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ORIGINAL ARTICLE

# NKp46-mediated killing of human and mouse hepatic stellate cells attenuates liver fibrosis

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## ABSTRACT

**Background** Liver fibrosis, which involves activation of hepatic stellate cells (HSC), is a major health problem and is the end outcome of all chronic liver diseases. The liver is populated with lymphocytes, among which are natural killer (NK) cells, whose activity is controlled by inhibitory and activating receptors. NKp46, one of the major NK activating receptors expressed by NK cells, is also a specific NK marker that discriminates NK cells from all other lymphocyte subsets. It recognises viral haemagglutinins and unknown cellular ligands.

**Methods** The anti-fibrotic activity of the NKp46 receptor was assessed in vivo and in vitro using NKp46-deficient mice ($NCR1^{gfp/gfp}$), the carbon tetrachloride model and in vitro NK killing assays. Primary murine and human HSC were stained for the expression of the NKp46 ligand using fusion proteins composed of the extracellular portions of the murine and human NKp46 receptors fused to human IgG1.

**Results** It was shown that murine HSC express a ligand for the murine orthologue of the NKp46 receptor, NCR1. NCR1 inhibited liver fibrosis in vivo; in vitro, murine HSC were killed in an NCR1-dependent manner. In humans it was shown that human HSC also express a ligand for the human NKp46 receptor and that the killing of human HSC is NKp46 dependent.

**Conclusions** In addition to NKG2D, NKp46/NCR1 play an important role in inhibition of liver fibrosis. This suggests that fibrosis can be better controlled through the manipulation of NKp46 activity.

### Significance of this study

**What is already known on this subject?**

- Activated hepatic stellate cells (HSC) are susceptible to natural killer (NK) cell mediated killing in vitro, suggesting that NK cells play a protective role in liver fibrosis.
- NK cell depletion experiments using the anti-asialo GM-1 antibody (which also deletes other immune cells) led to acceleration of liver fibrosis.
- Apart from NKG2D (ie, expressed by several immune cell subsets including NK and T cells), no other NK cell receptors are implicated in the control of liver fibrosis.
- NKp46 is considered to be an NK cell specific marker and is the only natural cytotoxicity receptor for which an orthologous protein (NCR1) has been found in mice.

**What are the new findings?**

- Primary human and mouse HSC express unknown ligands for human NKp46 and mouse NCR1 receptors.
- NKp46 is directly involved in the control of liver fibrosis through the killing of primary murine and human HSC.
- In the absence of NKp46, liver fibrosis is exacerbated.

**How might it impact on clinical practice in the foreseeable future?**

- Manipulation of NKp46 activity during liver fibrosis may lead to the development of new drugs for alleviation of liver fibrosis.

## INTRODUCTION

The liver, which is situated at a strategic junction between the gastrointestinal tract and the systemic circulation, is continuously exposed to bacterial products, viruses and toxins. Therefore, immune functions in general and innate immune activities in particular, are predominantly important in this organ. In this regard, the liver is populated with natural killer (NK) cells, which are central components of the innate immune system.[1] In humans, NK cells comprise about 30–50% of all liver lymphocytes, and 10–20% of all mouse intrahepatic lymphocytes are NK cells.[1–3] NK cells mediate early protection against viruses and are also involved in the killing of intracellular bacteria, tumours[4] and self pancreatic β cells.[5] In addition to their potent killing activity, NK cells produce several cytokines, such as interferon (IFN)-γ and tumour necrosis factor α (TNF-α).[4] The activity of NK cells is controlled by an array of activating and inhibitory receptors.[6] Some of the activating NK receptors are unique to NK cells, while others are not and are expressed by additional immune cells. For example, while the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46, are expressed almost exclusively on NK cells,[4 7] NKG2D is also expressed on other lymphocytes, such as CD8+ and γδ T-cells.[8] Several ligands have been identified for the NK activating receptors. NKG2D recognises numerous stress-induced ligands (MICA, MICB and ULBP 1–6[8]), NKp30 recognises viral ligands, tumour ligands and self ligands,[9–11] and NKp46 and NKp44 recognise viral hemagglutinins[12 13] and unknown tumour and self ligands.[4 5 12 14–16] In addition, as we and others


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have shown, NKp46 is also considered to be a specific NK cell marker and it is the only NCR for which an orthologue protein (NCR1) has been found in mice.[7 17–20]

Accumulating evidence suggests that NK cells also play an important role in liver injury and subsequent liver fibrosis.[21–23] Liver cirrhosis that results from chronic liver disease is characterised by replacement of normal liver tissue by a fibrotic scar. Activation of myofibroblast-like cells, mainly hepatic stellate cells (HSC), is a key step in the development of liver fibrosis.[21–23] During liver injury, HSC become activated and participate in liver repair. However, following chronic liver injury the HSC differentiate into fibrogenic myofibroblasts, which express α-smooth muscle actin (αSMA) and produce collagen (the hallmark of the fibrotic scar[24]). As liver fibrosis is associated with severe complications including the development of liver tumours, it is essential to investigate the mechanisms controlling HSC activation and elimination.

It has previously been shown that activated HSC are susceptible to NK cell-mediated killing in vitro and hence it was suggested that NK cells play a protective role in liver fibrosis.[21–23] Indeed, NK cell depletion experiments using the anti-asialo GM-1 antibody (which also deletes other immune cells) led to acceleration of liver fibrosis.[23 25] Furthermore, clinical evidence suggests that NK cells are important in the development of human cirrhosis, because, in the blood, low NK cell numbers and activity have been correlated with accelerated liver fibrosis in patients suffering from hepatitis C virus (HCV) infection.[26–28] Finally, it was recently shown that reducing NK cell inhibition leads to increased HSC killing, thereby reducing fibrosis.[29]

However, the molecular mechanisms by which NK cells recognise HSC and control liver fibrosis are largely unknown, and apart from NKG2D[23] (ie, expressed by several immune cell subsets including NK and T cells), no other NK cell receptors were implicated in the control of liver fibrosis. Here, we demonstrate for the first time that primary human and mouse HSC express an unknown ligand for the human NKp46 and the mouse NCR1 receptors. We further show that NKp46 is directly involved in controlling liver fibrosis through the killing of primary murine and human HSC, and we show in vivo that in the absence of NKp46, liver fibrosis is exacerbated.

## MATERIAL AND METHODS

### Mice

All experiments were performed using 8–12 week-old male C57BL/6 mice. The generation of the NCR1$^{gfp/gfp}$ mice was described previously.[18] All experiments were performed in the specific pathogen-free unit of the Hadassah Medical School (Ein-Kerem, Jerusalem) according to guidelines of the ethical committee.

