-hepatic NK cells were then isolated as described in the part 'NK cell isolation'.

### Primary Human HSCs

Primary activated human HSCs obtained from ScienCell (San Diego, CA, USA) have been described before.<sup>15–17</sup> HSCs were cultured for 2–4 passages in defined Stellate Cell Medium (SteCM, ScienCell) supplemented with 2% fetal bovine serum, 5 ml stellate cell growth supplement, 10 U/ml

penicillin and 10  $\mu$ g/ml streptomycin (all ingredients obtained from ScienCell) at 37 °C with 5% CO<sub>2</sub> and cryopreserved until further use.

Two days before HSCs were used in an experiment the cells were thawed and cultured in SteCM medium. Then the cells were harvested, washed, checked for viability using Trypan blue, and then used in the respective experiments. Activated status of HSCs was verified by immunofluorescence staining of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; Figure 1a).

### NK Cell Isolation

Circulating NK cells were isolated from total PBMC by depletion of non-NK cells using 'EasySep human NK cell enrichment kit' following the manufacturer's protocol (STEMCELL Technologies, Vancouver, BC, Canada). The purity of NK cells was >95% as determined by flow cytometric analysis of cells stained with anti-CD56-APC and anti-CD3-PerCP. CD3 contamination in purified NK cells was <1%.

Purified NK cells were cultured in RPMI-medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO<sub>2</sub> over night. Purified NK cells were stimulated with recombinant human IFN- $\alpha$  (100–5000 U/ml; PBL InterferonSource, Piscataway, NJ, USA) as further indicated in the manuscript.

### CFSE Staining of HSCs

To differentiate NK cells and HSCs after coincubation, HSCs were labeled with CFSE (carboxyfluorescein succinimidyl ester; Sigma-Aldrich, St Louise, MO, USA). Sixteen hours before coincubation, HSCs were removed from cell culture flask with 0.02% EDTA solution. The cells were washed twice with 10 ml sterile PBS to remove the medium completely. Afterwards,  $1 \times 10^6$  HSCs were resuspended in 1 ml PBS with 5  $\mu$ M CFSE and were incubated for 10 min at RT. The staining reaction was stopped by adding 5 ml SteCM for 5 min at RT. After washing the cells one more time with 5 ml SteCM,  $1.5 \times 10^4$  HSC/well were seeded in a 48-well plate.

### Apoptosis Assay

Cryopreserved HSCs were thawed and cultured in SteCM at 37 °C with 5% CO<sub>2</sub> for 2 days.

Isolated NK cells and HSCs were coincubated in SteCM for 6 h at an E:T ratio of 10:1 in a 48-well plate. Then HSCs were harvested and HSC apoptosis was studied by intracellular staining of active caspase-3 using an active caspase-3 apoptosis kit (BD Bioscience, Franklin Lakes, NJ, USA). In brief, HSCs were washed with PBS, resuspended in 500  $\mu$ l BD Cytotfix/Cytoperm and incubated for 20 min at 4 °C followed by additional washing steps with 1 ml BD Perm/Wash. Then 5  $\mu$ l rabbit anti-active caspase-3-PE antibody (BD Bioscience) was added and incubated for 30 min at 4 °C. Next, the cells were washed again with 2 ml BD Perm/Wash. Finally, cells

**Table 1 Patient characteristics**

	HCV-RNA (+)	HCV under therapy	HCV-RNA(-) after therapy	Healthy controls
Number	33	17	6	18
Female sex <sup>a</sup>	10 (30.3%)	2 (11.8%)	4 (67%)	7 (38.9%)
Age (years) <sup>b</sup>	56.1 (28–87)	45.2 (28–57)	49.7 (27–77)	35.9 (28–50)
<i>Clinical data</i>				
ALT U/L <sup>b</sup>	89.1 (13–384)	42.8 (9–141)	22.3 (15–36)	NA <sup>c</sup>
AST U/L <sup>b</sup>	72.1 (11–297)	34.9 (11–121)	18.7 (12–30)	NA <sup>c</sup>
γ-GT <sup>b</sup>	105.3 (21–327)	51.7 (25–146)	29.7 (22–43)	NA <sup>c</sup>
<i>HCV status</i>				
HCV load ( × 10 <sup>5</sup> IU/ml) <sup>b</sup>	31.3 (<0.0001–400)	<0.0001	<0.0001	NA <sup>c</sup>
<i>HCV genotypes</i>				
Genotype 1 <sup>a</sup>	20 (60.6%)	10 (58.8%)	—	—
Genotype 2 <sup>a</sup>	1 (3%)	—	—	—
Genotype 3 <sup>a</sup>	1 (3%)	4 (23.5%)	—	—
Genotype 4 <sup>a</sup>	1 (3%)	—	—	—
Undetermined genotype <sup>a</sup>	10 (30.3%)	3 (17.6%)	—	—

<sup>a</sup>Number of cases (number/total in %).

<sup>b</sup>Mean (range).

<sup>c</sup>NA, not analyzed.

were resuspended in 200 μl CellFIX (BD Bioscience) analyzed via flowcytometry (BD FACS Calibur).

Alternatively, apoptosis was studied via flowcytometry by using the APO-BRDU Kit (BD Bioscience) or a cleaved PARP antibody (eBioscience; following the manufacturers' recommendations).

### Antibodies

For blocking experiments the following antibodies were used: anti-NKG2D (clone #149810; 5 μg/ml) and anti-FasL (clone #100419; 5 μg/ml) obtained from R&D Systems (Minneapolis, MN, USA), anti-TRAIL (clone 2E5; 5 μg/ml; Alexis Biochemicals; San Diego, CA, USA).

Anti-ULBP-1, 2, 3 and anti-MICA/B were acquired from R&D Systems.

