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ORIGINAL ARTICLE

hIFN-α gene modification augments human natural killer cell line anti-human hepatocellular carcinoma function

W Jiang, C Zhang, Z Tian and J Zhang

Natural killer (NK) cells are characterized by an efficient antitumor activity, and this activity has been exploited as the basis of cancer immunotherapy strategies. Interferon-α (IFN-α) is an important cytokine required for induction of the durable antitumor immune response and is an important stimulator of NK cells. In this study, to augment the efficiency of NK cell cytotoxicity to tumor cells, human *IFN-α* gene-modified natural killer cell line (NKL) (NKL-IFNα) cells, which could stably secrete IFN-α, were established. We investigated the natural cytotoxicity of NKL-IFNα cells against human hepatocarcinoma cells (HCCs) *in vitro* and *in vivo*. NKL-IFNα cells displayed a significantly stronger cytolytic activity against both human HCC cell lines and primary human hepatoma cancer cells compared with parental NKL cells. The increased cytolytic activity of NKL-IFNα cells was associated with the upregulation of cytotoxicity-related genes, such as *perforin*, *granzyme B* and *Fas ligand*, in the NK cells. Moreover, cytokines secreted by NKL-IFNα cells, such as tumor necrosis factor-α and IFN-γ, induced increased expression of Fas on the target HCC cells, and resulted in increased susceptibility of the HCC cells to NK-mediated cytolysis. Encouragingly, NKL-IFNα cells could significantly inhibit HCC tumor growth in a xenograft model and prolonged the survival of tumor-bearing nude mice. These results suggest that *IFN-α* gene-modified NKL cells could be suitable for the future development of cell-based immunotherapeutic strategies for hepatocellular carcinoma.

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INTRODUCTION

Natural killer (NK) cells are an important component of the innate immune system, and characterized by a strong cytolytic activity against various types of tumor cells and virus-infected cells via non-major histocompatibility complex (MHC)-restricted and non-T-cell receptor-restricted mechanisms.[1,2] As an effector cell population, the intrinsic advantages of NK cells have led to the introduction of innovative strategies for cancer treatment. NK cell-based immunotherapy has been in development since the 1980s, and can potentially be implemented through the administration of cytokines or immunomodulatory drugs to activate endogenous NK cells,[3,4] or the adoptive transfer of induced alloreactive NK cells developed from allogeneic stem cells, *ex vivo* expanded autologous NK cells[5] or donor-derived allogenic NK cells.[6] Owing to the difficulties of generating sufficient numbers of highly purified NK cells to meet clinical requirements, NK cell lines have become increasingly attractive for their unlimited ability to proliferate under good manufacturing practice conditions.[7] More importantly, gene modification, by which NK cell-acquired specificity and the cytolytic activity against tumor cells can be augmented, is more feasible in NK cell lines than primary NK cells.[8] Therefore, NK cell lines could be a useful tool for clinical adoptive immunotherapy.

The natural killer cell line (NKL) is a well-characterized malignant NK cell line, which was established from the peripheral blood of a patient with $CD3^{-}CD16^{+}CD56^{+}$ large granular lymphocyte leukemia. The morphology of NKL cells resembles that of normal activated NK cells, with a $CD16^{+}CD56^{dim}$ phenotype and very similar natural killing, antibody-dependent cellular cytotoxicity and proliferative responses.[9] Of all NK cell lines, the NKL cell line had retained most of the original features of NK cells and could therefore potentially be used as a source of effector cells for adoptive immunotherapy.[10]

NK cells not only produce a number of cytokines, through which they can suppress tumor cell proliferation, but they also respond to numerous cytokines, such as interleukin (IL)-1, IL-2, IL-12, IL-15, IL-18, IL-21 and type I interferon (IFN) α and β, which increase the cytolytic activity, cytokine production, proliferation and antitumor effects of NK cells.[11] Previously, we established *IL-15* gene-modified NKL and NK-92 cells with augmented antitumor ability.[12,13] However, although the antitumor effects of NKL cells against HCC were enhanced by *IL-15* gene modification, only some types of HCC cells were susceptible to NKL-IL-15 cells.[12] Since then, we have been trying to establish a novel gene-modified NK cell line to act as a supplement for adaptive immunotherapy in patients with HCC.

IFN-α, a pleiotropic type I IFN, is one of the most important cytokines that activates NK cells. IFN-α is extensively used in the treatment of patients with hematological malignancies or certain solid tumors, such as melanoma and renal carcinoma.[14] IFN-α not only affects tumor cell function, for example, by downregulating oncogene expression and inducing tumor suppressor genes, but can also promote the differentiation and activity of host immune cells, including T cells, dendritic cells and NK cells, resulting in IFN-α-induced antitumor immunity.[15,16] IFN-α has also been widely used in the gene modification of dendritic cells for cancer immunotherapy.[17]

On this basis, we used electroporation technology to modify genetically NKL cells with the human IFN-α gene (*hIFNα*) and

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explored the potential of NKL-IFNα cells for HCC adaptive immunotherapy. Here, we report that NKL-IFNα cells exerted efficacious cytolysis against HCC cells, including HCC cell lines and primary HCC cells. Further investigation demonstrated that the expression of Fas on HCC cells was increased by tumor necrosis factor-α (TNFα) and IFNγ secreted by NKL-IFNα cells, which augmented NKL-IFNα-mediated cytolysis against HCC cells. Furthermore, adoptive transfer of NKL-IFNα cells significantly inhibited the growth of HepG2 xenograft tumors and prolonged the survival of mice bearing HCC xenograft tumors. This study provides evidence to indicate that human IFN-α gene-modified NKL cells may represent a potentially effective immunotherapy strategy for patients with liver cancer.