### Cells, monoclonal antibodies and immunoglobulin fusion proteins

The generation of the BW and BW transfectants has been described peviously.[13 18] Primary human HSC were isolated from normal human liver derived from brain dead normal donors. Isolation was performed according to earlier methodology described by Fridman *et al*.[30] Primary human and murine stellate cells were grown in complete Dulbecco modified Eagle medium supplemented with 1% foetal calf serum (FCS), or with 10% FCS for in vitro activation. The murine mastocytoma P815 and the 721.221 carcinoembryonic antigen (CEA) transfectant cell lines were grown in complete RPMI medium supplemented with 10% FCS. The NCR1-Ig, NKp46-Ig, NKp46D2-Ig and NKp46D1-Ig fusion proteins were generated in COS-7 cells and purified by affinity chromatography using a protein G column, as previously described.[13] For the proteinase K (PK) treatment, cells were incubated for 20 min with 10 µl of PK in 5% $CO_2$, 37°C, washed twice and stained with the various fusion proteins. For the trypsin (Try) treatment, cells were incubated for 1 min with 500 µl of trypsin in room temperature, washed twice and stained with the various fusion proteins. Staining with all fusion proteins was detected using a secondary PE-conjugated goat anti-human antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). For the identification of stellate cells, we used the anti-αSMA mouse monoclonal antibody conjugated to Cy-5 (Jackson ImmunoResearch).

### Induction of hepatic fibrosis

Hepatic fibrosis was induced using carbon tetrachloride ($CCl_4$). $CCl_4$ was diluted to 10% in corn oil, and 5 µl/g body weight of $CCl_4$ (0.5 µl of pure $CCl_4$) was introduced by intraperitoneal injections twice a week, for 4 weeks. All animals were killed 3 days after the final dose of $CCl_4$ following ketamine/xylazine anaesthesia; sera and livers were harvested.

### Histological assessment of liver injury

Mice liver biopsy specimens were obtained from naïve and fibrotic mice. The posterior one-third of the liver was fixed in 10% formalin for 24 h and then paraffin-embedded in an automated tissue processor. Sections (7 mm) were cut and stained in 0.1% Sirius red F3B in saturated picric acid (both from Sigma).

### Fibrosis quantification

Fibrotic area was assessed based on 63–180 fields from four Sirius red-stained liver sections per animal. Each field was acquired at magnification ×40 and then analysed using a computerised Bioquant-morphometry system.

### BW reporter assay

The BW reporter system and the generation of BW-NKp46-ζ, is described elsewhere.[13 18] For measurements of interleukin (IL)-2 secretion, 15 000 BW and BW-transfectant cells were co-incubated with 30 000 irradiated (6000 rad) target cells for 48 h at 37°C and 5% $CO_2$. Supernatants were collected and the IL-2 level was quantified using anti-mouse IL-2 monoclonal antibody (mAb) (BioLegend, Petach-Tiqva, Israel) and standard ELISA.

### In vitro cytotoxicity assays

Target cells were labelled with $^{35}$S-methionine and incubated for 5 h at 37°C with effector NK cells at various effector:target ratios as previously described.[31] Bulk NK cells were pre-incubated with anti-NKp46 sera and with control sera produced by immunising mice with NKp46-Ig and CEA-Ig as previously described.[5]

### αSMA immunoblot

Immunoblot analysis of αSMA in liver extracts was performed with modifications, as previously described.[21 32]

### Murine hepatic stellate cell isolation

HSC were isolated from normal and fibrotic C57Bl/6 mice using sequential pronase/collagenase digestion followed by Nycodenz density gradient centrifugation as described previously.[21]

### Immunofluorescence

Mouse liver biopsy specimens were incubated at room temperature with 4% paraformaldehyde solution and then were incubated overnight with 10% sucrose. They were then frozen at −80°C and

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7 mm-thick frozen sections were prepared using Cryostat (Leica CM 3000). For identification of HSC, the sections were treated with anti-αSMA mouse mAb that was conjugated to Cy-5 (Jackson ImmunoResearch). A confocal laser scanning microscope (Zeiss Axiovert 200 M; Carl Zeiss MicroImaging, Thornwood, New York, USA) was used to analyse the stained sections.

## RESULTS

### NCR1 protects from $CCL_4$-mediated liver fibrosis

Because NK cell depletion experiments demonstrated the essential role of NK cells in controlling liver fibrosis,[21 23] and since NKp46 is one of the major NK killer receptor expressed both in humans and in mice,[18 33] we hypothesised that NKp46 and its mouse orthologue, NCR1, will be involved in the control of liver fibrosis. To test this, we initially tested whether murine HSC (mHSC) express a ligand for NCR1. Since the cellular ligands for human NKp46 and mouse NCR1 are unknown, we stained mHSC with a fusion protein composed of the extracellular portion of NCR1 fused to human IgG1. As a negative control we used a truncated version of NKp46 lacking the ligand-binding domain, named NKp46D1-Ig.[5] As can be seen in figure 1, the αSMA-positive stellate cells that were isolated from the livers of $CCL_4$-injected fibrotic mice (purity above 90%, figure 1A; the insert depicts the negative control), express an unknown ligand for NCR1 (detected by NCR1-Ig, figure 1B), while NKp46D1-Ig (the control Ig fusion protein) demonstrated no binding (figure 1B). Immunohistochemical and immunofluorescence analysis of healthy mouse liver sections performed with NKp46-Ig demonstrated no specific staining of hepatocytes (data not shown). Because healthy mouse and human β cells are stained with NKp46-Ig,[5 34] we concluded that liver hepatocytes do not express the NKp46 ligand. To demonstrate that NCR1 participates in controlling liver fibrosis in vivo, we used the NCR1 knockout mice ($NCR1^{gfp/gfp}$) that we had previously generated[18] and the $CCL_4$ model. $NCR1^{gfp/gfp}$, knockout (K/O) and $NCR1^{+/+}$ wild-type (WT) mice were injected intraperitoneally, twice a week, for four consecutive weeks with $CCL_4$; liver fibrosis was assessed by staining the liver sections with Sirius red. Importantly, as can be seen in figure 1C, we observed that in the absence of NCR1, liver fibrosis is exacerbated. Quantification of the average areas of relative fibrosis showed that NCR1 knockout mice had a significantly increased relative fibrosis area compared with the WT group (mean fibrotic area 8360, SEM-988.5 vs 5291, SEM- 446.4, respectively, figure 1D). To further confirm these results we performed immunoblot analysis for the expression of the αSMA protein whose expression is proportional to fibrosis severity. In agreement with the above results, αSMA levels were increased in the livers derived from the NCR1 knockout mice, compared with the WT, or naïve untreated mice. The levels of β-actin were almost equal in all