### Transwell Experiments

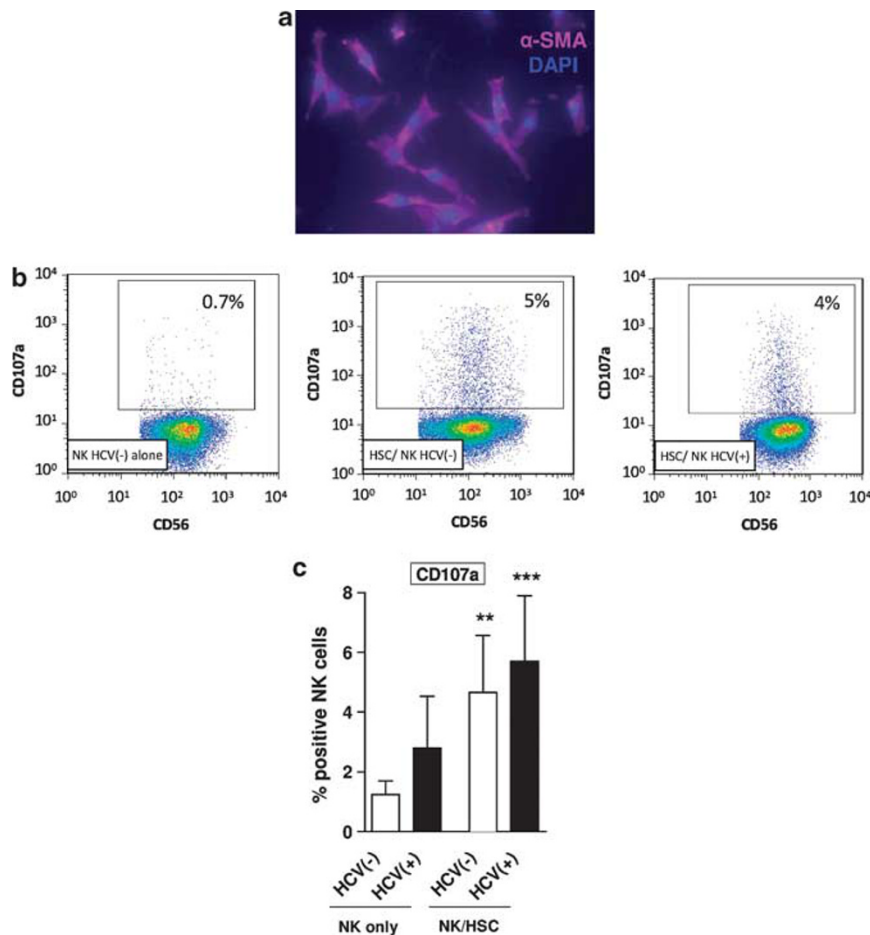
For transwell experiments plates with 0.4 μm pore diameter (Corning Life Science, Lowell, MA, USA) were used. NK cells were cocultured with HSCs on the upper chamber of the transwell. HSCs alone were seeded in the lower chamber. After 6 h HSCs were harvested from the lower chamber and analyzed by intracellular staining of active caspase-3 as described above.

### IFN-γ and TNF-α Secretion

Isolated NK cells were cocultured with HSCs (E:T ratio 1:1) in SteCM at 37 °C for 1 h. Then, brefeldin A (5 μg/ml; Sigma-Aldrich) was added for another 4 h. Next, cells were harvested and washed using PBS. Finally, cells were stained with anti-CD56-APC and anti-CD3-PerCP, fixed and permeabilized using Cytotfix/Cytoperm (BD Biosciences), followed by intracellular staining with an anti-IFN-γ-PE or TNF-α-PE mAb (R&D Systems, 1:40 dilution) and FACS analysis (BD FACS Calibur).

### CD107a Degranulation Assay

Cytotoxic activity of NK cells was assessed by a CD107a degranulation assay as described before.<sup>18</sup> In brief, purified NK cells were cocultured with HSCs at an effector:target (E:T) ratio of 1:1 in 48-well plates in SteCM in the presence of PE-conjugated CD107a mAb (R&D Systems, 1:40 dilution) at 37 °C. After 1 h GolgiStop (0.5 μl/250 μl; BD Biosciences) was added and the cells were cultured for an additional 4 h. Then, cells were stained using anti-CD56-APC and anti-CD3-PerCP, washed, and resuspended in CellFix (BD Biosciences) followed by flow cytometric analysis.



**Figure 1** Natural killer (NK) cell degranulation following coincubation with activated primary hepatic stellate cells (HSCs). **(a)** Immunofluorescence analysis of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in HSCs as a marker of HSC activation. **(b)** Example dot plots of CD107a expression on isolated NK cells from hepatitis C virus-negative (HCV(-)) and HCV(+) individuals following coincubation with HSCs. **(c)** Quantitative analysis of CD107a expression on purified NK cells from HCV(+) (black bars,  $n = 9$ ) or healthy donors (white bars,  $n = 6$ ) after interaction with HSCs. The columns indicate means and s.d.  $**P < 0.001$ ;  $***P < 0.0001$ .

### Immunohistochemistry

Slices of frozen HCV(+) liver specimens were made with a microtome (Leica Microsystems GmbH, Deutschland). The slices were dried over night, fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100. Antibody incubation was done in blocking buffer (5% donkey serum, 0.1% BSA in PBS) at 4 °C over night.

Following antibodies were used: anti- $\alpha$ -SMA (1:200, Abcam, England); anti-active-caspase-3 (1:500, Abcam); anti-mouse NL637 (1:200) and anti-rabbit NL557 (1:200; both R&D Systems).

### Statistical Analysis

Statistical analysis was done by Mann–Whitney *U*-test or paired *t*-test using GraphPad Prism 4.0 software (GraphPad, USA). Mann–Whitney *U*-test was used to compare HSC apoptosis induction through different NK cell groups. Paired *t*-tests were used in stimulation, blocking and transwell

experiments. *P*-values  $\leq 0.05$  were considered statistically significant. Data are shown as mean  $\pm$  s.d. (standard deviation).

