

# Simultaneous Knockdown of Multiple Ligands of Innate Receptor NKG2D Prevents Natural Killer Cell-Mediated Fulminant Hepatitis in Mice

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NKG2D activation plays an important role in initiating and maintaining liver inflammation, and blockade of NKG2D recognition becomes a promising approach to alleviate liver inflammation. Treatment by silencing NKG2D ligands on hepatocytes, but not NKG2D on circulating immune cells, is more liver-specific, and simultaneous knockdown of multiple NKG2D ligands on hepatocytes will be more efficient in liver disease intervention. Here, we constructed a single vector that could simultaneously express multiple short hairpin RNAs (shRNAs) against all murine NKG2D ligands including Rae1, Mult1, and H60. After hydrodynamic injection of plasmid containing the three shRNA sequences (shRae1-shMult1-shH60), also called pRNAT-shRMH, we found the expression of all three NKG2D ligands on hepatocytes was downregulated both on messenger RNA and protein levels. Moreover, natural killer (NK) cell-mediated NKG2D-dependent fulminant hepatitis of the mice was alleviated, along with inactivation of hepatic NK cells, by pRNAT-shRMH if compared with its counterpart RNA interference vectors against single or double ligands. The therapeutic efficacy of pRNAT-shRMH was equivalent to that of injecting three monoclonal antibodies against Rae1, Mult1, and H60. For better *in vivo* application, we constructed a recombinant adenovirus containing pRNAT-shRMH (called Ad-RMH) with efficient hepatotropic infection capacity and observed that Ad-RMH intravenous injection exerted a similar therapeutic efficiency as plasmid pRNAT-shRMH hydrodynamic injection. Noticeably, simultaneous knockdown of multiple human NKG2D ligands (MICA/B, ULBP2, and ULBP3) also significantly attenuated NK cell cytolysis against human NKG2D ligand-positive hepatocyte L-02 cells, suggesting a possible translation into human settings. **Conclusion:** Simultaneous knockdown of multiple ligands of NKG2D prevents NK cell-mediated fulminant hepatitis and is a potential therapeutic approach to treat liver diseases. (HEPATOLOGY 2013;57:277-288)

NKG2D, a lectin-like type-2 transmembrane protein, is reported to be a stimulatory immunoreceptor expressed by natural killer (NK) cells, NK1.1<sup>+</sup> T cells,  $\gamma\delta$ T cells, CD8<sup>+</sup> T cells, and some myeloid cells.<sup>1-5</sup> NKG2D recognizes various ligands that are distantly related to major histocompatibility complex class I molecules, including MICA/B,<sup>1</sup> ULBPs in human,<sup>6,7</sup> and Rae1, Mult1, and H60 in mice.<sup>8-10</sup> These ligands are not constitutively expressed, but could be stress-induced, such as through

viral infection, DNA damage or malignant transformation.<sup>11-14</sup> Expression of NKG2D ligands renders target cells susceptible to NK cells cytolysis and induces interferon- $\gamma$  (IFN- $\gamma$ ) production by NK cells.<sup>1,7,8,10</sup> It was reported that NKG2D was involved in NK cell-mediated allergic airway inflammation, and blocking antibody for NKG2D receptor delayed airway allergy.<sup>15</sup> In a renal ischemia model, NKG2D ligand recognition induced local inflammation and leukocyte accumulation.<sup>16</sup> Blockade of NKG2D signaling is

Abbreviations: ALT, alanine aminotransferase; ConA, concanavalin A; D-GalN, D-galactosamine; DMEM, Dulbecco's modified Eagle's medium; HBsAg, hepatitis B surface antigen; IFN- $\gamma$ , interferon- $\gamma$ ; MNC, mononuclear cell; mRNA, messenger RNA; NK, natural killer; PCR, polymerase chain reaction; poly(I:C), polyinosinic:polycytidylic acid; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA; Tg, transgenic; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ .

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beneficial in cell transfer–induced colitis in severe combined immunodeficient mice,<sup>17,18</sup> and ameliorates collagen-induced arthritis by administration of a nondepleting anti-NKG2D monoclonal antibody (CX5).<sup>19</sup> A few years ago, we and other group reported that blockade of NKG2D–ligand interaction could attenuate NKG2D-mediated acute hepatitis in hepatitis B viral infected subjects.<sup>3,20</sup> So, NKG2D activation plays an important role in initiating and maintaining inflammation, and blockade of NKG2D recognition becomes a potential approach to treat a variety of tissue injury.