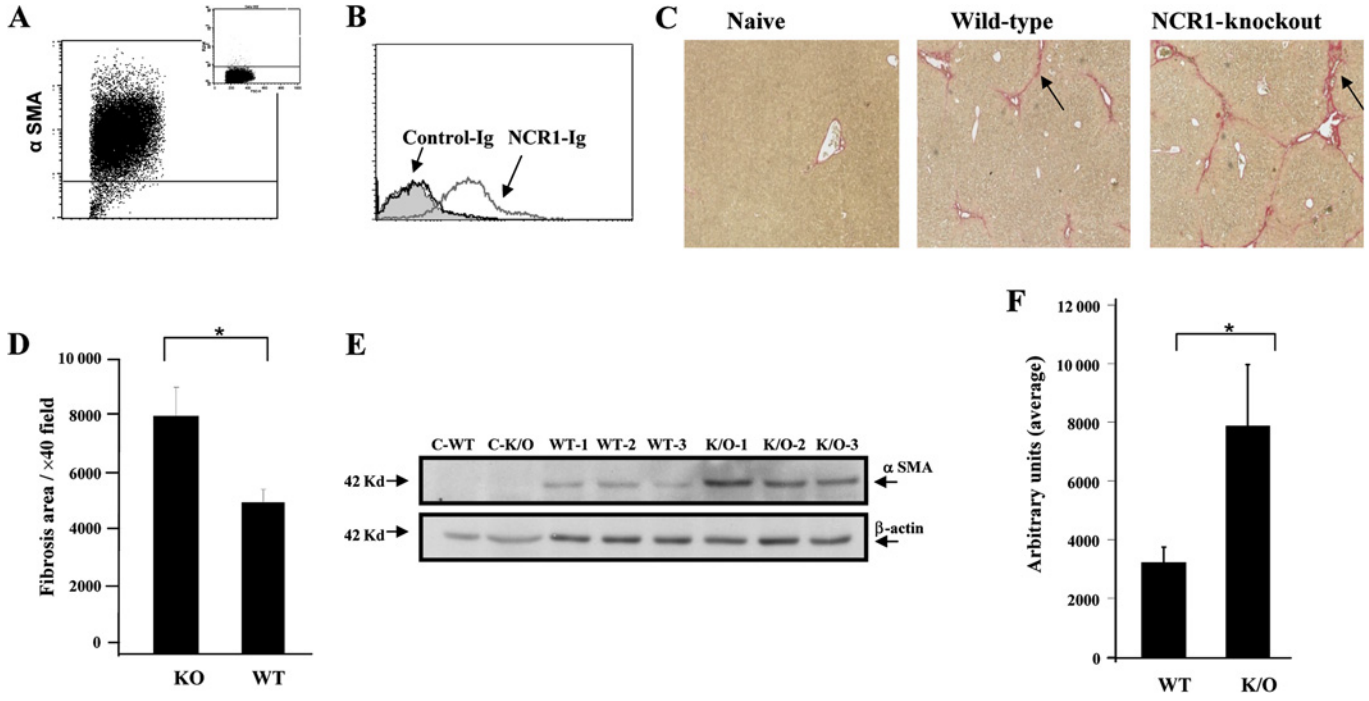


**Figure 1** Enhancement of liver fibrosis in the absence of NCR1. (A) Fluorescence-activated cell sorter (FACS) analysis of α-smooth muscle actin (αSMA) expression in primary murine hepatic stellate cells (mHSC) obtained from fibrotic livers after carbon tetrachloride ($CCl_4$) induction. The insert is the staining of the primary murine HSC with isotype control only. (B) FACS analysis of primary mHSC cells stained with the NCR1-Ig fusion protein (grey line) and with a control NKp46D1-Ig fusion protein (black line). The filled grey histogram is the background staining with secondary antibody only. The figure presents data obtained from one of three independent stainings. (C) $NCR1^{gfp/gfp}$ (NCR1-knockout) and wild-type (WT) mice were treated with $CCl_4$; liver tissue sections were harvested and stained with Sirius red. Representative tissue sections (magnification ×40) from naïve (un-injected), fibrotic WT and NCR1-knockout mice are shown. Fibrotic septa in fibrotic animals are highlighted by black arrow. (D) Quantification of the fibrotic areas in (C) was performed using a computerised morphometry system. The analysis was performed on 63-180 high power field (hpf, ×40) of liver sections derived from fibrotic WT and NCR1-knockout (K/O) mice. The final fibrotic areas were determined by subtracting the control value from every mouse sample; mean and standard errors were calculated for each mice group (*$p<0.02$). (E) Immunoblot analysis for the expression of αSMA (upper) and β actin (lower) in the livers of mice treated with $CCL_4$ and in naïve mice. Representative whole-liver protein lysates extracts were obtained from three fibrotic wild type (WT) mice, from three NCR1-knockout (K/O) mice, from untreated wild type (C-WT) and from NCR1-knockout (C-K/O) mice. (F) Densitometrical analysis of the average of the αSMA expression presented in (E). *$p<0.02$. The findings presented in (C–F) are representative of two independent experiments that included 7–8 animals in each mice group.

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lysates taken from the treated mice (figure 1E). Densitometric analysis showed that the differences observed in αSMA levels between fibrotic and naïve mice are statistically significant (figure 1F). Thus, primary mHSC express an unknown ligand for NCR1 and the NCR1 receptor plays an important protective role during liver fibrosis.

### Mechanism of NCR1 protection

To investigate the mechanisms by which NCR1 protects against the development of liver fibrosis, we initially investigated whether the NK cell percentages in the liver were altered following $CCL_4$-mediated fibrosis induction, in the presence and in the absence of NCR1. For this, we used $NCR1^{gfp/gfp}$ (K/O) mice and NCR1 heterozygous ($NCR1^{gfp/+}$, HET) mice as NK cells in both mice are green fluorescence proteins (GFP) positive and the NCR1 heterozygous mice function normally.[18] As can be seen in figure 2A, a slight reduction in NK cell percentages was observed in both the HET and the K/O mice following fibrosis induction. These reductions however were not statistically significant (figure 2B). In addition, the absolute NK cell numbers were not significantly altered in both the HET and the K/O mice before and after fibrosis induction (data not shown).