## RESULTS

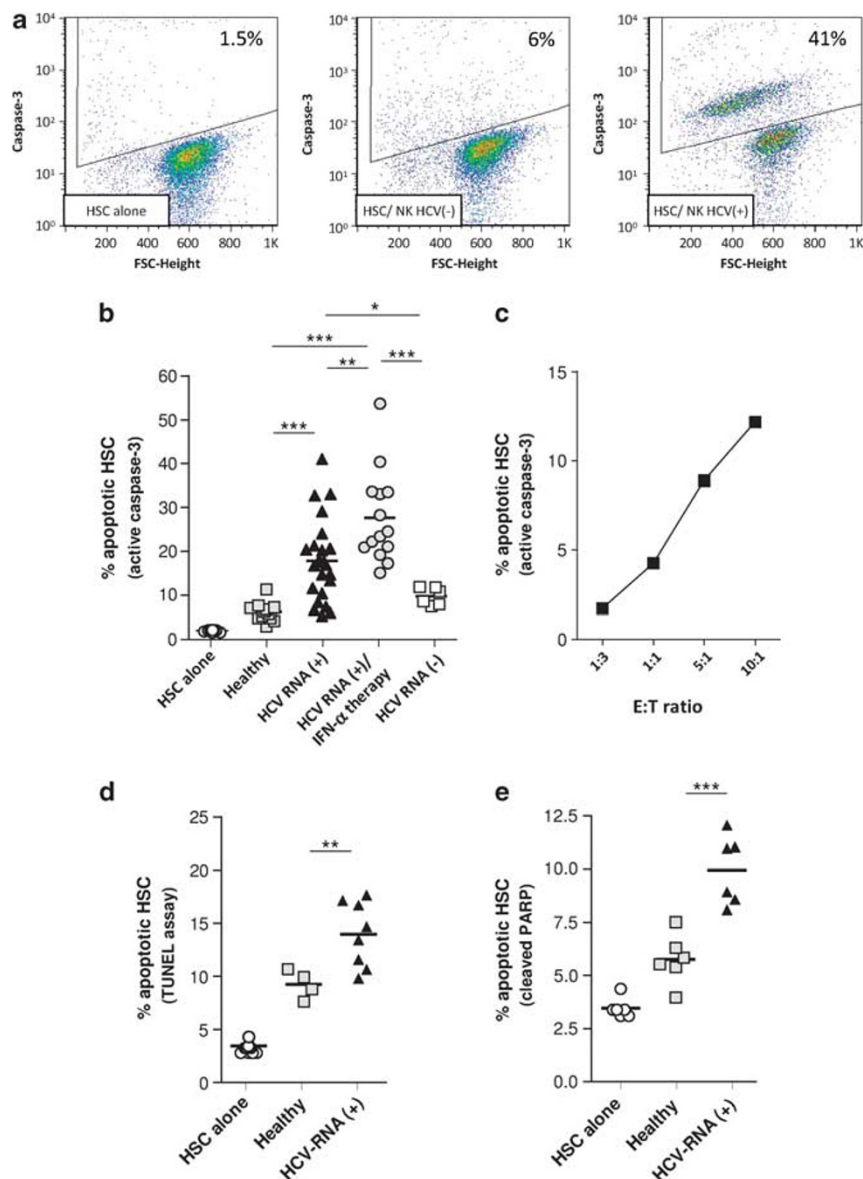
### NK Cells from Patients with Chronic Hepatitis C Induce Apoptosis in Primary Human Hepatic Stellate Cells

Following coincubation with activated primary human HSCs, NK cells from both healthy individuals and HCV RNA(+) patients showed a significant increase in CD107a expression (healthy:  $1.2 \pm 0.5\%$  vs  $4.7 \pm 1.9\%$ ;  $P = 0.005$ ; HCV RNA(+):  $2.8 \pm 1.8\%$  vs  $5.7 \pm 2.2\%$ ;  $P < 0.001$ ; Figures 1b and c), indicating activation of NK cells irrespective of HCV infection. Moreover, NK cell frequency within the lymphocyte pool did not differ significantly between HCV(+) and HCV(-) individuals ( $11.2 \pm 5.3$  vs  $15.4 \pm 8.1\%$ ;  $P = 0.08$ ).

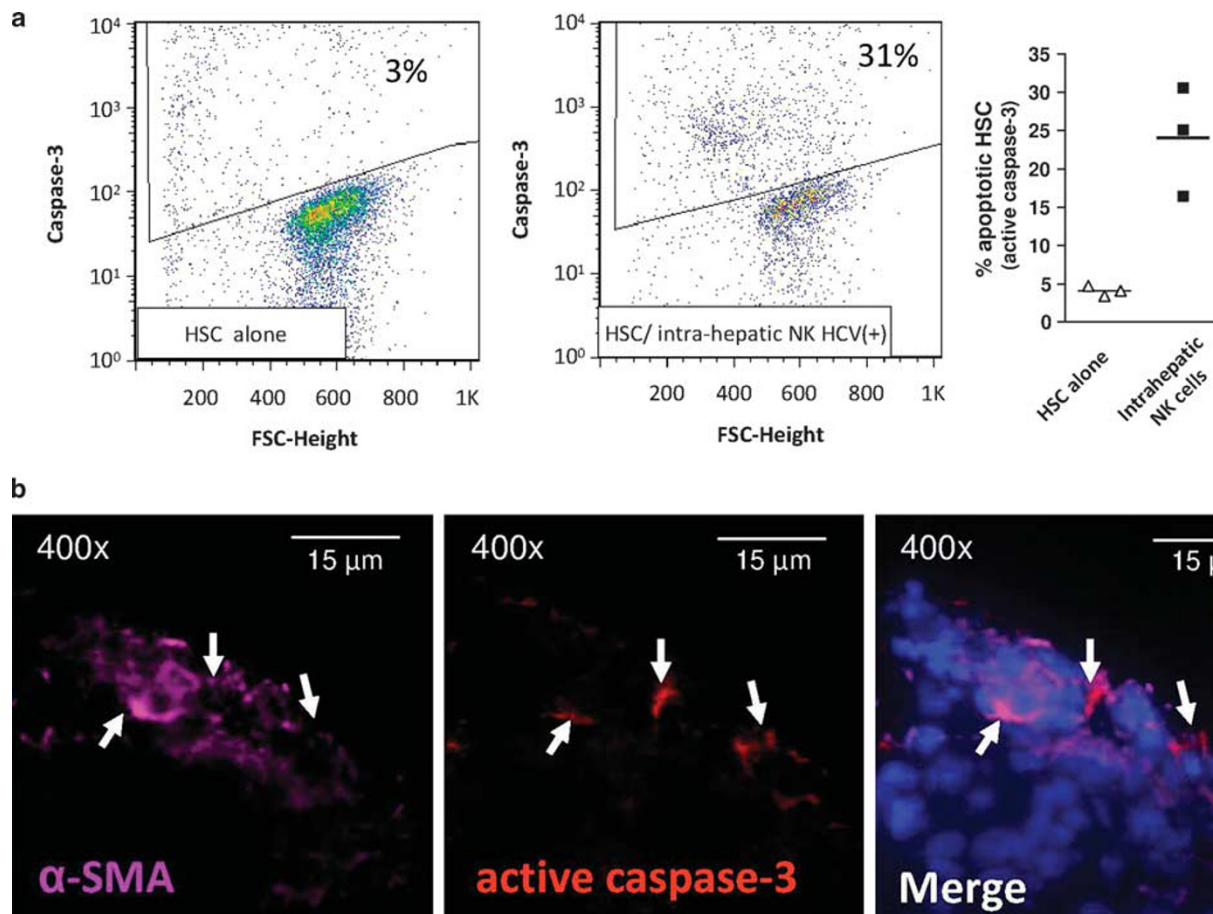
However, using a caspase-3 activation assay we observed only little cell death of primary human HSCs after coincubation with NK cells from healthy HCV-negative individuals ( $6.2 \pm 2.1\%$ ). In contrast, NK cells from HCV RNA(+) patients induced apoptosis in a substantial number of HSCs ( $17.8 \pm 9.2\%$ ,  $P < 0.0001$  vs healthy controls; Figures 2a and b). It is noteworthy that NK cell-induced HSC apoptosis was dependent on the NK cell/HSC ratio (Figure 2c) and could also be confirmed when TUNEL assays (Figure 2d) or detection of cleaved PARP (Figure 2e) was used to