Because treatment by silencing NKG2D ligands on hepatocytes, but not NKG2D on circulating immune cells, is more liver-specific, and considering the short lifetime of antibodies *in vivo* (hence repeat administrations) and the high expense thereafter, we chose gene knockdown approach via liver-specific RNA interference (RNAi) to suppress expression of multiple NKG2D ligands on hepatocytes. In this study, we constructed a triple short hairpin RNA (shRNA)-expressing vector against all murine NKG2D ligands and observed that this vector effectively alleviated polyinosinic:polycytidylic acid [poly(I:C)]/D-galactosamine (D-GalN)-induced NK cell-mediated hepatitis after delivery either by hydrodynamic injection or infection with adenovirus. In addition, the triple shRNA expressing vector could also efficiently attenuate concanavalin A (ConA)-induced NKG2D-dependent acute hepatitis in hepatitis B virus–infected subjects. It was proved that triple shRNA-expressing vector against murine NKG2D ligands has effective protection *in vivo* by silencing NKG2D ligands on hepatocytes, which were upregulated in acute hepatitis models, including poly(I:C)/D-GalN and ConA stimulation. A triple shRNA-expressing vector against all human NKG2D ligands was constructed, which also significantly attenuated NK cell cytotoxicity against human NKG2D ligand–positive hepatocytes, indicating a possible translation into human settings. In these experimental systems, a triple shRNA-expressing vector exerted much better anti-inflammatory effects than double or single shRNA-expressing vector, demonstrating simultaneous silence of multiple ligands of activating NK cell receptor may be promising approach to more efficiently treat liver diseases.

## Materials and Methods

**Preparation of Multiple shRNA Expressing Vectors Against Mouse and Human NKG2D Ligands.** To construct the multiple shRNA-expressing vectors, single shRNA-expressing vectors were constructed first. Small interfering RNA (siRNA) sequences corresponding to the *Mus musculus* Rae1  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$  gene (NCBI accession number: NM\_009016, 009017, 009018, 020030, 198193) common sequences, *Mus musculus* Mult1 gene (NM\_029975) and *Mus musculus* H60  $\alpha$ ,  $\beta$ ,  $\gamma$  (NM\_010400.2, 001177775.1, 001204916.2) common sequences, *Homo sapiens* MICA (NM\_000247.1), *Homo sapiens* ULBP2 (NM\_025217.2), and *Homo sapiens* ULBP3 (NM\_024518.1) were introduced into pRNAT-H1.1/shuttle vector (Genscript, SD1216) using the sense and anti-sense strand oligonucleotides. The chosen siRNA sequences were shown as follows: Rae1, 5'-CAG CAA GTG CTC TTT GCT A-3'; Mult1, 5'-AGC TGA CTG CCA GTA ACA A-3'; H60, 5'-CCT GGA GAG AAA GTC ATT C-3' (sense); MICA, 5'-AGG AGA TTA GGG TCT GTG A-3'; ULBP2, 5'-AGG CCA GGA TGT CTT GTG A-3'; ULBP3, 5'-TGG ATA CAT CCG TGG ATC T-3'; and negative control sequence, 5'-GAG ACC CTA TCC GTG ATT A-3'. The shRNA-expressing cassettes, including the H1.1 promoter and corresponding oligonucleotides, were amplified from the single shRNA-expressing vectors, using the primers listed in Supporting Table 2. Further ligation was performed via the same restriction enzyme site to construct the multiple shRNA-expressing vectors.

**Preparation of Multiple shRNA Expressing Adenovirus Vectors Against Murine NKG2D Ligands.** The multiple shRNA-expressing vectors, with the pRNAT-H1.1/shuttle framework, were recombined with a PI-Sce I/I-Ceu I digested lineared Adeno-X expression system (Clontech) to prepare recombinant adenovirus expressing multiple shRNA against murine NKG2D ligands. The viruses were packaged and propagated in HEK293 cells and then purified by CsCl discontinued density gradient centrifugation. The virus titer was determined via Adeno-X Rapid Titer Kit (Clontech).