RESULTS

Establishment of the *hIFN-α* gene-modified NKL cell line

IFN-α, a type I IFN, exerts a variety of biological effects, including antitumor activity, and has been used in clinical oncology.[14] NKL cells are a typical immature NK cell line with a $CD16^{+}CD56^{dim}$ phenotype, which resemble activated NK cells.[9] In this study, we transferred the human *INF-α* (*hIFN-α*) gene into NKL cells using an electroporation method followed by Zeocin selection. As shown in Figure 1a, the levels of *IFN-α* mRNA in *hIFN-α* gene-modified NKL (NKL-IFNα) cells was significantly higher than the parental NKL cells. To confirm whether *IFN-α* gene-modified NKL cells secrete soluble IFN-α, an enzyme-linked immunosorbent assay (ELISA) was performed to quantify the levels of IFN-α secreted into the supernatant of NKL-IFNα cells and NKL cells transfected with the empty vector (NKL-vec cells). As shown in Figure 1b, the concentration of IFN-α in the supernatant of NKL-IFNα cells cultured for 24 h was 241.38 ± 42.55 pg ml^{-1} per million cells, which increased to 305.51 ± 17.07 pg ml^{-1} per million cells after culture for 48 h. In contrast, the levels of IFN-α in the supernatant of NKL-vec cells and NKL cells cultured for 48 h were only 48.62 ± 22.97 and 59.25 ± 17.52 pg ml^{-1} per million cells, respectively. Moreover, NKL-IFNα cells exhibited similar morphologic characteristics to the parental NKL cells and were suitable for long-term culture *in vitro*. Collectively, these results indicated that we had successfully established an *hIFN-α* gene-modified NKL cell line.

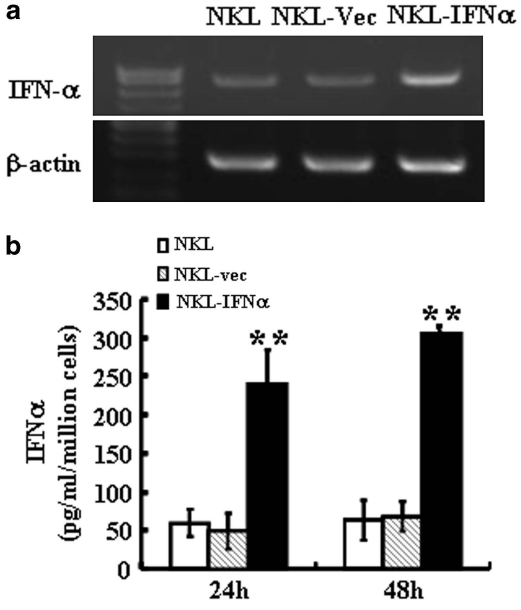

Figure 1. Human *IFN-α* gene modification of the NKL cell line. As described in the Materials and methods section, NKL cells were transfected with the human (h) *IFN-α* gene and selected using Zeocin (NKL-IFNα), while psectagB vector-transfected NKL cells (NKL-vec) were used as a control. (**a**) Total cellular RNA was isolated and the mRNA levels of the *hIFN-α* gene were analyzed by RT-PCR. (**b**) After washing with PBS, *IFN-α* gene-modified NKL cells and parental NKL cells were cultured in complete RPMI-1640 medium with 100 U ml^{-1} IL-2 for 24 or 48 h. The supernatants were harvested and an ELISA was performed to analyze the levels of IFN-α in the supernatants. Data shown are mean ± s.d. of at least three independent experiments. $^{**}P < 0.01$ in comparison with NKL-vec cells.

IFN-α gene modification enhances NKL cell-mediated natural cytotoxicity against HCCs

To investigate the function of NKL-IFNα cells, three human HCC cell lines, HepG2, H7402 and HepG2.2.15, were used as target cells. As shown in Figure 2, NKL-IFNα cells were significantly more cytotoxic to HepG2 and H7402 cells than NKL-vec cells at effector:target (E:T) ratios of 10:1 and 5:1. In addition, NKL-IFNα cells were also significantly more cytotoxic to HepG2.2.15 cells, a hepatitis B virus-positive (HBV+) HCC cell line, than NKL-vec cells. To further evaluate the cytotoxicity of NKL-IFNα cells, primary human hepatoma cancer cells isolated from three HCC patients were used as target cells. NKL-IFNα cells were significantly more cytotoxic to all three primary human hepatoma cancer cell lines than NKL-vec cells; the cytotoxicity of NKL-IFNα cells was $82.6 \pm 5.1\%$, while the cytotoxicity of NKL-vec cells was $52.4 \pm 12.4\%$ at the lowest E:T ratio (2.5:1). These data indicated that NKL-IFNα cells displayed an augmented cytotoxicity against HCC. In addition, as shown in Figure 2, the difference between NKL-IFNα cell- and NKL-vec cell-mediated cytolysis against HCC

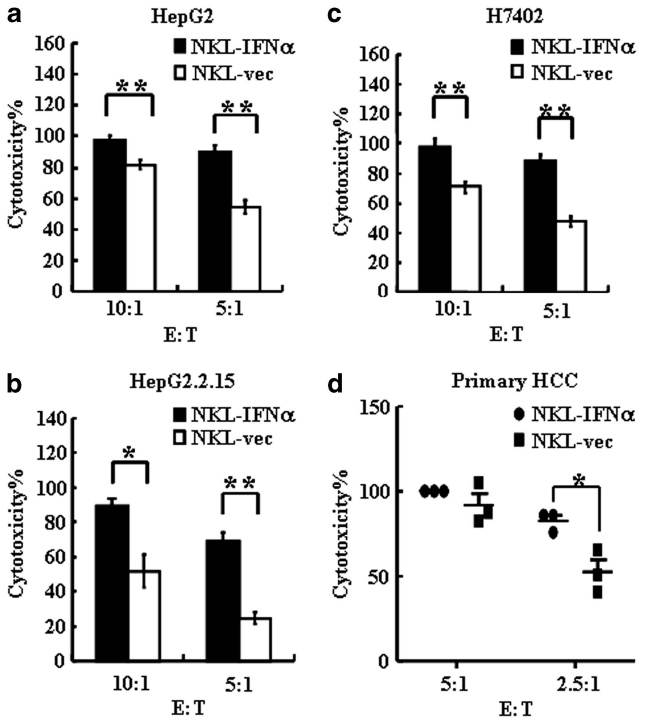

Figure 2. Cytotoxicity of *IFN-α* gene-modified NKL cells against human HCC cells. (**a**–**c**) NKL-IFNα cells or NKL-vec cells were used as effector (E) cells and human HCC cell lines were used as target (T) cells. NK cell-mediated lysis of HCC cells was assessed by the MTT assay at various E:T ratios. Data shown are mean ± s.d. of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus NKL-vec cells. (**d**) Primary human hepatoma cancer cells isolated from three hepatocellular carcinoma patients were used as target cells. NK cell-mediated lysis of the HCC cells by NKL-IFNα cells or NKL-vec cells was assessed by the MTT assay. $^{*}P < 0.05$ versus NKL-vec cells, Wilcoxon–Mann–Whitney test.