study apoptosis. Interestingly, the ability of NK cells to induce HSC apoptosis was markedly reduced in HCV-infected patients who had successfully cleared the virus following treatment with peg-interferon- $\alpha$ /ribavirin ( $9.8 \pm 2\%$ ,  $P = 0.046$  vs HCV RNA(+)), suggesting an HCV-associated effect (Figure 2b).

To show the intrahepatic relevance of our findings, we analyzed the interaction of intrahepatic HCV(+) NK cells with HSCs, which also resulted in a strong NK cell-induced HSC apoptosis induction (Figure 3a). In addition, we found



**Figure 2** Natural killer (NK) cells from hepatitis C virus (HCV)-infected patients are highly efficient in inducing hepatic stellate cell (HSC) apoptosis. (a, b) After coincubation of isolated peripheral NK cells with activated primary HSCs, apoptosis of HSCs was determined by intracellular analysis of active caspase-3 via flowcytometry (healthy  $n = 12$ , HCV(+)  $n = 24$ , HCV(+) + IFN- $\alpha$  therapy  $n = 14$ , HCV-RNA(-)  $n = 6$ ). (c) NK cell-induced HSC apoptosis at different effector:target ratios. To confirm induction of HSC apoptosis by NK cells, we repeated these experiments using either TUNEL assays (d) or analysis of cleaved PARP (e) instead of studying active caspase-3 to detect HSC apoptosis. \* $P < 0.05$ ; \*\* $P < 0.001$ ; \*\*\* $P < 0.0001$ .



**Figure 3** Interaction of intrahepatic natural killer (NK) cells with hepatic stellate cells (HSCs). **(a)** Induction of HSC apoptosis by liver NK cells isolated from hepatitis C virus-positive (HCV(+)) liver samples ( $n = 3$ ). **(b)** Immunohistochemistry of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; violet), active caspase-3 (red) and cell nucleus (blue) in HCV(+) liver slice indicating apoptotic HSCs. Arrows indicate  $\alpha$ -SMA and active caspase-3 positive cells.

colocalization of  $\alpha$ -SMA and active caspase-3 in the liver biopsy specimens from HCV(+) patients, indicating that HSC apoptosis indeed occurs in HCV(+) livers (Figure 3b).

#### NK Cell Activity Against HSCs *In Vitro* Inversely Correlates with Fibrosis Stage

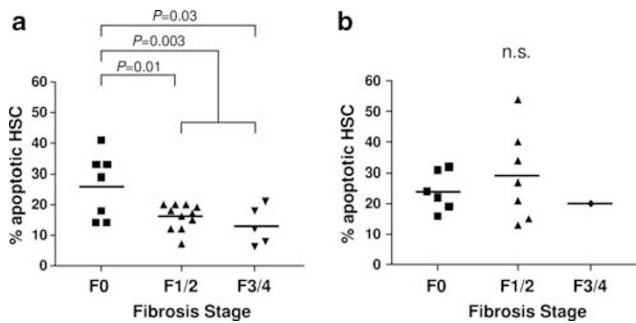
The level of HSC apoptosis induced by peripheral NK cells from HCV-infected patients was not correlated to ALT, AST,  $\gamma$ -GT, gender, HCV genotype or HCV RNA levels (data not shown). Staging of fibrosis was available in 37 HCV-infected patients (23 treatment-naïve individuals and 14 patients under IFN- $\alpha$  therapy), who had distributions of age, sex, transaminase serum levels and serum HCV RNA loads identical with the rest of our cohort. NK cell-mediated apoptosis of primary human HSCs was inversely associated with stage of liver fibrosis, so that significantly higher apoptosis induction was found in patients with minimal or no liver fibrosis as compared to patients with fibrosis stages Metavir F1 and F2 ( $P = 0.01$ ) or F3 and F4 ( $P = 0.03$ ) fibrosis (Figure 4a). No such associations were found in patients under therapy (Figure 4b).

#### NK cell-Induced HSC Apoptosis Is Contact-Dependent and Involves TRAIL, FasL and NKG2D

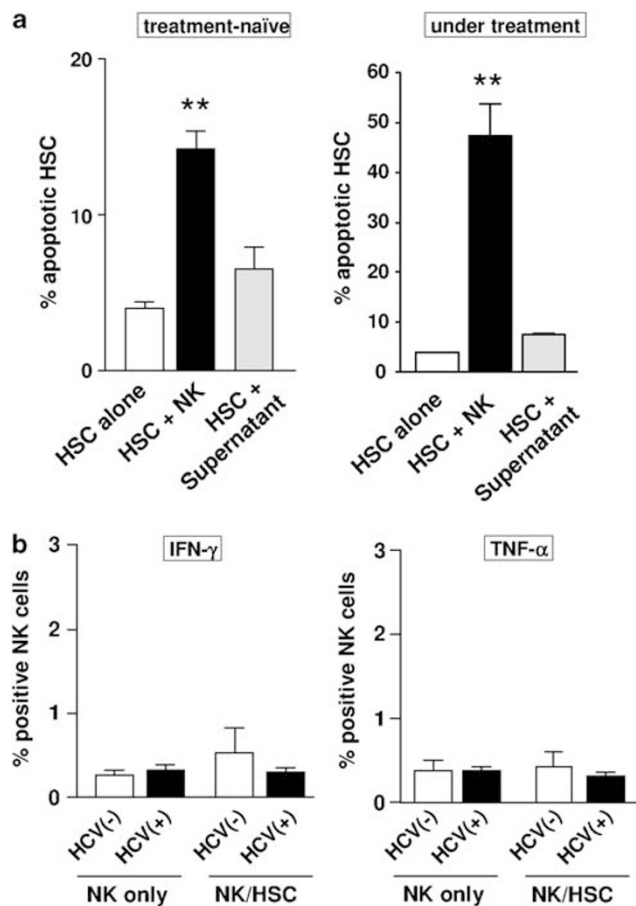
To find out, whether the enhanced activity of NK cells from patients with chronic hepatitis C to induce cell death in primary HSCs was mediated by soluble factors, we performed transwell experiments. However, supernatants of HSCs stimulated NK cells from both treatment-naïve HCV(+) individuals (Figure 5a, left panel) and HCV patients under treatment with IFN- $\alpha$  (Figure 5a, right panel) had little effect on HSC apoptosis, suggesting a contact-dependent mechanism.