Next, we tested whether the anatomical location of NK cells within the liver changes following fibrosis induction and whether the absence of NCR1 would affect this distribution. We performed confocal microscopy analysis prior to fibrosis induction (naïve), and 4 weeks later, following $CCL_4$ injection (fibrotic). NK cells were detected by their intrinsic GFP expression and activated HSC were detected by using anti αSMA mAb. As can be seen in figure 2C (which shows the NK cells of the NCR1 knockout mice), in naïve mice, αSMA expression could barely be detected and the green NK cells are scattered throughout the liver. In contrast, following fibrosis induction, NK cells were observed mainly in the peri-portal spaces near the portal canal, and extend along the active side of the fibrotic septa formations adjacent to the HSC (which are labelled red, figure 2C). Similar observations were noted in the NCR1 heterozygous mice (data not shown). These results demonstrate that following fibrosis induction, NK cells redistribute and are found in close proximity with HSC, suggesting that such proximity might enable a direct, NCR1-mediated interaction with mHSC.

Activation of NKp46/NCR1 leads to IFN-γ secretion,[14] killing induction,[5 15] or both.[18] It has been reported that IFN-γ deficient mice are more susceptible to liver fibrosis induced by administration of $CCl_4$.[35] Therefore, we tested whether incubation of NK cells with primary HSC will lead to the production of cytokines, but did not observed any effect on IFN-γ levels (data not shown). We next performed in vitro cytotoxicity assays with NK cells derived from NCR1 K/O and HET mice and with primary mHSC. The intrinsic GFP expression of HET and K/O mice (figure 3A) enabled us to accurately perform these killing experiments by using similar numbers of NK cells. Peripheral blood lymphocytes derived from the NCR1 knockout and heterozygous mice that were injected with or without polyinosine-polycytidylic acid (Poly I.C) were isolated and the NK cell percentages were determined. As can be seen, the GFP intensity of the KO mice is higher than that of the HET mice (figures 2A and 3A,B). This is because in the KO mice the two

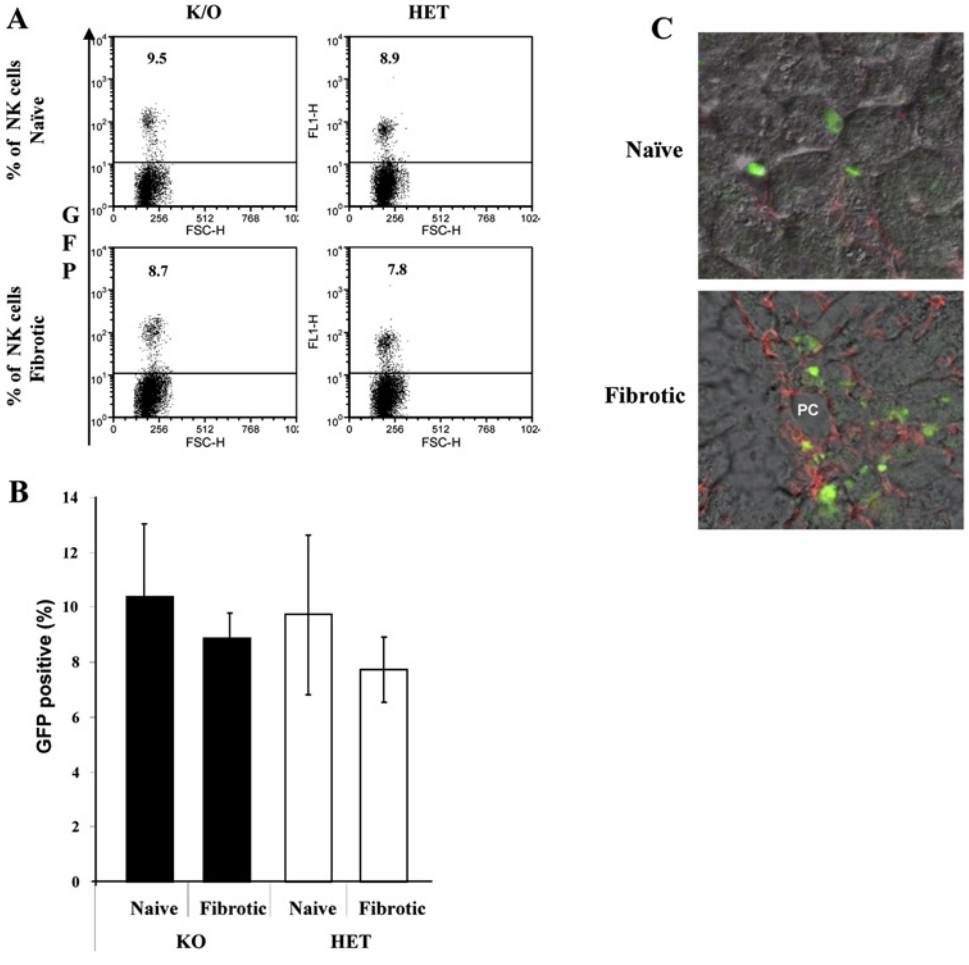


**Figure 2** Percentages and location of natural killer (NK) cells during liver fibrosis. (A) Fluorescence-activated cell sorter (FACS) analysis of GFP-positive NK cells in $NCR1^{gfp/gfp}$ (K/O) and in the $NCR1^{gfp/+}$ mice (HET), derived from naïve, untreated (upper dot plots), and fibrotic (lower dot plots) mice. The NK cell (GFP+ cells) percentages are depicted in the upper quadrants. The figure shows a pool of liver lymphocytes derived from 2–4 mice in each group. (B) Summary of the percentages of liver NK cell (GFP-positive cells) obtained from naïve and fibrotic mice of three independent experiments (2–4 mice in each group). There is no significant statistical difference. (C) Liver sections derived from naïve (upper), and fibrotic (lower) $NCR1^{gfp/gfp}$ mice were stained with antibodies against αSMA. NK cells were visualised using the endogenous GFP expression. The portal canal is indicated. The figure shows one representative experiment of three performed, each with three different animals.