**Animals.** Five-week-old male C57BL/6 mice were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Science (Shanghai, China). Eight-

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week-old male hepatitis B surface antigen (HBsAg)-transgenic (Tg) mice were purchased from Vital River Laboratories (Peking, China). All mice were housed in a specific pathogen-free, temperature-controlled micro-environment with 12-hour day/night cycles. All procedures performed were in compliance with the animal care regulations of the University of Science and Technology of China.

**Cell Lines.** The NKG cell, a new human NK cell line, was from a Chinese male patient with rapidly progressive non-Hodgkin's lymphoma. It was established and characterized by other colleagues in our group.<sup>21</sup> Cells were cultured in minimal essential medium alpha in 12.5% fetal bovine serum and 12.5% donor equine serum with 100 U/mL recombinant human IL-2 (rhIL-2; PeproTech). The mouse macrophage cell line RAW264.7, mouse embryonic fibroblast cell line NIH/3T3, and human embryonic kidney 293 cells were obtained from the Shanghai Cell Bank. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) in 10% fetal bovine serum, with 100 U/mL penicillin, and 100 g/mL streptomycin within a humidified incubator containing 5% CO<sub>2</sub> at 37°C.

**Reagent.** To induce fulminant hepatitis, C57BL/6 were injected intravenously with poly(I:C) (1 µg/mouse, Amersham) and intraperitoneally with D-GalN (10 mg/mouse, Sigma-Aldrich) at the same time, and HBsAg-Tg mice were injected intravenously with ConA (3 µg/g, Sigma-Aldrich). All constructed multiple shRNA-expressing vectors (10 µg/mouse) were injected hydrodynamically 3 days before poly(I:C)/D-GalN injection. Purified recombinant adenovirus were injected hydrodynamically ( $4 \times 10^9$  ifu/mouse) or intravenously ( $1 \times 10^{11}$  ifu/mouse).

**Serum Alanine Aminotransferase Assay.** Mice were anesthetized and then bled from the retro-orbital venous plexus. Serum alanine aminotransferase (ALT) activities were determined with a serum aminotransferase test kit (Rongsheng, Shanghai, China) following the manufacturer's instructions.

**Isolation of Hepatocytes.** The liver was perfused with two kinds of solution sequentially. Solution I was composed of 0.5 mM ethylene glycol tetraacetic acid, 5.3 mM KCl, 4.4 mM KH<sub>2</sub>PO<sub>4</sub>, 136 mM NaCl, 0.13 mM Na<sub>2</sub>HPO<sub>4</sub>, and 19.5 mM Tricine. Solution B was composed of DMEM and 0.075% collagenase I (Sigma-Aldrich). Hepatocytes were then separated with 40% Percoll by centrifugation at 400g for 10 minutes.

**Preparation of Peripheral Blood Mononuclear Cells and Isolation of NK Cells.** Peripheral blood mononuclear cells were isolated from freshly heparinized venous blood from healthy adults (Anhui Prov-

ince Blood Center) by Ficoll-Hypaque. NK cells were purified by depletion of magnetically labeled non-NK cells with an NK Cell Isolation Kit (Miltenyi Biotec).

**Flow Cytometric Analysis.** The monoclonal antibodies used for flow cytometry in this study included APC-anti-CD3ε, PerCP-CY5.5-NK1.1, PE-Rae1, PE-IFNγ, and PE-MICA/B (BD Pharmingen); PE-Mult1 (eBioscience); and PE-H60, PE-ULBP3, and APC-ULBP2 (R&D Systems). For intercellular cytokine staining, liver mononuclear cells (MNCs) were stained with APC-anti-CD3ε and PerCP-CY5.5-NK1.1. After surface markers became stained, cells were fixed, permeabilized, and stained with PE-IFN-γ. Stained cells were analyzed by FACSCalibur, and data were analyzed using WinMDI2.9 software.