Accordingly, NK cells secreted only negligible amounts of IFN- $\gamma$  and TNF- $\alpha$  after coincubation with HSCs, without any significant differences between NK cells from HCV(+) and HCV(-) patients (Figure 5b).

Thus, we next characterized HSCs with respect to surface expression of molecules involved in the induction of apoptosis. As is exemplarily shown in Figure 6a we observed robust expression of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-receptor II (but not TRAIL-R I). Accordingly, pre-treatment of NK cells with a TRAIL-specific



**Figure 4** Natural killer (NK) cell activity against hepatic stellate cells (HSCs) *in vitro* inversely correlates with fibrosis stage. Induction of HSC apoptosis by NK cells from treatment-naïve hepatitis C virus (HCV)-positive patients (a) and HCV-infected patients under interferon- $\alpha$  (IFN- $\alpha$ ) treatment (b), respectively, was plotted against liver fibrosis stage.



**Figure 5** Natural killer (NK) cell-mediated induction of hepatic stellate cell (HSC) apoptosis requires cell-cell contact. (a) Primary HSCs were coincubated with either NK cells from hepatitis C virus-positive (HCV(+)) patients or with the supernatants from HCV(+)) NK cells with HSCs for 6 h. Then, induction of HSC apoptosis was studied (treatment naïve  $n=4$ , under treatment  $n=3$ ). (b) NK cells from HCV(+)) (black bars,  $n=9$ ) or healthy donors (white bars,  $n=6$ ) were coincubated with primary HSCs and then analyzed for secretion of interferon- $\gamma$  (IFN- $\gamma$ ) and TNF- $\alpha$ , respectively. The columns indicate means and s.d. \*\* $P<0.001$ .

antibody significantly reduced NK cell-mediated induction of HSC apoptosis (Figure 6b). It is noteworthy that NK cells obtained from HCV-infected patients displayed a significantly stronger ability to induce apoptosis of HSCs than NK cells from HCV(-) controls even after blocking of TRAIL (Figure 6c).

In addition, we detected a strong expression of Fas on activated HSCs, suggesting a role for Fas/FasL interactions in NK cell-mediated HSC apoptosis. In line with this hypothesis, we found that blocking of FasL on NK cells with a specific antibody significantly reduced induction of HSC apoptosis (Figure 6d).

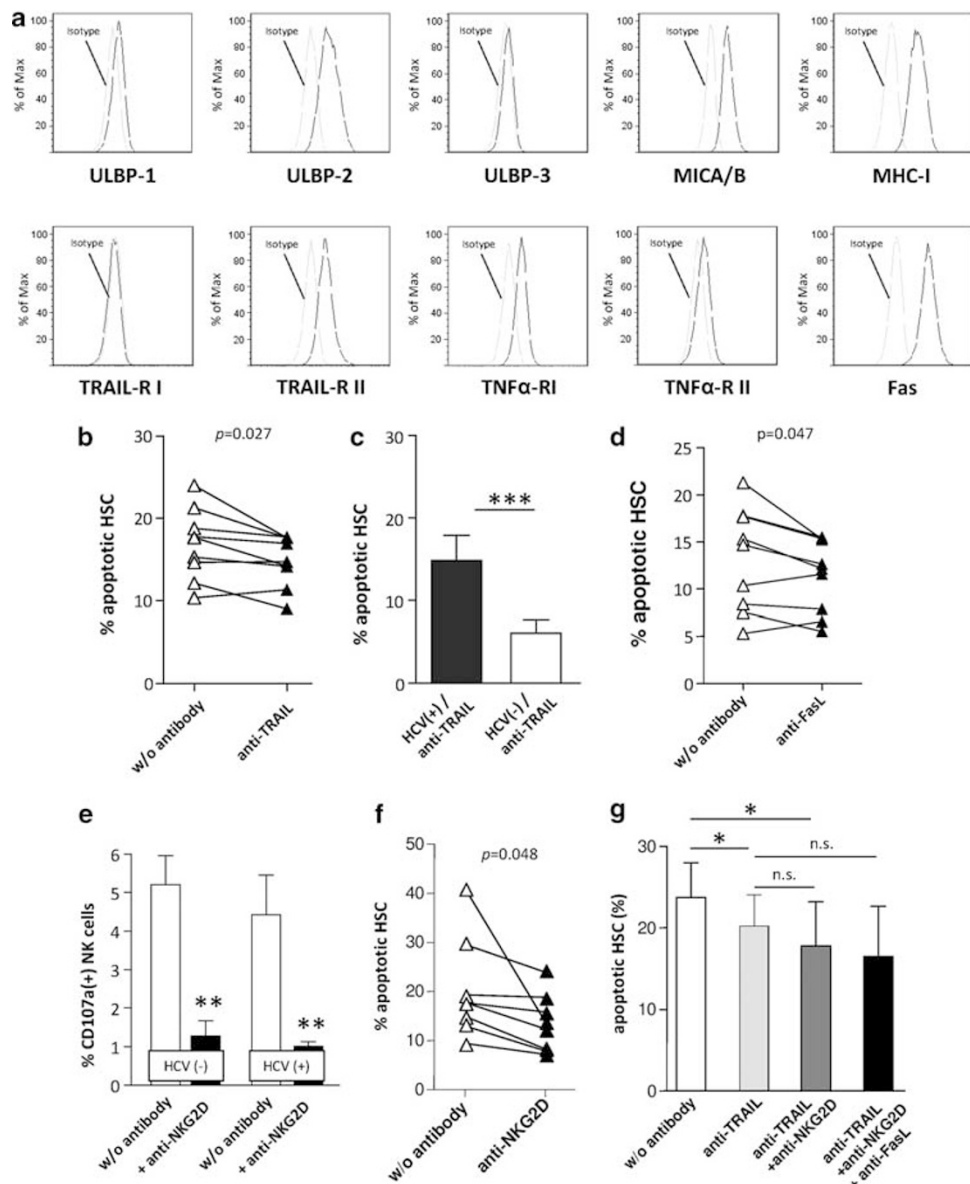
Finally, there was a substantial surface expression of the NKG2D ligands ULBP-1/2 and MICA/B on primary HSCs. Accordingly, we found that blocking of NK cell/HSC interactions with a NKG2D-specific antibody partly reduced both NK cell degranulation (Figure 6e) as well as NK cell-induced HSC apoptosis (Figure 6f).