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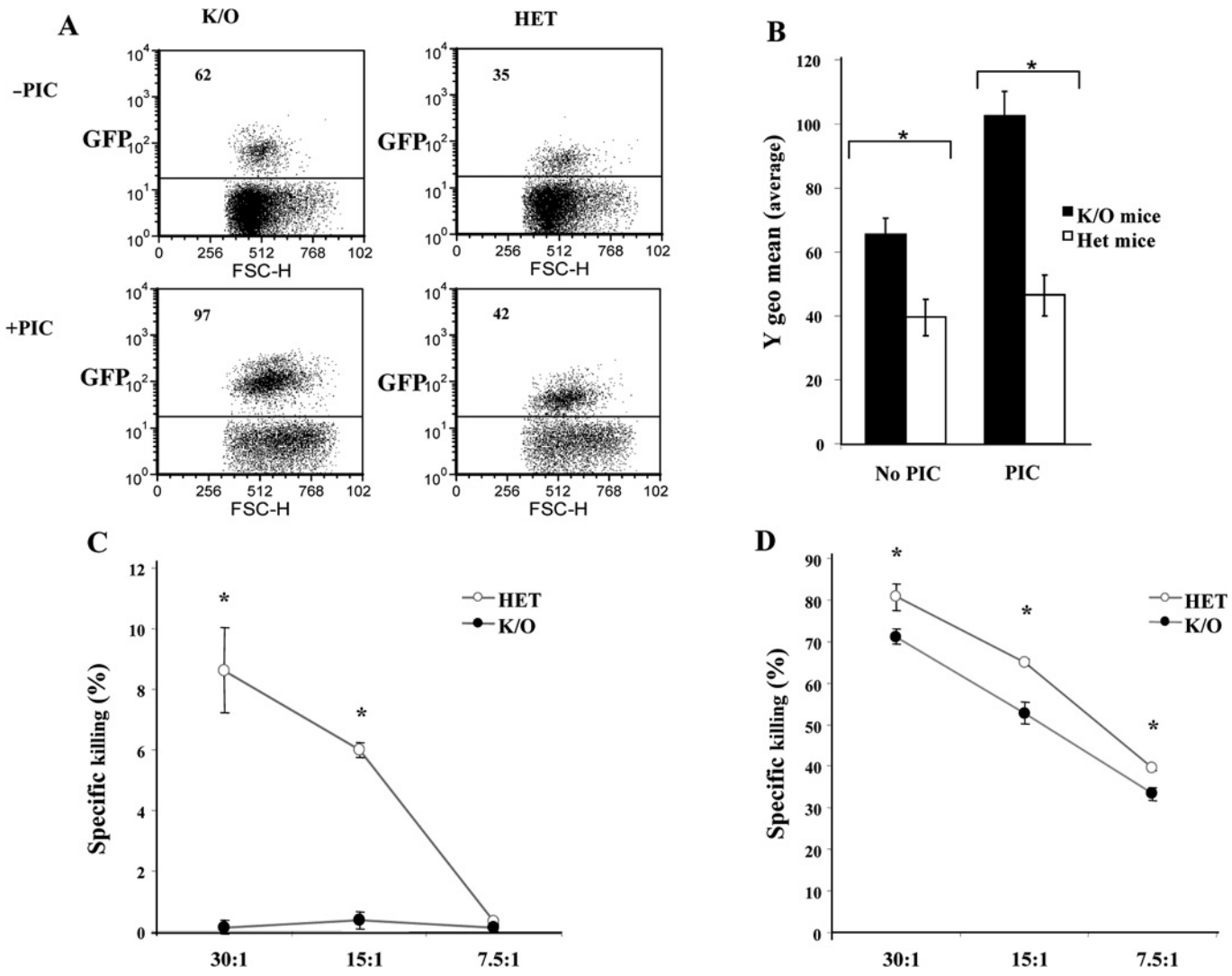


**Figure 3** Primary murine hepatic stellate cells are killed in an NCR1-dependent manner. (A) Fluorescence-activated cell sorter (FACS) analysis of GFP-positive natural killer (NK) cells derived from peripheral blood of five NCR1$^{gfp/gfp}$ (K/O) and NCR1$^{gfp/+}$ (HET) mice, with the injection (lower dot plots) or without (upper dot plots) the injection of Poly I.C. The Y geometric means of the GFP+ cells are indicated in the upper quadrants. (B) Average of the Y geometric (geo) means of the GFP expression in the NCR1$^{+/gfp}$ heterozygous (HET) and in the NCR1$^{gfp/gfp}$ knockout mice (K/O). Shown is the average of three independent experiments. *p<0.0005. (C—D) Murine HSC were labelled with $^{35}$S-methionine and then incubated with non-activated (C), or with Poly I.C activated (D) mouse NK cells derived from peripheral blood of the NCR1 heterozygous (HET), or homozygous (K/O) mice. *p<0.009. Representative of two independent experiments.

NCR1 alleles were replaced by GFP.[18] Furthermore, it seems that the GFP intensity increases following Poly I.C induction (figure 3A,B). The reasons for this are currently unknown.

Equal numbers of NK cells derived from the K/O and HET mice were incubated with primary mHSC. As can be seen in figure 3C, little or no killing of primary mHSC was observed when non-activated (without Poly I.C) NK cells were obtained from the NCR1 knockout mice, whereas moderate killing was detected with non-activated heterozygous NK cells. Following Poly I.C activation, the killing of NK cells was significantly enhanced (figure 3D), indicating that killer receptors, other than NKp46, are involved in the killing of mHSC by activated NK cells. Importantly, even in this setting, in the absence of NCR1 the killing of mHSC was significantly reduced (figure 3D). Thus, we concluded that NKp46 is the primary receptor involved in the killing of mHSC by non-activated NK cells, and that on NK cell activation, in addition to NKp46, other receptors such as NKG2D[23] are involved in the killing of mHSC.

### Primary human HSC express an unknown ligand for human NKp46

The above results demonstrate that NCR1 play an important role in protecting from liver fibrosis. To test whether these observations are relevant to humans, we next tested whether human primary HSC (hHSC) express an unknown ligand for NKp46. Primary hHSC that were isolated from healthy liver donors (see 'Material and methods'), were cultured with either 1% or 10% FCS for in vitro activation. Cells were then stained for the expression of αSMA (figure 4A) and for the expression of the NKp46 ligand. The NKp46 receptor is composed of two extracellular Ig-like domains (a membrane distal domain named D1 and a membrane proximal domain named D2), a stalk region and an intracellular domain.[34] We have previously demonstrated that the binding of NKp46 to viral haemagglutinins, to tumour cells and to β cells is mediated via the membrane proximal domain and the stalk region of NKp46.[12 34] To test whether primary hHSC are recognised by NKp46 and to identify the

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NKp46 domain that is involved in this interaction, we stained hHSC with NKp46D2-Ig (that contains the D2 domain and the stalk region), with NKp46D1-Ig (that contains the membrane distal domain), and with the intact NKp46-Ig protein. As can be seen in figure 4B, primary hHSC express a putative ligand for NKp46 and the NKp46 binding is confined, as before,[12 34] to the membrane proximal domain and the stalk region (NKp46D2-Ig, figure 4B). Furthermore, because human NKp46-Ig binds to mHSC (data not shown) and mouse NCR1 binds the hHSC (figure 4B), we concluded that the HSC ligands of NKp46 and