**CD107a Degranulation Analysis.** The frequency of degranulating NK cells were quantitated by CD107a staining.<sup>22</sup> NKG cells were resuspended at  $1 \times 10^6$  cells/mL in minimal essential medium alpha (HyClone) containing 10% fetal bovine serum, 10% donor equine serum, 4 mM L-glutamine, and 100 IU/mL penicillin. Cells were then stimulated with stable transfected L-02 cells at an effector-to-target ratio of 6:1; medium alone served as the negative control. Cells were stimulated with phorbol-12-myristate-13-acetate (2.5 µg/mL, Sigma) and ionomycin (0.5 µg/mL, CalBioChem) as the positive control. PE-CD107a antibody (BD Bioscience) was added directly to the tubes at 20 µL/mL. Cells were incubated for 1 hour at 37°C in 5% CO<sub>2</sub>, and then 6 µg/mL monensin (Sigma) was added for an additional 5 hours at 37°C in 5% CO<sub>2</sub>. Cells were stained for the surface NK cell marker PE-CY5-CD56 (BD Bioscience) for 30 minutes. A total of 20,000 events were acquired and analyzed using Winmd software. The analysis was performed on gated CD56<sup>+</sup> cells.

**Histopathological Analysis.** The livers of mice were harvested at the indicated time points, fixed in formalin, and then embedded in paraffin. Six-micrometer-thick sections were cut from each paraffin block and used for hematoxylin and eosin staining to evaluate liver injury via standard techniques.

**Quantitative Polymerase Chain Reaction.** Total RNA was extracted via the phenol/chloroform method using TRIzol reagent (Invitrogen). Quantitative polymerase chain reaction (PCR) was performed using SYBR Premix Ex Taq (Takara) with an RG-3000 detector (Rotor gene) according to the manufacturer's instructions. The primer sequences used were as follows: GAPDH, 5'-Cgg AgT CAA Cgg ATT Tgg TC-3' (sense), 5'-ATT CAT ATT ggA ACA TgT AAA CCA TgT AgT-3' (anti-sense); Rae1, 5'-gCT gTT



gCC ACA gTC ACA TC-3' (sense), 5'-CCT ggg TCA CCT gAA gTC AT-3' (anti-sense); Mult1, 5'-CAA TgT CTC TgT CCT Cgg AA-3' (sense), 5'-CTg AAC ACg TCT CAg gCA CT-3' (anti-sense); H60, 5'-gTg TgA TgA CgA TTT gTT gAg-3' (sense), 5'-ATT gAT ggA TTC Tgg gCC ATC-3' (anti-sense). All expression levels of interested genes were normalized to the housekeeping gene GAPDH. Gene expression values were then calculated based on the  $\Delta\Delta C_t$  method as described.<sup>23</sup>

**Enzyme-Linked Immunosorbent Assay of Cytokines.** Serum samples were harvested 18 hours after poly(I:C)/D-GalN coadministration for measurement of the cytokines IFN- $\gamma$  and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ). Cytokine concentration was determined using a corresponding commercial enzyme-linked immunosorbent assay kit (Senxiong Biotech, Shanghai, China). The data were analyzed with Origin 8.0 software.

**<sup>51</sup>Cr Release Cytotoxicity Assay.** <sup>51</sup>Cr release cytotoxicity assay was performed to assay the cytotoxicity of human NKG cells against L-02 cells. L-02 cells ( $10^6$  cells) were labeled with 200  $\mu$ Ci of sodium chromate for 1 hour at 37°C and washed three times. Effector cells (human NKG cells) and target (L-02 cells) were incubated for 4 hours at the indicated effector-to-target ratios. Spontaneous release of <sup>51</sup>Cr was determined by incubating target cells with plain medium alone and was always <20%. Maximum release of <sup>51</sup>Cr was determined by incubating target cells with 2% Triton X-100. The result was calculated as follows:  $100 \times ((\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release}))$ .

**Statistical Analysis.** All data are presented as the mean  $\pm$  SEM. Differences between the groups were analyzed using the Mann-Whitney U test;  $P < 0.05$  was considered statistically significant.

## Results

**Construction of an shRNA Vector (pRNAT-shRMH) for Knockdown of Multiple NKG2D Ligands.** We selected RNAi target sequences for silencing murine NKG2D ligands genes (Rae1, Mult1, and H60), using siRNA designer software based on various algorithms as described.<sup>24,25</sup> These RNAi target sequences were efficient in silencing surface expression of Rae1, Mult1, and H60, respectively, after delivery of siRNA or shRNA-expressing vector into corresponding positive expression cell lines Raw264.7 and NIH-3T3 (Supporting Fig. 1). Three single shRNA-expressing vectors against murine NKG2D