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cells was reduced by increasing the E:T ratio, which suggests that a strategy of increasing the number of NK cells could be a useful therapeutic option.

To establish the effects of INF-α on NK cells themselves and/or the tumor cells, HepG2 cells were pretreated with or without 200 pg ml^{-1} IFN-α for 6 h. IFN-α was removed by washing, and the cells were then exposed to parental NKL cells for the cytotoxicity assay. There was no significant difference between the cytotoxicity of NKL cells against IFN-α-treated and -untreated HepG2 cells at E:T ratios of 10:1 and 5:1 (Supplementary Figure S1). These results indicate that the enhanced natural cytotoxicity of NKL-INFα cells against human HCC cells is mediated directly by the effects of IFN-α on the NKL cells, rather than by IFN-α acting on the tumor cells.

IFN-α gene modification increases the activation of NKL cells

The activation and cytotoxicity of NK cells are influenced by the expression of cytotoxicity-associated molecules, including NKG2D, NKG2A, TNF-α, IFN-γ, perforin, granzyme B and Fas ligand (FasL). The expression of *TNF-α*, *IFN-γ*, *perforin*, *granzyme B* and *FasL* mRNAs were markedly increased in NKL-IFNα cells, while the expression of *NKG2D* increased slightly and *NKG2A* was not different compared with NKL-vec cells (Figure 3a). Next, the protein levels of TNF-α, perforin, granzyme B and FasL were determined by fluorescence-activated cell sorter (Figure 3b). As shown in Figure 3c, NKL-IFNα cells expressed higher levels of IFN-γ than NKL-vec cells. Stimulation with phorbol 12-myristate 13-acetate (PMA)/ionomycin for 48 h increased the concentration of IFN-γ secreted by NKL-IFNα cells from 1786 ± 115 to 7299 ± 447 pg ml^{-1} (Figure 3d). As PMA/ionomycin did not have a significantly different effect on the number of NKL-IFNα cells and NKL-vec cells, the increased production of IFN-γ was mainly associated with the activation of NKL-IFNα and NKL-vec cells by PMA/ionomycin. These results indicate that the *hIFN-α* gene modification altered the expression of cytotoxicity-associated genes, which in turn enhanced the natural cytotoxicity of NKL cells against HCC cells.

Elevated Fas on HCC cells contributes to the augmented sensitivity of HCC cells to NKL-IFNα-mediated cytolysis

The interactions between receptors expressed on NK cells and the ligands expressed on target cells determine the activation of NK cells. As NKG2D is an important activating receptor in NK cells, firstly we analyzed the expression of NKG2D ligands in HCC cells by quantitiative reverse transcription-polymerase chain reaction (RT-PCR). However, the transcript levels of NKG2D ligands, including *MICA/B* and *ULBP1-4*, did not alter in HCC cells after coculture with NKL-IFNα cells (data not shown). The expression of MHC I molecules on tumor cells can be induced by IFNα, which is expected to decrease the activity of NK cells.[18] Therefore, we analyzed the levels of MHC I molecules in HepG2 cells cocultured with NKL-IFNα or NKL-vec cells for 24 h at a ratio of 1:1 in a Transwell system. As shown in Supplementary Figure S2, NKL-IFNα cells did not obviously increase the expression of HLA-ABC on HepG2 cells compared with NKL-vec cells.

Next, to determine whether NKL-IFNα cells had enhanced natural cytotoxic effects by inducing apoptosis in HCC cells, we quantified the levels of three apoptosis-associated genes, *Bcl-2*, *Mcl-1* and *Fas*, in HCC cells. As shown in Figure 4a, *Fas* mRNA level increased markedly after coculture with NKL-IFNα cells, whereas the expression of *Bcl-2* and *Mcl-1* was not obviously different

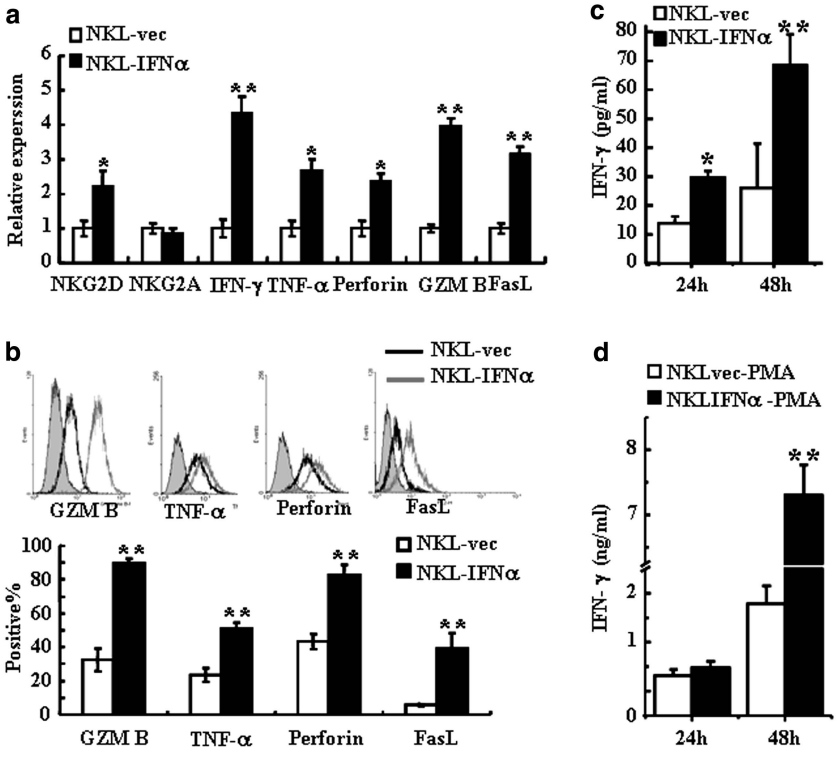

Figure 3. Expression of cytotoxicity-associated genes by *IFN-α* gene-modified NKL cells. NKL-IFNα and NKL-vec cells were harvested, and the expression of cytotoxicity-associated genes was determined by qRT-PCR (**a**) and flow cytometry (**b**). Data are expressed as the fold change in mRNA expression normalized to NKL-vec cells, or as the percentage of positively-stained cells. One representative experiment of at least three independent experiments is shown (**b**, upper), data are the mean ± s.d. of at least three independent experiments (**b**, lower). (**c**) Concentration of IFN-γ produced by NKL-IFNα and NKL-vec cells, as determined by an ELISA assay. (**d**) Production of IFN-γ by NKL-IFNα and NKL-vec cells after stimulation with PMA/ionomycin for 24 or 48 h, as evaluated by an ELISA. Data shown are mean ± s.d. of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus NKL-vec cells.