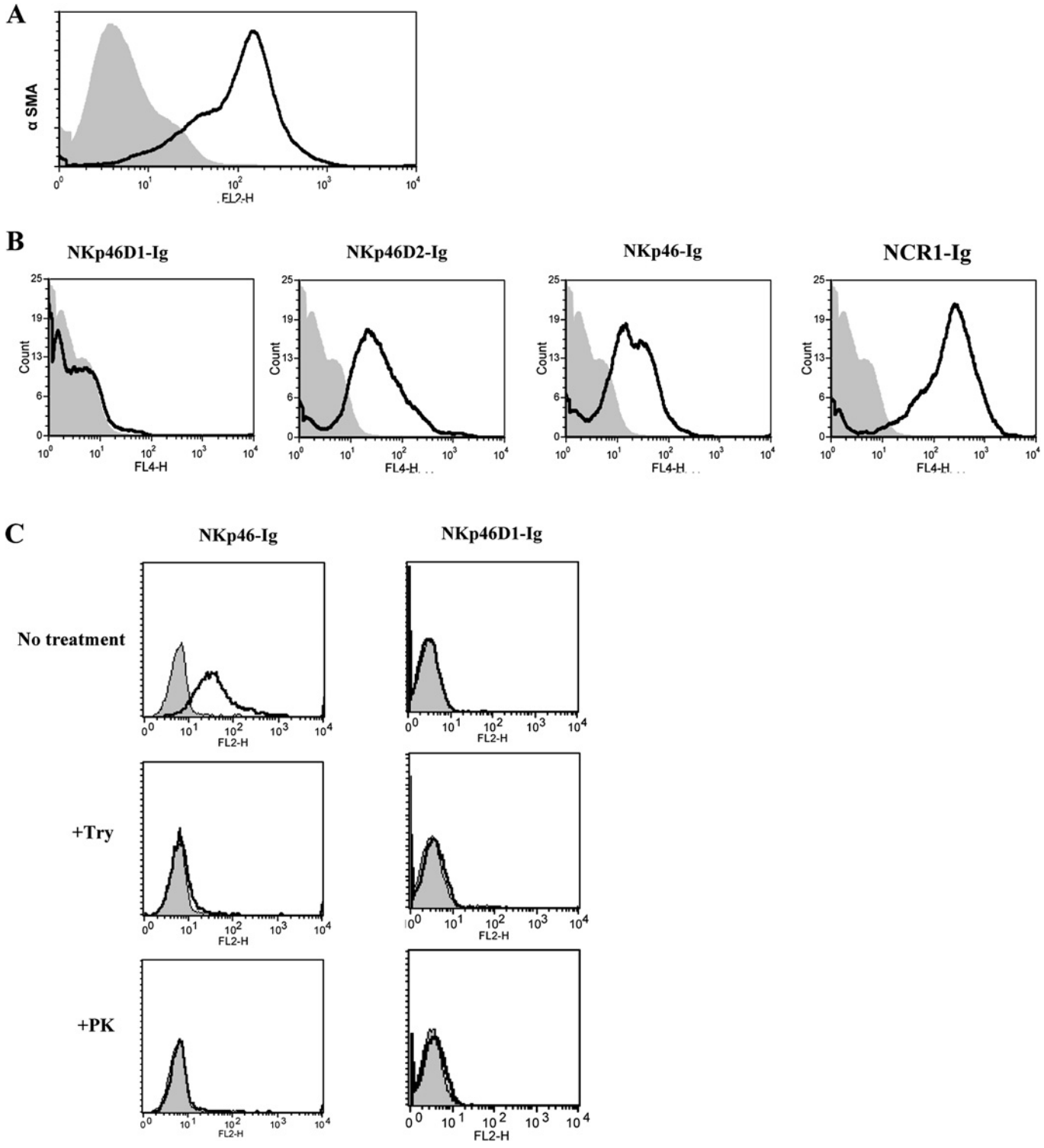


**Figure 4** Expression of NKp46 ligand on primary human hepatic stellate cells (hHSC). (A) Fluorescence-activated cell sorter (FACS) analysis of α-smooth muscle actin (αSMA) expression on primary hHSC. (B) Staining of primary hHSC with various variants of NKp46 (indicated above the histograms, black histograms). The filled grey histogram in (A) and (B) is the background staining performed with secondary monoclonal antibodies only. Representative of four independent experiments. (C) FACS staining of primary hHSC with NKp46-Ig and D1-Ig (black empty histograms) before (upper row) and after treatment with trypsin (+Try, middle row), or with proteinase K (+PK, lower row). The grey filled histogram is the background staining with secondary antibody only. The figure shows one representative experiment of the two performed.

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NCR1 are probably conserved. Similar results were obtained with non-activated primary hHSC (data not shown).

To obtain further information about the identity of the NKp46 ligand expressed by hHSC, we treated the primary hHSC with trypsin and proteinase K (PK), and then stained the treated cells with NKp46-Ig and NKp46D1-Ig fusion proteins. As shown in figure 4C, both treatments completely abrogated the NKp46-Ig binding, indicating that the NKp46 ligand is either a protein or a modification present on a protein. Similar observations were noted with mHSC (data not shown).

Next, we tested whether the triggering of NKp46 by its hHSC ligand will lead to NKp46 activation. For that purpose we used a cell-based reporter assay system in which BW thymoma cells are transfected with chimeric proteins composed of the extracellular domain of the NKp46 receptor, fused to the transmembrane domain and intracellular region of the mouse CD3-ζ (BWNKp46ζ). In this system the triggering of the NKp46 by its ligands leads to IL-2 secretion, thus reporting for the presence of functional NKp46 ligands. The expression of the NKp46 receptor on the BW cells was verified using specific mAbs (figure 5A).