ligands Rae1, Mult1, and H60 (known as pRNAT-shRae1, pRNAT-shMult1, and pRNAT-shH60) were constructed by subcloning chemically synthesized oligonucleotides into pRNAT-H1.1/shuttle, a shuttle plasmid for adenovirus. With the constructed shRNA-expressing vectors as PCR templates, the shRNA expression cassettes—including the H1.1 promoter, RNAi oligonucleotides, and terminator—were amplified together with designed restricted enzyme sites. These shRNA expression cassettes were finally inserted with pRNAT-H1.1/shuttle to be multiple shRNA-expressing vectors (Supporting Fig. 2; Fig. 1). A serial of multiple shRNA-expressing vectors were constructed, including single, double, or triple shRNA-expressing vector against murine NKG2D ligands (Fig. 1A). The shRNA expression sequences of Rae1 (400 bp), Mult1 (200 bp), H60 (200 bp), and negative control (400 bp) were amplified from corresponding single shRNA-expressing vectors by PCR primers with designed restricted enzyme sites. These shRNA expression cassettes were then sequentially ligated to be a large shRNA fragment of 1.4 kb shown by the electrophoretic results (known as shRMH), which was further inserted into pRNAT-H1.1/shuttle (Fig. 1B,C). The clones (including clones 3, 4, and 6) expressed positive insertion, and we selected clone 3 of pRNAT-shRMH for further study (Fig. 1D).

**Multiple Expressions of NKG2D Ligands and Their Simultaneous Knockdown by a pRNAT-shRMH Vector In Vivo.** In our previous report, poly(I:C) in D-GalN-sensitized mice caused severe liver injury in an NK cell-dependent NKG2D-mediated manner.<sup>23</sup> In order to test the efficacy of pRNAT-shRMH vector on simultaneous knockdown of multiple NKG2D ligands, we examined expression of murine NKG2D ligands in this mouse model and found poly(I:C) and D-GalN coadministration markedly stimulated multiple expression of NKG2D ligands (Rae 1, Mult1, and H60) on hepatocytes (Supporting Fig. 3), indicating NK cells may interact with hepatocytes through multiple NKG2D ligand recognition. The data were not consistent with those of poly(I:C) from Sigma, and we presumed that the two poly(I:C) differ in lengths of nucleotides, and may mimic different kind of RNA virus infection, causing distinct NKG2D ligands expression on hepatocytes. We hydrodynamically administered the multiple shRNA-expressing plasmids in order to efficiently deliver the gene into hepatocytes *in vivo*<sup>26-28</sup> and found that expression of all three NKG2D ligands was simultaneously inhibited in hepatocytes in both messenger RNA (mRNA) (Fig. 2A) and protein (Fig. 2B) levels by injection with