Thus, these findings suggest that NKG2D, TRAIL as well as the Fas/FasL system, may be involved in NK cell-mediated apoptosis of HSCs. However, our finding that combined blocking of NKG2D together with FasL and TRAIL did not completely prevented HSC apoptosis (Figure 6g) may indicate that additional mechanism(s) also may have a role.

### Regulation of NK Cell-Mediated Apoptosis of Primary Human HSCs

IFN- $\alpha$  has been shown to effectively trigger NK cell activity.<sup>19,20</sup> Thus, we studied whether pre-stimulation of NK cells with IFN- $\alpha$  affects their ability to induce apoptosis in primary human HSCs. As depicted in Figure 7a, we found that prior exposure to IFN- $\alpha$  significantly increased the ability of HCV(-) NK cells to induce apoptosis in HSCs in a dose-dependent manner, whereas incubation of HSCs alone with IFN- $\alpha$  had no effect on HSC cell death (data not shown). In line with these *in vitro* findings, we observed that NK cells derived from patients under treatment with IFN- $\alpha$  were significantly more effective in inducing apoptosis of primary human HSCs ( $27.6 \pm 10.5\%$ ,  $P=0.0029$ ) than the NK cells from treatment-naïve individuals (Figure 2b).

Further experiments suggested a role for TRAIL in IFN- $\alpha$ -triggered induction of HSC apoptosis by NK cells, because we observed a significantly upregulated surface expression of TRAIL on IFN- $\alpha$ -stimulated NK cells (Figure 7b, left graph) whereas expression of Fas and NKG2D, respectively, was not affected (Figure 7b, left graph; data not shown). Accordingly, we could show that pre-incubation of primary HSCs with recombinant TRAIL resulted in dose-dependent apoptosis induction of primary human HSCs (Figure 7c). In contrast, coincubation with recombinant FasL had only minimal effects (Figure 7c). A role for TRAIL in IFN- $\alpha$ -triggered NK cell-induced HSC apoptosis was further confirmed in blocking experiments, as we found that pre-incubation with a neutralizing TRAIL antibody significantly reduced induction of HSC apoptosis (Figure 7d).



**Figure 6** Natural killer (NK) cell-mediated induction of hepatic stellate cell (HSC) apoptosis involves tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), FasL and NKG2D. **(a)** Phenotypic characterization of activated primary HSCs by flow cytometric analysis (ULBP: UL16 binding proteins). **(b)** The effect of TRAIL blockade on NK cell-induced HSC apoptosis in the hepatitis C virus (HCV)-infected patients. **(c)** Comparison of residual apoptosis induction by NK cells from HCV(+) patients and healthy controls after TRAIL blockade. **(d)** HCV(+) NK cell-mediated HSC apoptosis following pre-treatment of NK cells with a FasL-specific antibody. **(e)** Blocking NKG2D on NK cells during coincubation with primary HSCs significantly reduced NK cell degranulation of HCV(+) ( $n=3$ ) and healthy NK cells ( $n=3$ ). **(f)** The effect of NKG2D blocking on HSC apoptosis induced by NK cells from HCV RNA(+) patients. **(g)** The effect of combined blockade of TRAIL together with NKG2D and FasL ( $n=5$ ). The columns indicate means and s.d.  $*P < 0.05$ ;  $**P < 0.001$ ;  $***P < 0.0001$ .

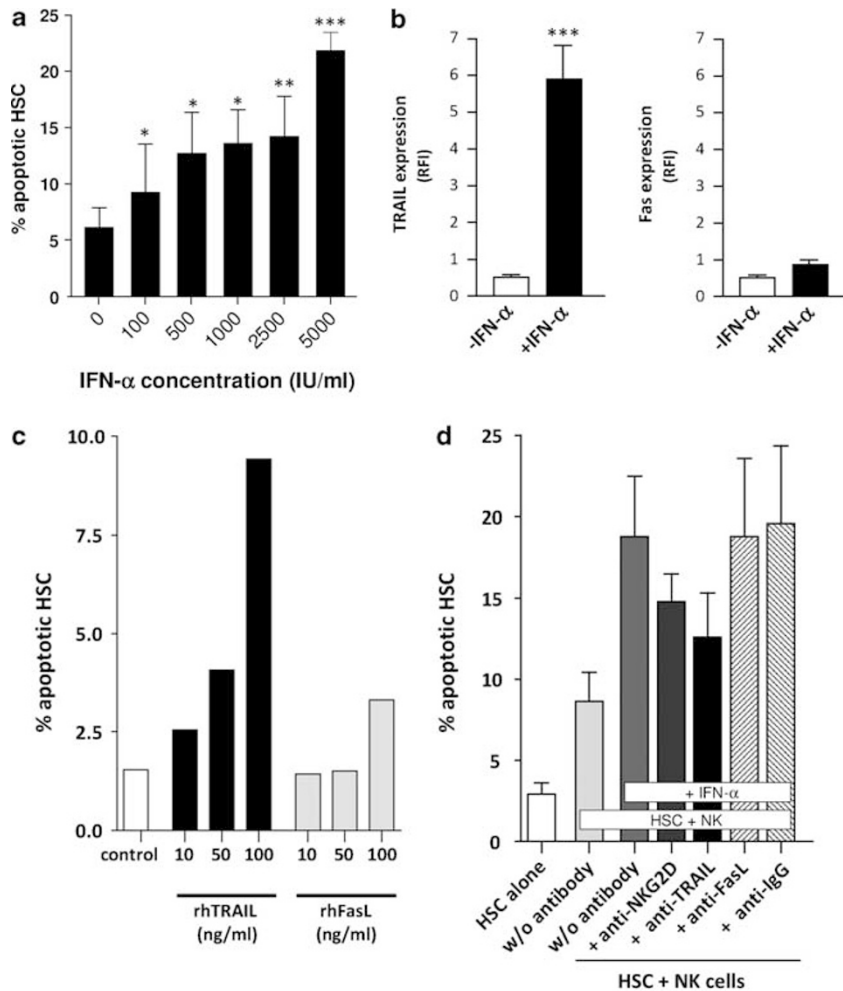
## DISCUSSION

In murine models of liver fibrosis, NK cells have been shown to display anti-fibrotic activity by killing of activated HSCs.<sup>7–14</sup> However, the potential role of human NK cells in hepatic fibrogenesis has remained largely unclear.