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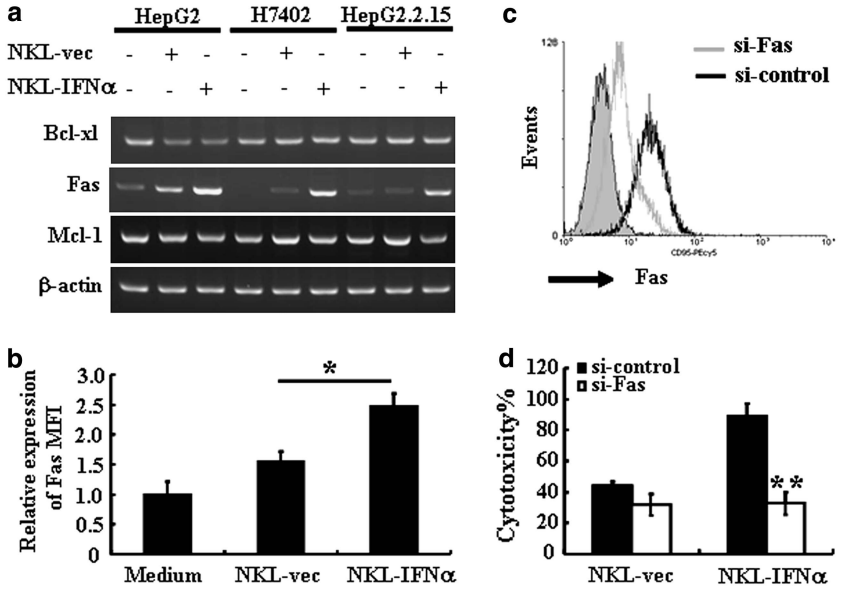

Figure 4. Upregulation of Fas contributes to the susceptibility of HCC cells to *IFNα* gene-modified NKL cell cytolysis. (**a**) HepG2, H7402 or HepG2.2.15 cells were cocultured with NKL-IFNα or NKL-vec cells using Transwell inserts. After 24 h, the expression levels of *Bcl-xl*, *Fas* and *Mcl-1* in the HCC cells were determined by qRT-PCR. One representative experiment of at least three independent experiments is shown. (**b**) HepG2 cells were cocultured with NKL-IFNα cells or NKL-vec cells using Transwell inserts. After 24 h, the expression of Fas protein by HepG2 cells was determined by flow cytometry. (**c**) Following transfection with either 50 nM *Fas* siRNA (si-Fas) or si-control using Lipofectamine 2000 for 24 h, the expression of Fas on HepG2 cells was determined by flow cytometry. (**d**) The cytolytic activity of NKL-IFNα or NKL-vec cells against HepG2 cells transfected with si-Fas or si-control was assessed at various E:T ratios. Data shown are mean ± s.d. of at least three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ versus NKL-vec cells.

compared with HCC cells cocultured with NKL-vec cells. Furthermore, a flow cytometry assay was used to quantify the protein expression level of Fas in HepG2 cells. As shown in Figure 4b, HCC cells cocultured with NKL-IFNα cells for 24 h expressed higher levels of Fas than HCC cells cocultured with NKL-vec cells. To further confirm the contribution of Fas in HCC cells to the cytotoxicity of *IFN-α* gene-modified NKL cells, a small interfering RNA (siRNA) interference experiment was performed (Figure 4c). We observed that the sensitivity of HepG2 cells, which were treated with *Fas* siRNA for 24 h, to NKL-IFNα cell-mediated cytolysis was significantly inhibited, and NKL-IFNα cell-mediated cytolysis against *Fas* siRNA-treated HepG2 cells was similar to the levels as NKL-vec cell-mediated cytolysis at an E:T ratio of 5:1 (Figure 4d), suggesting that elevated Fas expressed on HCC cells has a critical role in NKL-IFNα cell-mediated cytolysis.

TNF-α and IFN-γ secreted by NKL-IFNα cells induce the expression of Fas on HCC cells

It has been documented that expression of Fas can be induced on the surface of HCC cells by stimulation with TNF-α and IFN-γ.[19] To investigate whether these cytokines contribute to the upregulated expression of Fas on HCC cells, NK cells were treated with TNF-α- or/and an IFN-γ-blocking mAb before coincubation with HepG2 cells. As shown in Figure 5a, blocking both TNF-α and IFN-γ significantly decreased the expression of Fas on HepG2 cells, concomitant with a suppression of NK cell-mediated lysis against HepG2 cells (Figure 5b). These data demonstrate that cytokines produced by *IFN-α* gene-modified NKL cells, such as TNF-α and IFN-γ, are also involved in NKL-IFNα cell-mediated cytolysis, possibly by inducing increased expression of Fas on the target cells.

IFN-α gene-modified NKL cells exert an augmented antitumor effect in a xenograft model of HCC in nude mice

To further assess the application of *IFN-α* gene-modified NKL cells in adoptive cellular immunotherapy, different therapeutic schedules were investigated in a xenograft model of HCC in nude mice. Firstly, HepG2 cells were intraperitoneally injected into the nude mice, and then 5×10^7 irradiated NKL-IFNα cells, 5×10^7 irradiated NKL-vec cells or phosphate-buffered saline (PBS) were intravenously injected on day 0 (Figure 6a), or on days 7 and 14 after tumor cell inoculation (Figure 6b). In this experiment, the survival of mice treated with NKL-IFNα cells was significantly prolonged, compared to mice injected with NKL-vec cells or PBS. As shown in Figure 6a, 60% of the HepG2-bearing mice treated with NKL-IFNα cells survived longer than 90 days (the last follow-up time), whereas all of the PBS-treated mice died within 60 days after HepG2 cell inoculation. The percentage of mice surviving for 90 days increased from 17 to 50% in the tumor-bearing mice, which were treated two times with NKL-IFNα cells on days 7 and 14 after tumor cell inoculation, and the specific survival effect of NKL-INFα cells was approximately 30% higher than in NKL-vec cells (Figure 6b). These data suggest that *IFN-α* gene-modified NKL cells inhibited the growth and development of HCC at both the early and advanced stages of disease.