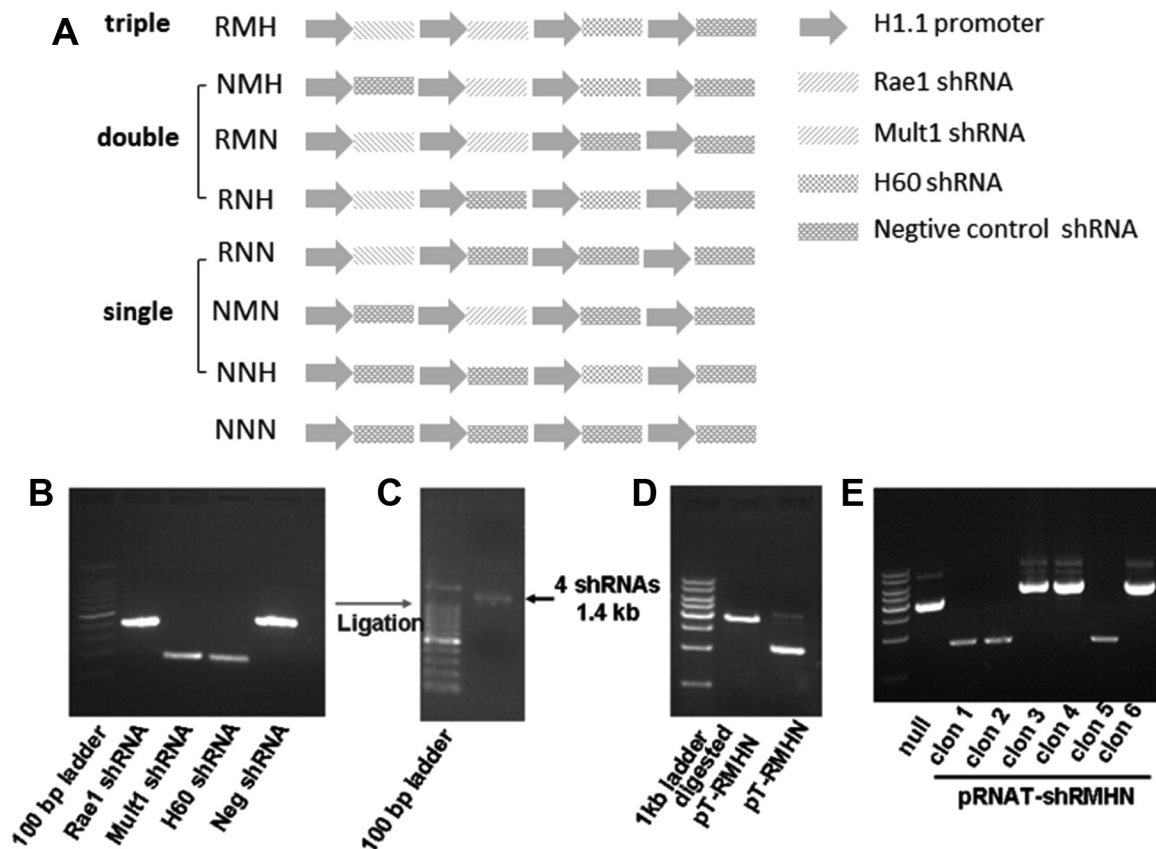


Fig. 1. Construction of multiple shRNA-expressing vectors against murine NKG2D ligands. (A) Triple, double, and single shRNA-expressing vectors against murine NKG2D ligands and negative control shRNA-expressing vectors were designed. (B) shRNA transcripts of Rae1, Mult1, H60, and negative control were amplified from corresponding single shRNA-expressing vectors, including the H1.1 promoter, shRNA-expressing nucleotides, and terminator. More transcript sequences, including the BglII restriction site (5,775 bp) to H1.1 promoter site (6,064 bp) and terminator (1 bp) to NdeI restriction site (261 bp), were amplified, respectively, with Rae1 and negative control shRNA transcript from corresponding single shRNA-expressing vectors for ligation with BglII and NdeI double-digested pRNAT-H1.1 vector. (C) shRNA transcripts of Rae1, Mult1, H60, and negative control were sequentially ligated, and a large shRNA fragment shRMH (1.4 kb) was obtained. (D) pRNAT-shRMH was generated by combination of shRMH and pRNAT-H1.1/shuttle via BglII and NdeI restriction enzyme sites. (E) Clone 3 of pRNAT-shRMH was selected for further study.

a triple shRNA-expressing vector pRNAT-shRMH (10  $\mu$ g/mice) 3 days prior to poly(I:C)/D-GalN coadministration. As shown in Fig. 2A, pRNAT-shRMH vector simultaneously markedly reduced mRNA expression of Rae1, Mult1, and H60, the efficiency of which was almost equivalent to a single or double RNAi vector targeting at a common ligand, demonstrating the specificity of RNAi against each ligand. Consistent with knockdown of mRNA expression, pRNAT-shRMH inhibited the surface expression of Rae1, Mult1, and H60 on hepatocytes via flow cytometry from 11.03% to 5.09%, 14.62% to 5.89%, and 16.38% to 6.45%, respectively (Fig. 2B). Overall, triple shRNA-expressing vector (pRNAT-shRMH) simultaneously suppressed expression of three kinds of murine NKG2D ligands, whereas single or double shRNA-expressing vectors only suppressed one or two kinds of corresponding ligands, further proving that simultaneous knockdown by pRNAT-shRMH was ligand-specific.