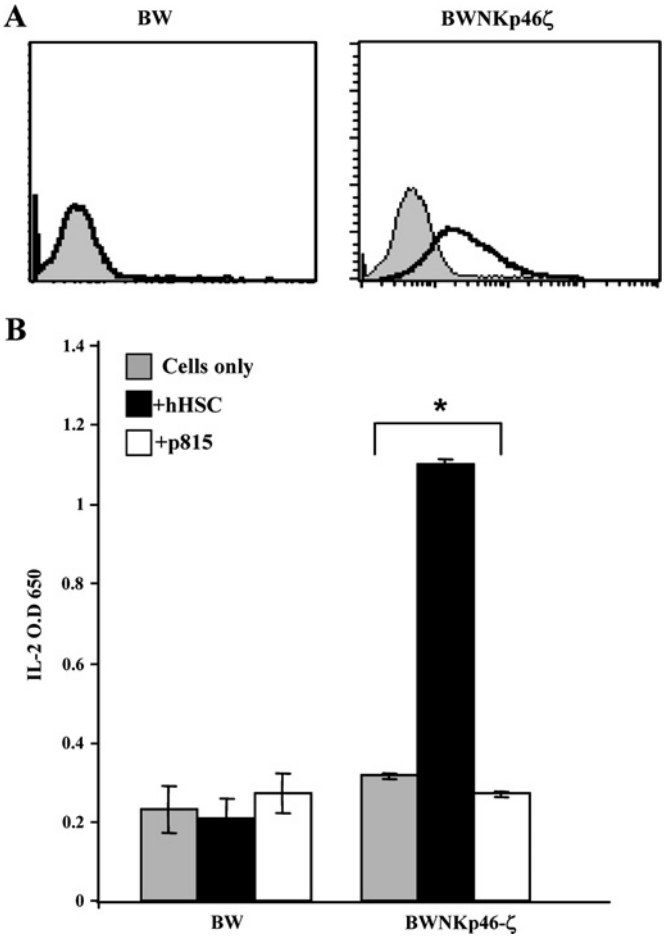


**Figure 5** Functional activation of the NKp46 receptor by its human hepatic stellate cells (hHSC) ligand. (A) Fluorescence-activated cell sorter (FACS) analysis of the BW and BW transfectant expressing the chimeric receptor NKp46-ζ using specific monoclonal antibodies. The filled grey histogram is the background staining with a secondary antibody only. Black lines represent the specific anti-NKp46 staining. Data from one of two independent staining is shown. (B) ELISA for IL-2 secretion by BW and the BWNKp46ζ (indicated on the x axis), following 48-h incubation with primary irradiated hHSC, with p815, or with no target cells. Values are mean ± SD for triplicate samples. Data from one of three independent experiments are shown. *$p<0.00014$.

Parental BW and BWNKp46ζ cells were incubated for 48 h with hHSC, as well as with p815 (known not to express the NKp46 ligand[18]). As can be seen in figure 5B, a significant increase of mouse IL-2 secretion was observed when the BWNKp46ζ were incubated together with the primary hHSC, but not with p815 cells. This suggests that primary hHSC express a functional ligand for NKp46.

### Primary hHSC are killed in an NKp46-dependent manner

To investigate the functional outcome of the interaction between NKp46 and hHSC, we initially generated polyclonal antibodies against NKp46 to block its activity. Mice were injected with NKp46-Ig and with a control CEA-Ig; the sera obtained were analysed for specific recognition using transfectants expressing NKp46 (BWNKp46ζ) and CEA (721.221 CEA) (figure 6A). The parental untransfected BW or 721.221 cells do not express the appropriate proteins (either NKp46 or CEA, data not shown). We validated the specificity of NKp46-Ig and the CEA-Ig sera and observed that the anti-NKp46 sera stained the BWNKp46ζ cells and the anti-CEA-Ig sera stained the 721.221 CEA cells (figure 6B). Then, we pre-incubated primary bulk human NK cells with no sera, with the anti-NKp46 sera, and with the control anti-CEA sera for 1 h, added the radioactive labelled primary hHSC, and evaluated the killing of the radioactive labelled cells 5 h later. Importantly, we observed that NKp46 is involved in the killing of primary hHSC, as the killing of these cells was significantly reduced by the anti-NKp46 sera but not by the anti-CEA sera (figure 6C). Thus, human and mouse HSC express unknown ligands for NKp46 and are killed in an NKp46-dependent manner.

## DISCUSSION

The liver is a special organ, not only because of its anatomical location and its function, but also because of its unique lymphocyte composition. Indeed, NK cells constitute 30–50% of all human hepatic lymphocytes,[1 2] while peripheral blood NK cell percentages are limited up to about 15%.[1] These large percentages of hepatic NK cells are reminiscent of the NK cell percentages that are present in the decidua during pregnancy (50–80%).[36] However, decidual NK cells lack CD16 and are almost unable to kill, whereas liver NK cells are potent killers, although almost all of them lack CD16.[2] Another interesting and unique aspect of mouse liver NK cells is that they acquire and retain antigen-specific memory against haptens and virus-derived antigens.[37] A possible explanation for the presence of such substantial numbers of NK cells in the liver might be the constitutive exposure of the liver to various pathogens such as gut-derived bacteria and viruses, for example HCV.[26]

Here we demonstrate for the first time, both in vivo and in vitro, in humans and in mice, that NKp46 protects from liver fibrosis through the killing of primary HSC. The function of NKp46 is most critical in non-activated NK cells as the killing of primary mHSC was completely abolished in the absence of NKp46. Following NK activation, the killing of primary mHSC was also significantly enhanced in the knockout mice, suggesting that apart from NKp46, other receptors are also involved in the killing of mHSC by activated NK cells. Indeed, it was previously demonstrated in both mice and in humans that stellate cells express several ligands for NKG2D and that the interaction between NKG2D and its ligands leads to the killing of primary HSC by activated NK cells.[23]

The findings that liver NK cells as well as decidual NK cells are involved in tissue repair and remodelling mechanisms and that NKp46 is involved in both activities (as shown in this paper, and