Alternatively, the female mice were subcutaneously injected with HepG2 cells and treated intravenously with 5×10^7 irradiated NKL-IFNα cells or NKL-vec cells, or PBS on days 7 and 14 after tumor cell inoculation. Photographs of the tumors dissected from the female nude mice and the tumor growth curves are presented in Figure 6c. After approximately 40 days, the tumor volumes in the PBS control group and NKL-vec cell-treated group were 862 ± 55 and $621\pm30\,mm^3$, respectively, whereas the tumor volume of the NKL-IFNα cell-treated group was only $216\pm33\,mm^3$ ($P<0.01$), indicating that NKL-IFNα cells inhibited tumor growth by 74.9%. The same trend was obtained in the tumor growth curves of the male nude mice (Figure 6d). These results indicate that treatment with NKL-IFNα cells is effective in suppressing the tumor growth and prolonging the survival time of mice bearing HCC xenograft tumors.

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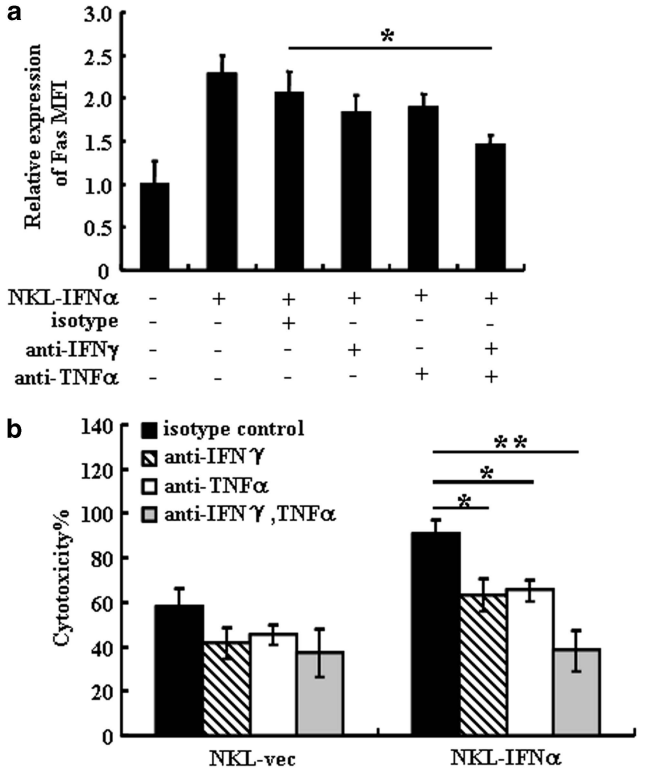

Figure 5. Secretion of the cytokines TNF-α and IFN-γ by *IFN-α* gene-modified NKL cells mediates the induction of Fas expression on HCC cells. NKL-IFNα cells or NKL-vec cells were treated with an anti-TNF-α mAb, anti-IFN-γ mAb or the isotype control mAbs. (**a**) HepG2 cells were incubated with the NKL cells in the presence or absence of the anti-TNF-α mAb and/or anti-IFN-γ mAb for 24 h, and then the expression of Fas by HepG2 cells was determined by flow cytometry. (**b**) The cytotoxicity of NKL-IFNα cells or NKL-vec cells against HepG2 cells (at an E:T ratio of 5:1) in the presence or absence of the anti-TNF-α mAb, anti-IFN-γ mAb or isotype control mAbs was assessed by the MTT method. Data are mean ± s.d. of at least three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ versus NKL-vec cells.

DISCUSSION

NK cells have an important role in the first line of defense against viral infection and tumor cells, and act as an essential part of the link between the adaptive and innate immune systems.[20] Therefore, the application of NK cells in human cancer immunotherapy is gaining increasing attention, and treatments using NK cells have recently entered clinical trials.[21] Owing to their unlimited ability to proliferate under good manufacturing practice conditions, NK cell lines provide an attractive source of cells for NK cell-based cellular immunotherapy.[22] Most importantly, the antitumor activities of NK cell lines can be further enhanced by genetic engineering, to make the cells more cytotoxic or more easily expanded and maintained *in vitro*.[7] In previous work, several cytokine genes, including *IL-2*, *IL-15* and stem cell factor (*SCF*), were adopted for gene modification to augment the activation of NK cells and increase their cytotoxicity towards tumor cells.[12,13,23,24] However, the spectra of target cells, which are susceptible to cytolysis by different gene-modified NK cells, can vary. Therefore, it is necessary to research and develop various kinds of gene-modified NK cells for application in clinical cancer therapy.

As an important factor linking innate and adaptive immunity, IFN-α acts directly on T cells and NK cells to induce phenotypic activation, and prevent activation-induced cell death of cytotoxic T-lymphocytes.[25,26] IFN-α, a pleiotropic cytokine, can also exert important effects on the differentiation and function of dendritic cells,[27,28] and has been applied for gene modification of dendritic cells.[29,30] In addition, IFN-α can directly inhibit the proliferation of tumor cells *in vitro* and *in vivo*.[14] Therefore, in this study, we selected hIFN-α for gene modification of the NKL cell line, and established *hIFN-α* gene-modified NKL cells, which retained many of the original phenotypic features of NK cells (Figure 1). The expression of human IFN-α in NKL-IFNα cells significantly augmented the natural cytotoxicity of NKL cells towards the human HCC cell lines HepG2, H7402 and HepG2.2.15, as well as primary human hepatoma cancer cells isolated from three hepatocellular carcinoma patients (Figure 2). HepG2.2.15 cells are HBV^{+} HCC cells. It has been reported that HBV-specific immune responses are either absent or suppressed, with a deficiency of innate and adaptive immune cells, in chronic hepatitis B (CHB) patients.[31,32] Therefore, *IFN-α* gene-modified NKL cells might represent a potential therapeutic strategy for HBV^{+} HCC. In addition, low ratios of NKL-INFα cells had a similar or even higher cytotoxic effect towards HCC cells compared with high ratios of NKL-vec cells. Therefore, the administration of even a low number of NKL-INFα cells could potentially achieve the same treatment efficacy as high numbers of NKL-vec cells. Furthermore, we demonstrated that adoptive transfer of IFN-α gene-modified NKL cells could significantly enhance survival and inhibit tumor growth in HCC tumor-bearing mice (Figure 6). As NKL-INFα cells increased the survival of mice bearing HCC tumors by approximately 30% compared to mice injected with NKL-vec cells, the adoptive transfer procedure could be optimized further. For example, the number of injections or the number of NKL-IFNα cells injected could be increased. Overall, these results indicate that *IFN-α* gene modification enhanced the natural cytotoxicity of NKL cells towards HCC cells both *in vitro* and *in vivo*.