In the present study we analyzed interactions of human NK cells with HSCs and studied whether chronic HCV infection may alter these interactions.

We found that NK cells displayed only little cytotoxic activity and cytokine secretion following coincubation against primary HSCs, irrespective of HCV infection. However, NK cells from HCV RNA(+) patients were highly efficient in inducing HSC apoptosis. This was in sharp contrast to NK cells from both healthy donors and successfully treated HCV RNA(-) patients, indicating that NK cell-mediated induction of HSC apoptosis is an HCV-associated phenomenon.





**Figure 7** Effect of interferon- $\alpha$  (IFN- $\alpha$ ) on natural killer (NK) cell-induced hepatic stellate cell (HSC) apoptosis. **(a)** Purified NK cells from healthy donors ( $n = 3$ ) were cultured in the presence of increasing doses of recombinant IFN- $\alpha$  followed by coincubation with primary HSCs for 6 h. Then, apoptosis of HSCs was determined by intracellular staining of active caspase-3. **(b)** The effect of recombinant IFN- $\alpha$  (5000 U/ml) on surface expression of the death ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; left graph) and Fas (right graph). **(c)** Induction of HSC apoptosis by coincubation of primary HSCs with increasing concentrations of recombinant human TRAIL (0, 10, 50 and 100 ng/ml) and FasL (0, 10, 50 and 100 ng/ml) for 6 h. **(d)** Purified NK cells were stimulated with IFN- $\alpha$  *in vitro* and then coincubated with primary HSCs in the presence or absence of blocking antibodies specific for NKG2D, TRAIL and FasL, respectively ( $n = 5$ ). As control an IgG-specific antibody was used. Columns indicate means and s.d.; \* $P < 0.05$ ; \*\* $P < 0.001$ ; \*\*\* $P < 0.0001$ . RFI, relative fluorescence intensity.

However, HCV does not directly activate NK cells,<sup>21</sup> suggesting an indirect effect. Recent data by Ahlenstiel *et al*<sup>19</sup> reported that chronic exposure to HCV-induced IFN- $\alpha$  may critically modulate NK cell function in hepatitis C resulting in a polarized NK cell phenotype. Consistent with this hypothesis, we found that both *in vitro* and *in vivo* stimulation with IFN- $\alpha$  significantly increased NK cell-mediated induction of HSC apoptosis. It is noteworthy that IFN- $\alpha$  has been shown to induce surface expression of TRAIL on NK cells.<sup>19,20</sup> Furthermore, TRAIL-mediated killing of activated HSCs is considered as a mechanism how NK cell accomplish their anti-fibrotic activity.<sup>13</sup> Accordingly, we found increased TRAIL expression on IFN- $\alpha$ -treated NK cells and could demonstrate that recombinant human TRAIL dose-dependently

induced HSC apoptosis *in vitro*. Moreover, we found that pre-incubation with anti-TRAIL effectively blocked the induction of HSC apoptosis by *in vitro* IFN- $\alpha$ -stimulated NK cells. In addition, blocking of TRAIL on NK cells from HCV RNA(+) patients at least in part prevented NK cell-induced cell death of HSCs.

Moreover, we detected a strong expression of Fas on activated HSCs, suggesting a role for Fas/FasL interactions in NK cell-mediated HSC apoptosis. Accordingly, we found that pre-incubation of NK cells with anti-FasL in part (but significantly) blocked the induction of HSC apoptosis.

Besides TRAIL- and FasL-mediated NK cell activity, killing of activated HSCs by NK cells has been shown to involve

NKG2D- and granzyme-dependent mechanisms as well as secretion of IFN- $\gamma$ .<sup>9,11,13</sup>

However, cocubation of NK cells from both healthy and HCV-infected individuals did not result in substantial IFN- $\gamma$  production, suggesting that this pathway may only have a minor role in the induction of HSC apoptosis.

It is noteworthy that cocubation of NK cells with HSCs resulted in significant NK cell degranulation. Moreover, our data suggest a role for NKG2D, as we found that blocking of this activating NK cell receptor reduced the induction of HSC apoptosis in both un-stimulated and IFN- $\alpha$ -stimulated NK cells and reduced NK cell degranulation as well. In addition, the NKG2D ligands, ULBP-2 and MICA/B, were strongly expressed on HSCs.

The role of NKG2D in HCV infection is discussed controversially as some authors reported downregulated expression of this NK cell receptor,<sup>22</sup> whereas other studies found an increased surface expression in hepatitis C.<sup>23,24</sup> Upregulated expression of NKG2D would be an intriguing explanation for our observation of increased NK cell activity against HSCs. However, in line with our previous findings,<sup>25</sup> we did not observe any significant differences regarding NKG2D expression between HCV-positive and healthy individuals. In addition, *in vitro* treatment of NK cells with IFN- $\alpha$  did not affect NKG2D expression (data not shown). Thus, the pathways how NKG2D contributes to enhanced cytolytic activity of HSCs in hepatitis C remain unclear.

Interestingly, we found that combined blocking of NKG2D together with FasL and TRAIL did not completely prevent HSC apoptosis, indicating that additional not yet identified mechanism(s) also may have a role.

Finally, we asked for the potential *in vivo* role of NK cell-induced HSC apoptosis in chronic hepatitis C. Unfortunately, access to liver NK cells is limited. Thus, only a small number of intra-hepatic NK cells ( $n=3$ ) from HCV(+) patients could be studied. It is noteworthy that analyzing interactions of liver NK cells with HSCs confirmed our findings of strong NK cell-induced apoptosis induction. In addition, we found colocalization of  $\alpha$ -SMA (marker for activated HSCs) and active caspase-3 (marker for apoptosis) in the liver biopsy specimens from HCV-positive patients, indicating that HSC apoptosis indeed occurs in HCV(+) livers. Accordingly, Gonzalez *et al*<sup>26</sup> recently demonstrated intra-hepatic HSC apoptosis to be inversely correlated with the stage of fibrosis in chronic HCV. Finally, we observed an inverse correlation between NK cell-induced HSC apoptosis and the stage of liver fibrosis, with significantly higher anti-HSC activity of NK cells in patients without fibrosis than in patient with F1/F2 and F3/F4 fibrosis, respectively. These findings resemble data presented by Morishima *et al*<sup>27</sup> who found that cytolytic activity of NK cells was inversely associated with liver fibrosis stage.