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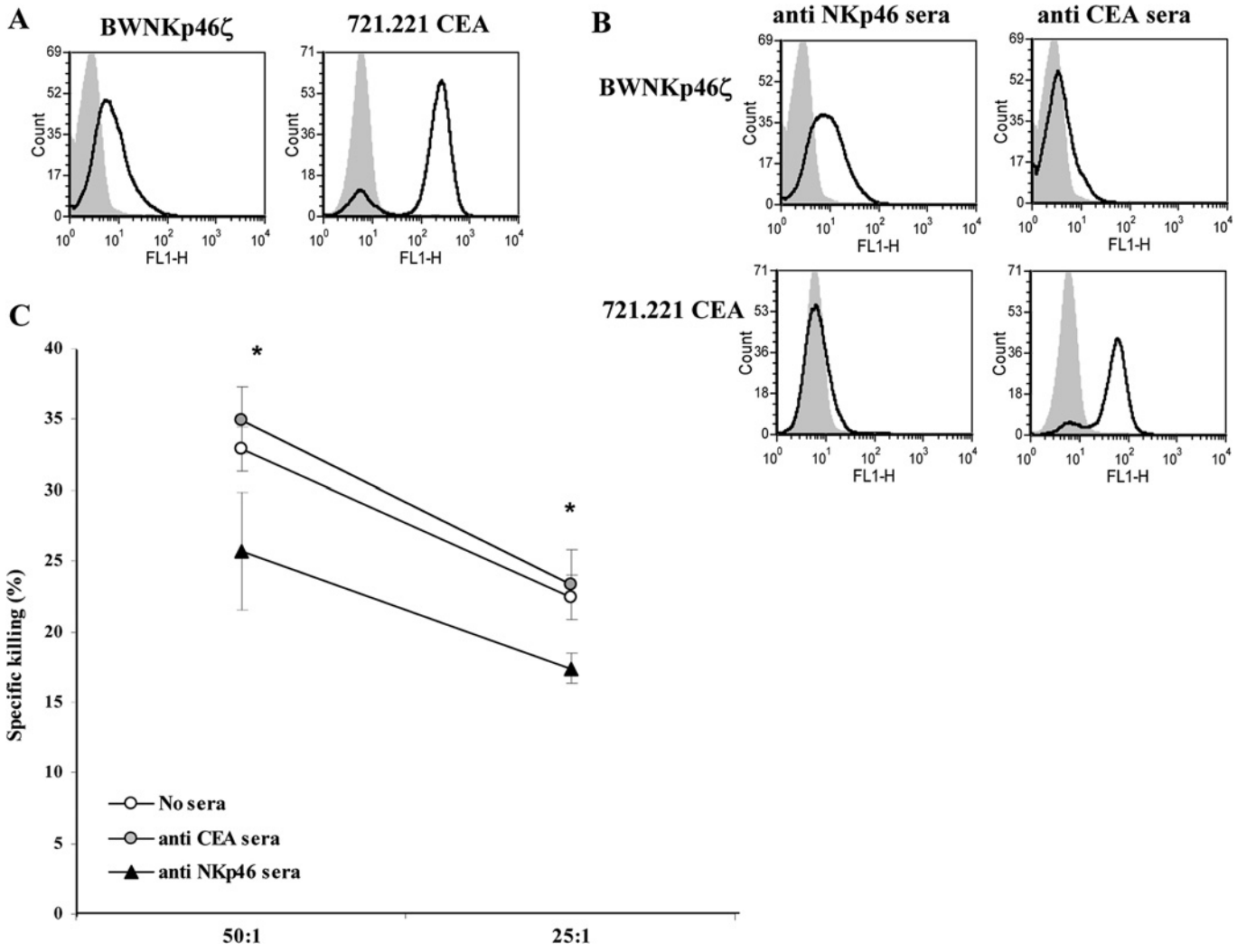


**Figure 6** Primary human hepatic stellate cells (HSC) are killed in an NKp46-dependent manner. (A-B) Fluorescence-activated cell sorter (FACS) analysis of the BW and 721.221 cells expressing the chimeric receptor NKp46-ζ and CEA, respectively, stained with specific monoclonal antibodies (mAbs) (A), and with sera derived from mice immunised with NKp46-Ig and CEA-Ig (B). The filled grey histogram is the background staining with a secondary antibody only. Data from one of two independent stainings is shown. (C) Bulk natural killer (NK) cells were pre-incubated with no Ab, anti-NKp46 sera or with control sera (CEA sera) and then co-incubated with human primary HSC at the indicated E:T ratios for 5 h. Shown are mean values of primary HSC killing (represent as percentages) ± SD. Error bars (SD) are derived from triplicates. *$p<0.05$ (anti-CEA sera and no Ab compared to anti-NKp46 sera in E:T 50:1, and 25:1). Representative of two independent experiments.

also by Hanna *et al*[38]) suggests that NK cells that are found in high numbers in other anatomical places might also have NKp46-related tissue remodelling and tissue repair functions. In this regard, lung NK cells are particularly interesting. They consist of around 20–30% of lung lymphocytes,[3] and it has been demonstrated that NKp46 is critical for fighting influenza virus infections in the lungs.[18] We therefore suggest that lung NK cells might also posses additional functions, such as protection from lung fibrosis.

We also demonstrate that the NK cell percentages and numbers do not significantly change following the induction of liver fibrosis. We showed that the anatomical location of liver NK cells within the tissue is altered and that following fibrosis induction, NK cells are seen in proximity to HSC. On the other hand, in NOD mice, a murine model for type 1 diabetes, NK cells are not found in the normal pancreas and are seen in proximity to β cells only during the pre-diabetic stage.[5] We suggest that these redistribution (liver NK cells) or accumulation (NOD NK cells) mechanisms were probably developed to prevent autoimmunity under normal, steady state conditions. Indeed, non-activated, quiescent, primary human HSCs (data not shown) and healthy β cells[5 34] express the unknown NKp46 ligand. A thorough investigation of the mechanisms leading to pancreatic NK cell accumulation in NOD mice and to the redistribution of liver NK cells during fibrosis might lead to improved treatment of both diseases.

Little is known about the involvement of NKp46 in other liver diseases. To the best of our knowledge, the interaction of NKp46 with hepatocellular carcinoma was not investigated. However, all hepatocellular carcinoma cell lines that we have tested, including HuH7, Hep3b and HepG2, express an unknown NKp46 ligand and are killed in an NKp46-dependent manner (data not shown). Contradictive results were obtained regarding the expression and the function of NKp46 in patients suffering from chronic hepatitis C infections. While some reports demonstrated reduced NKp46 expression,[39] others have shown that NKp46 expression is actually increased.[40] The reasons for these discrepancies are unknown. Nevertheless, regardless of

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whether NKp46 expression is increased or decreased, together with the observations presented here, it seems that NKp46 plays a critical role in various liver diseases.

NKp46 recognises various ligands: pathogen-derived ligands such as influenza hemagglutinins,[4 12 13] unknown tumour ligands,[4] and unknown self ligands expressed by β cells[5 34] and by HSC (as shown in this paper). The various ligands are different from each other and are recognised by NKp46 using various mechanisms[5 12 13 34] (also shown in this paper). The fact that NKp46 recognises different ligands is not surprising as NK cells have a limited number of killer receptors that are programmed to recognise different pathogens, tumours and even self cells. The identification of the NKp46 ligands is therefore of particular importance as it can lead to the development of new therapeutic modalities for the treatment of virus infections, tumour transformation, type I diabetes, and as shown here, liver fibrosis.

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**Contributors** CG performed the experiments, analysed data, wrote the manuscript; SD, SK-E, EH and LA-t provided essential reagents; RS and OM supervised the project.

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# NKp46-mediated killing of human and mouse hepatic stellate cells attenuates liver fibrosis

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