One mechanism implicated in NK cell cytotoxicity is the release of cytoplasmic granules, such as perforin and granzymes, which lyse the target cells. Indeed, we observed that the expression of cytotoxicity-associated molecules, including TNF-α, granzyme B, perforin, IFN-γ and FasL, was upregulated in *IFN-α* gene-modified NKL cells (Figure 3a). As the interactions of NKG2D and its ligands are important in NK-mediated cytolysis, we also quantified expression of the NKG2D ligands MICA/B and ULBP1-4 in the target cells after coculture with NK cells; however, no detectable differences were observed in NKL-IFNα cell- and NKL-vec cell-treated HCC cells (data not shown). The expression of MHC I molecules on tumor cells would disturb NK cell function. However, compared with NKL-vec cells, NKL-IFNα cells did not obviously increase the expression of HLA-ABC on HepG2 cells (Supplementary Figure S2). In comparison with the remarkably increased the expression of cytotoxicity-associated molecules by NKL-IFNα cells, and the induction of Fas expression on HepG2 cells by NKL-IFNα cells, it is unlikely that IFNα-induced MHC-I expression influences the activity of NK cells.

Another pathway of NK cell-induced cytolytic activity is mediated by the surface TNF ligand family members, including FasL, TNF-α and TNF-related apoptosis-inducing ligand. These ligands interact with specific receptors on the target cell surface, and can induce tumor-cell apoptosis. However, activated NK cells can also secrete a number of effector cytokines, such as IFN-γ, TNF-α, granulocyte–macrophage colony-stimulating factor, granulocyte-colony-stimulating factor and macrophage-colony-stimulating factor.[33–35] Indeed, we observed an increase in the expression of FasL by NKL-IFNα cells (Figure 3b). To determine whether *IFN-α* gene modification could enhance the natural cytotoxicity of NKL cells and lead to increased apoptosis in HCC cells, the expression of apoptosis-associated genes was quantified in the target HCC cells. Fas was upregulated in HCC cells exposed to NKL-IFNα cells, and we observed that Fas has a critical role in NKL-IFNα cell-mediated lysis of HCC cells (Figure 4).

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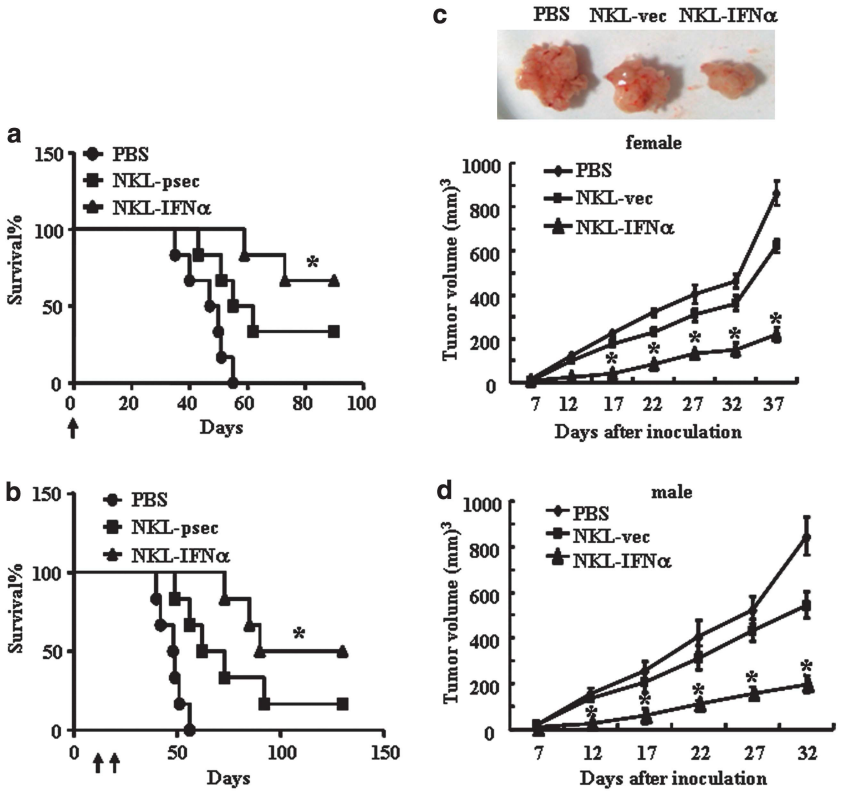

Figure 6. Antitumor effects of *IFNα* gene-modified NKL cells on HCC tumor-bearing nude mice. (**a**) Female nude mice were intraperitoneally injected with 5×10^6 HepG2 cells and then injected intravenously with 5×10^7 (2.5×10^9 cells per kg) irradiated NKL-IFNα cells or NKL-vec cells on day 0. (**b**) Female nude mice were intraperitoneally injected with 5×10^6 HepG2 cells and then intravenously injected with 5×10^7 irradiated NKL-IFNα cells or NKL-vec cells on days 7 and 14 after tumor cell inoculation. The survival curves are shown as percentages of the initial number of animals per group. $^*P < 0.05$ versus NKL-vec cells, Mann–Whitney *U*-test. Arrows indicate the days of NK cell transfer. (**c**, **d**) Male or female nude mice were subcutaneously injected with 5×10^6 HepG2 cells, and then intravenously injected with 5×10^7 irradiated NKL-IFNα cells or NKL-vec cells on days 7 and 14 after tumor cell inoculation. Photographs of dissected tumors from the female tumor-bearing mice are shown, and the tumor volumes of the female and male tumor-bearing mice were measured on the indicated days. $^*P < 0.05$ versus NKL-vec.

In line with other publications,[36,37] further investigation demonstrated that the cytokines TNF-α and IFN-γ, which were produced by NKL-IFNα cells, induced the expression of Fas on the surface of the HCC cells. As shown in Figure 5, the expression of Fas on HepG2 cells was significantly increased by coculture with NKL-IFNα cells; however, the expression of Fas on HCC cells in response to NKL-IFNα cells was suppressed by incubation with TNF-α- or IFN-γ-blocking mAbs, which was concomitant with an attenuation of the susceptibility of HCC cells to NKL-IFNα cell-induced cytolysis.