In line with data obtained in murine models of liver fibrosis, these findings suggest that in chronic HCV infection

the presence of active NK cells may be protective for the liver disease progression.

At the moment it remains unclear what mechanism(s) mediate loss of NK cell activity in progressive fibrosis. A recent study by Muhanna *et al*<sup>12</sup> suggests that upregulation of inhibitory NK cell receptors may be involved. An alternative explanation has been provided by Jeong *et al*.<sup>10</sup> These authors showed that in contrast to quiescent and early activated HSCs, respectively, intermediately activated HSCs produce high levels of TGF- $\beta$ . It is noteworthy that TGF- $\beta$  has been shown to inhibit NK cell activity.<sup>8,28,29</sup> Accordingly, Jeong *et al*<sup>10</sup> nicely showed that resistance of intermediately activated HSCs to killing by NK cells is likely mediated by TGF- $\beta$ . It is noteworthy that replication of HCV has been shown to induce secretion of TGF- $\beta$  and to activate HSCs. Thus, HCV-induced overproduction of TGF- $\beta$  might result in impaired NK cell function, which subsequently supports progression of fibrosis.

Taken together, we show that NK cells from HCV(+) patients effectively induce apoptosis of activated HSCs and that decreased NK cell activity is associated with advanced fibrosis stages in hepatitis C.

#### ACKNOWLEDGEMENT

This work was supported by the German Research Foundation (DFG SFB/TRR 57).

#### DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

- Spengler U, Nattermann J. Immunopathogenesis in hepatitis C virus cirrhosis. *Clin Sci* 2007;112:141–155.
- Guidotti LG, Chisari FV. Immunobiology and pathogenesis of viral hepatitis. *Annu Rev Pathol* 2006;1:23–61.
- Friedman SL, Rockey DC, Bissell DM. Hepatic fibrosis 2006: report of the third AASLD single topic conference. *Hepatology* 2007;45:242–249.
- Iredale JP. Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *J Clin Invest* 2007;117:539–548.
- Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest* 2005;115:209–218.
- Pasquinelli C, Shoenberger JM, Chung J, *et al*. Hepatitis C virus core and E2 protein expression in transgenic mice. *Hepatology* 1997;25:719–727.
- Gao B, Radaeva S, Jeong WI. Activation of natural killer cells inhibits liver fibrosis: a novel strategy to treat liver fibrosis. *Expert Rev Gastroenterol Hepatol* 2007;1:173–180.
- 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.
- Jeong WI, Park O, Radaeva S, *et al*. STAT1 inhibits liver fibrosis in mice by inhibiting stellate cell proliferation and stimulating NK cell cytotoxicity. *Hepatology* 2006;44:1441–1451.
- Jeong WI, Park O, Suh YG, *et al*. Suppression of innate immunity (natural killer cell/interferon-gamma) in the advanced stages of liver fibrosis in mice. *Hepatology* 2011;53:1342–1351.
- Melhem A, Muhanna N, Bishara A, *et al*. Anti-fibrotic activity of NK cells in experimental liver injury through killing of activated HSC. *J Hepatol* 2006;45:60–71.
- Muhanna N, Abu Tair L, Doron S, *et al*. Amelioration of hepatic fibrosis by NK cell activation. *Gut* 2011;60:90–98.
- Radaeva S, Sun R, Jaruga B, *et al*. 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.
- Radaeva S, Wang L, Radaev S, *et al*. 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–G816.
15. Coenen M, Nischalke HD, Kramer B, *et al*. Hepatitis C virus core protein induces fibrogenic actions of hepatic stellate cells via toll-like receptor 2. *Lab Invest* 2011;91:1375–1382.
  16. Martin-Vilchez S, Sanz-Cameno P, Rodriguez-Munoz Y, *et al*. The hepatitis B virus X protein induces paracrine activation of human hepatic stellate cells. *Hepatology* 2008;47:1872–1883.
  17. Semela D, Das A, Langer D, *et al*. Platelet-derived growth factor signaling through ephrin-b2 regulates hepatic vascular structure and function. *Gastroenterology* 2008;135:671–679.
  18. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. *J Immunol Methods* 2004;294:15–22.
  19. Ahlenstiel G, Titerence RH, Koh C, *et al*. Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an interferon- $\alpha$ -dependent manner. *Gastroenterology* 2010;138:325–335.e321–322.
  20. Stegmann KA, Bjorkstrom NK, Veber H, *et al*. Interferon- $\alpha$ -induced TRAIL on natural killer cells is associated with control of hepatitis C virus infection. *Gastroenterology* 2010;138:1885–1897.
  21. Yoon JC, Shiina M, Ahlenstiel G, *et al*. Natural killer cell function is intact after direct exposure to infectious hepatitis C virions. *Hepatology* 2009;49:12–21.
  22. Sene D, Levasseur F, Abel M, *et al*. Hepatitis C virus (HCV) evades NKG2D-dependent NK cell responses through NS5A-mediated imbalance of inflammatory cytokines. *PLoS Pathog* 2010;6:e1001184.
  23. Oliviero B, Varchetta S, Paudice E, *et al*. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. *Gastroenterology* 2009;137:1151–1160.e1151–1157.
  24. Varchetta S, Oliviero B, Francesca Donato M, *et al*. Prospective study of natural killer cell phenotype in recurrent hepatitis C virus infection following liver transplantation. *J Hepatol* 2009;50:314–322.
  25. Nattermann J, Feldmann G, Ahlenstiel G, *et al*. Surface expression and cytolytic function of natural killer cell receptors is altered in chronic hepatitis C. *Gut* 2006;55:869–877.
  26. Gonzalez SA, Fiel MI, Sauk J, *et al*. Inverse association between hepatic stellate cell apoptosis and fibrosis in chronic hepatitis C virus infection. *J Viral Hepat* 2009;16:141–148.
  27. Morishima C, Paschal DM, Wang CC, *et al*. Decreased NK cell frequency in chronic hepatitis C does not affect *ex vivo* cytolytic killing. *Hepatology* 2006;43:573–580.
  28. Yu J, Wei M, Becknell B, *et al*. Pro- and antiinflammatory cytokine signaling: reciprocal antagonism regulates interferon- $\gamma$  production by human natural killer cells. *Immunity* 2006;24:575–590.
  29. Dasgupta S, Bhattacharya-Chatterjee M, O'Malley Jr BW, *et al*. Inhibition of NK cell activity through TGF- $\beta$  1 by down-regulation of NKG2D in a murine model of head and neck cancer. *J Immunol* 2005;175:5541–5550.