Adoptive immunotherapy using NKL-IFNα cells could be applied in combination with other therapeutic strategies. For example, the androgen receptor has an important role in the growth of HCC and contributes significantly to the preferential development of HCC in men.[38] Combination therapy with the use of an androgen receptor-blocking agent could enhance the antitumor efficacy of NKL-IFNα cells in HCC patients.

Taken together, these findings demonstrate that *IFN-α* gene modification of NKL cells promoted the expression of granzyme B and perforin, which were responsible for enhanced NKL cell cytotoxicity. In addition, the *IFN-α* gene modification promoted the ability of NKL cells to induce HCC cell apoptosis, as NKL-IFNα cells induced the expression of Fas on the HCC cells. On the basis of these results, this study suggests that *hIFN-α* gene-modified NKL cells may represent a novel and potential immunotherapeutic strategy for HCC, which could complement the use of *IL-15* gene-modified or other gene-modified NK cells.

MATERIALS AND METHODS

Cell culture and cell lines

The human HCC cell lines HepG2 and H7402 were conserved in our laboratory and cultured in RPMI-1640 medium (GIBCO/BRL, Grand Island, NY, USA) supplemented with 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin and 10% fetal bovine serum (FBS). HepG2.2.15 cells (serotype ayw, genotype D), derived from HepG2 cells by transfection with a plasmid carrying two head-to-tail copies of HBV genomic DNA,[39] were maintained in complete Dulbecco's modified Eagle's medium (GIBCO/BRL) supplemented with 10% FBS. NKL cells, a kind gift of Professor BQ Jin (Department of Immunology, Fourth Military Medical University, Xi'an, People's Republic of China), are conserved in our laboratory and were cultured in RPMI-1640 medium containing 10% FBS and 100 U ml^{-1} IL-2. All cells were incubated at 37 °C in a 5% CO_2 atmosphere.

Patients and tumor tissue samples

Human primary HCC tissues were obtained from three HCC patients who underwent surgical resection at Shandong Provincial Hospital. None of these patients had received preoperative chemotherapy or radiotherapy. The HCC tissues were carefully washed with Hank's solution containing 2% FBS and 1% ethylenediaminetetraacetic acid, and then dissected into small pieces. Cell suspensions were obtained by mechanical dissociation, incubated in 0.25% trypsin at 37 °C for 20 min, and then the upper part of the cell suspension was carefully recovered and passed through a 70μm cell sieve. Primary human HCC cells were pelleted by centrifugation at 50 *g* for 10 min at 4 °C, resuspended at 60–70% confluence in RPMI-1640 containing 10% FBS and then used as target cells in the cytotoxicity assay.

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Gene modification of NKL cells

Total RNA was extracted from human peripheral blood mononuclear cells of healthy volunteers, and *IFN-α1* cDNA was amplified using an RT-PCR method and inserted into the expression vector pSecTag B (Invitrogen, Carlsbad, CA, USA). The resulting recombinant vector (pSecTag B-IFNα) was transferred into NKL cells by electroporation using a Bio-Rad Gene Pulser II (Bio-Rad, Hercules, CA, USA) at 200 mV and capacitance of 975 AFarads in a 0.4-cm electroporation cuvette.[40] The empty vector pSecTag B was electroporated to create control cells. Following electroporation, the cells were selected by incubation in the presence of 100 mg ml^{-1} Zeocin (Invitrogen). The *IFNα* gene-transferred NKL cells were cloned, expanded and confirmed by RT-PCR and ELISA. The resulting cell line was called NKL-IFNα, and the control cell line was called NKL-vec.

Cytotoxicity assay

NK cell-mediated cytotoxicity was determined via the MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. HepG2, H7402 or HepG2.2.15 cells were used as target cells and placed in 96-well plates at 1×10^4 cells per well. Primary human HCC cells were also used as target cells, and were placed in 96-well plates at 2×10^4 cells per well. The effector cells were added to the target cells at effector/target (E/T) ratios of 10:1 and 5:1, or 5:1 and 2.5:1. For the IFN-γ- and TNF-α-blocking assay, the NK cells were preincubated with 5 μg ml^{-1} anti-IFN-γ monoclonal antibody (mAb) and/or 2.5 μg ml^{-1} anti-TNF-α mAb (R&D Systems, Minneapolis, MN, USA) or the isotype control mAbs. For the RNA interference assays, HepG2 cells were seeded, incubated for 12 h and then transfected with 50 nM Fas-siRNA or control-siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The sense *Fas* siRNA strand used in this study was 5′-CGUAUGACACAUUGAUUAA-3′; all siRNAs were purchased from Ribobio (Guangzhou, China).

Finally, the E/T cell mixtures were incubated for 6 h at 37 °C, 20 μl of 5 mg ml^{-1} MTT solution was added and incubated for a further 4 h. The absorbance (*A*) values at 570 nm were determined using a microplate reader (Bio-Rad). Percentage (%) cytotoxicity was calculated as (1-(absorbance value of target plus effector cells − absorbance value of effector cells)/absorbance value of target cells) × 100%.

RT-PCR and real-time RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) as described by the manufacturer, and cDNA was synthesized from 2 μg RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and an oligo(dT) primer, as recommended by the manufacturer. The mRNA expression levels of *IFN-α* and the apoptosis-associated genes *Bcl-2*, *Mcl-1* and *Fas* were determined by RT-PCR. Each PCR program consisted of 30 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s and extension at 72 °C for 1 min, followed by a final 10 min extension at 72 °C.

The mRNA expression levels of cytotoxicity-associated genes were determined by real-time RT-PCR using a TransStart SYBR qPCR Kit (Toyobo Co. Ltd, Osaka, Japan) using a MyiQ thermocycler (Bio-Rad). Each PCR reaction mixture (20 μl) contained 10 μl of 2 × SYBR Green Master Mix and 7 μl of the forward and reverse primers (5 nM). The reactions were incubated for 10 min at 95 °C, followed by 45 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s. The expression levels of the target genes were normalized to *GAPDH*. The sequences of the PCR primers are listed in Table 1.

Enzyme-linked immunosorbent assay

Approximately 1×10^6 NKL-IFNα cells or NKL-vec cells were collected, washed three times with PBS, cultured in 10 ml RPMI-1640 medium for 24 or 48 h, and the supernatants were harvested. The levels of IFN-α in the cell culture supernatants were detected using an ELISA kit (R&D Systems), following the manufacturer's instructions. To induce secretion of IFN-γ, the NKL-IFNα cells or NKL-vec cells were stimulated with or without PMA (5 μg ml^{-1}; Sigma, St Louis, MO, USA) and ionomycin (50 μg ml^{-1}; Sigma) for 24 and 48 h, and the levels of IFN-γ were quantified using the ELISA.

Flow cytometry analysis

To detect Fas protein expression on the cell surface, the HCC cells were stained with a phycoerythrin (PE)/cy5-conjugated Fas antibody (R&D Systems) or isotype control antibody (eBioscience, San Diego, CA, USA) at 4 °C for 45 min. To detect FasL protein expression on the cell surface, NKL cells were stained with a PE-conjugated FasL antibody (eBioscience) or an isotype control antibody (eBioscience) at 4 °C for 45 min. For intracellular staining of TNF-α, perforin and granzyme B, NKL cells were treated with monensin (Sigma) for 4 h to inhibit the secretion of cytokines, harvested, washed once, fixed in 0.4% paraformaldehyde at 4 °C for 30 min, permeabilized in buffer containing 0.01% saponin (Sigma) and 0.09% NaN_3 (Sigma), followed by incubation for 1 h with PE-conjugated IFN-γ, PE-conjugated TNF-α or fluorescein isothiocyanate-conjugated granzyme B antibodies (eBioscience). All stained cells were analyzed using a flow cytometer (FACScalibur; BD Biosciences, San Jose, CA, USA), and the data were processed with WinMDI 2.9 software (Scripps Research Institute, La Jolla, CA, USA).

Table 1. Primers used for qRT-PCR

Transcript	*Sequence (5′–3′)*	*Product size (bp)*
β-actin	F: 5′-ATCATGTTTGAGACCTTCAACA-3′ R: 5′-CATCTCTTGCTCGAAGTCCA-3′	300
IFN-α	F: 5′-TTAGGATCCATGGCCTCGCCCTTT-3′ R: 5′-CGCGAATTCGTTATTCCTTCCTCC-3′	569
Bcl-2	F: 5′-CGACTTCGCCGAGATGTCCAGCCAG-3′ R: 5′-ACTTGTGGCCCAGATAGGCACCCAG-3′	364
Fas	F: 5′-GCCATTAAGATGAGCACCAAGG-3′ R: 5′-CCCAAATAGGAGTGTATGCAGAGG-3′	525
Mcl-1	F: 5′-AAAGTCAGCTCCACTGAAGCTGTG-3′ R: 5′-AGTCCTCCACCTCGTTGTCCGTGA-3′	436
GAPDH	F: 5′-GAAGGTGAAGGTCGGAGT-3′ R: 5′-CATGGGTGGAATCATATTGGAA-3′	155
NKG2D	F: 5′-TGCATGCAAAGGACTGTGTAAAG-3′ R: 5′-GCAGTGTTACCGCTGGTGTAATC-3′	86
NKG2A	F: 5′-GAGGCACAACAATTCTTCCC-3′ R: 5′-CAGGCCAGCAAACTCTCTTC-3′	143
IFN-γ	F: 5′-CTTGGCTTTTCAGCTCTGCATC-3′ R: 5′-CTTCAAAATGCCTAAGAAAAGAGTTCC-3′	151
TNF-α	F: 5′-ATCTTCTCGAACCCCGAGTGA-3′ R: 5′-CGGTTCAGCCACTGGAGCT-3′	83
Perforin	F: 5′-AGTACAGCTTCAGCACTGACA-3′ R: 5′-ATGAAGTGGGTGCCGTAGTT-3′	175
GZM-B	F: 5′-GCGGTGGCTTCCTGATACAAG-3′ R: 5′-CCCCCAAGGTGACATTTATGG-3′	82
FasL	F: 5′-CACTTTGGGATTCTTTCCAT-3′ R: 5′-GTGAGTTGAGGAGCTACAGA-3′	159

Abbreviations: FasL, Fas ligand; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GZM-B, granzyme B; IFN, interferon; qRT-PCR, quantitative reverse transcription polymerase chain reaction; TNF, tumor necrosis factor.

Coculture assays

HepG2 cells and NKL-IFNα or NKL-vec cells were cocultured at a ratio of 1:1 using a 0.4-μm porous Transwell system (Corning Costar, Tewksbury, MA, USA) in 12-well plates for 24 h; complete RPMI-1640 medium served as the control.

Nude mouse xenograft assay

Female and male nude BALB/c mice (6 weeks-old) were obtained from the Shanghai Experimental Animal Center (Chinese Academy of Sciences, Shanghai, China), and housed under pathogen-free conditions. All handling and experimental procedures were conducted in accordance with experimental animal guidelines. To induce tumor growth, 5×10^6 HepG2 cells (200 μl) were subcutaneously injected into the right flank of female or male mice. When the tumor volumes reached approximately 50 mm^3 (at 7 days after inoculation), the mice were randomly assigned to three groups with six mice in each group (PBS treatment control, NKL-vec treatment control and NKL-IFNα treatment group). After irradiation at 800 cGy (200 cGy min^{-1}), 5×10^7 (2.5×10^9 cells per kg) NKL-IFNα or NKL-vec cells (200 μl) were harvested and intravenously injected into the mice on days 7 and 14 after tumor cell inoculation. Tumor growth was assessed every 5 days by measuring the tumor volume, calculated as $V = lw^2/2$, where *l* is the length and *w* is the width.

In addition, groups of six female mice were injected intraperitoneally with 5×10^6 HepG2 cells (200 μl) to determine the survival of the xenograft mice. The mice were injected intravenously with PBS, 5×10^7 irradiated

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NKL-IFNα cells or 5×10^7 NKL-vec cells on day 0 or on days 7 and 14 after tumor cell inoculation.

Statistical analyses

All data are expressed as the mean ± s.d. of at least three independent experiments. Statistical analysis was performed using a paired Student's *t*-test in studies with two groups and one-way analysis of variance in the studies with more than two groups; significance was defined as $^*P<0.05$ and $^{**}P<0.01$. Mouse survival was analyzed using the Mann–Whitney *U*-test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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