**Simultaneous Knockdown of Multiple Ligands of Mouse NKG2D Prevents NK Cell-Mediated Fulminant Hepatitis In Vivo.** Coadministration of poly(I:C) and D-GalN induced severe liver injury with significant elevation of serum ALT levels, as described by us.<sup>23</sup> We hydrodynamically injected multiple shRNA-expressing vectors (against murine NKG2D ligands) 3 days before poly(I:C)/D-GalN coadministration (Fig. 3A) at 10  $\mu$ g/mouse dose, which was identified to be the lowest concentration to obtain the best efficacy (Supporting Fig. 4). Mice treated with shRNA-expressing vectors against single, double, or triple murine NKG2D ligands showed a decrease in ALT levels and alleviation of liver injury when compared with pRNAT-shNNN-treated mice; however, the most efficient protective effect occurred only in pRNAT-shRMH-treated mice (Fig. 3B,C). Levels of inflammatory cytokines, including IFN- $\gamma$  and TNF- $\alpha$ , was consistent with ALT levels and histopathological results of

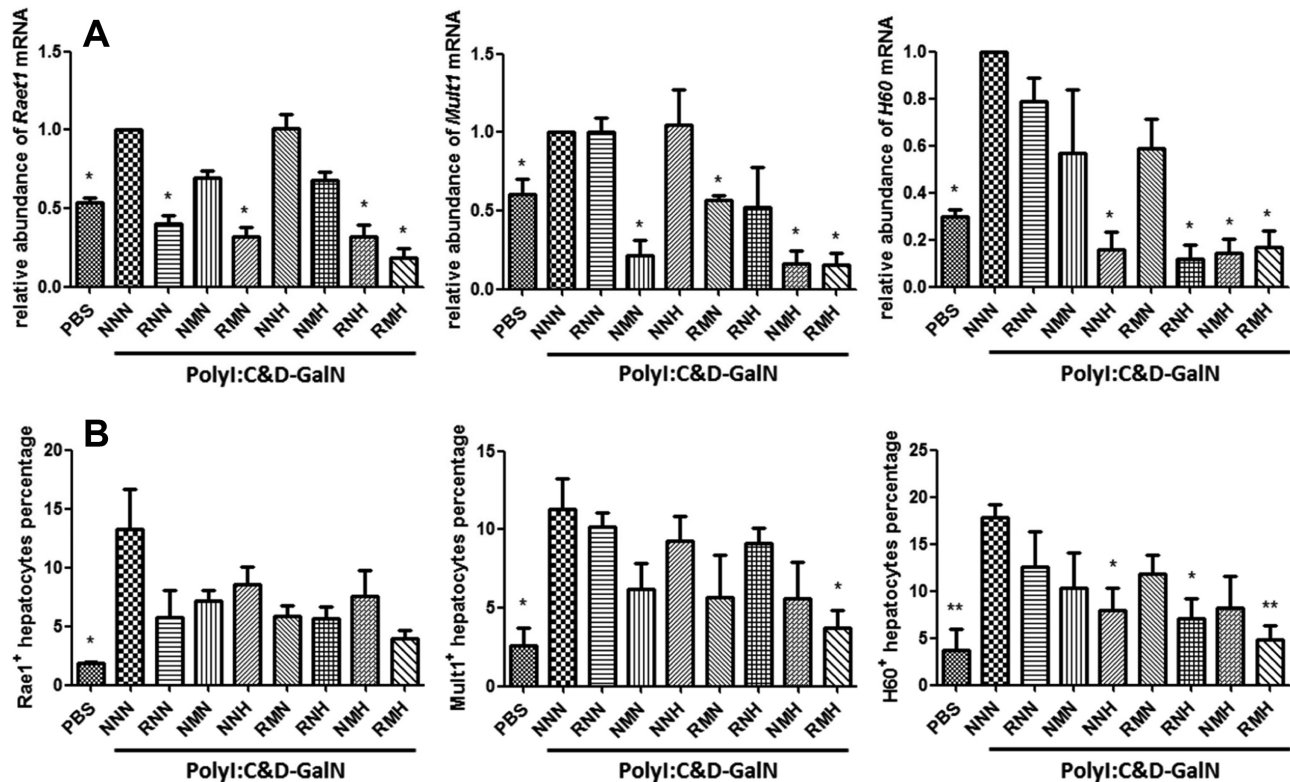


Fig. 2. Knockdown efficiency and specificity of multiple shRNA-expressing vectors against murine NKG2D ligands. Hepatocytes were prepared 18 hours after poly(I:C)/D-GalN coadministration of multiple RNAi plasmid preinjected mice for (A) quantitative reverse-transcription PCR analysis of *Rae1*, *Mult1*, and *H60* mRNA and (B) flow cytometry analysis of *Rae1*, *Mult1* and *H60* expression. Values are shown as the mean  $\pm$  SEM from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  versus pRNAT-NNN-preinjected mice.

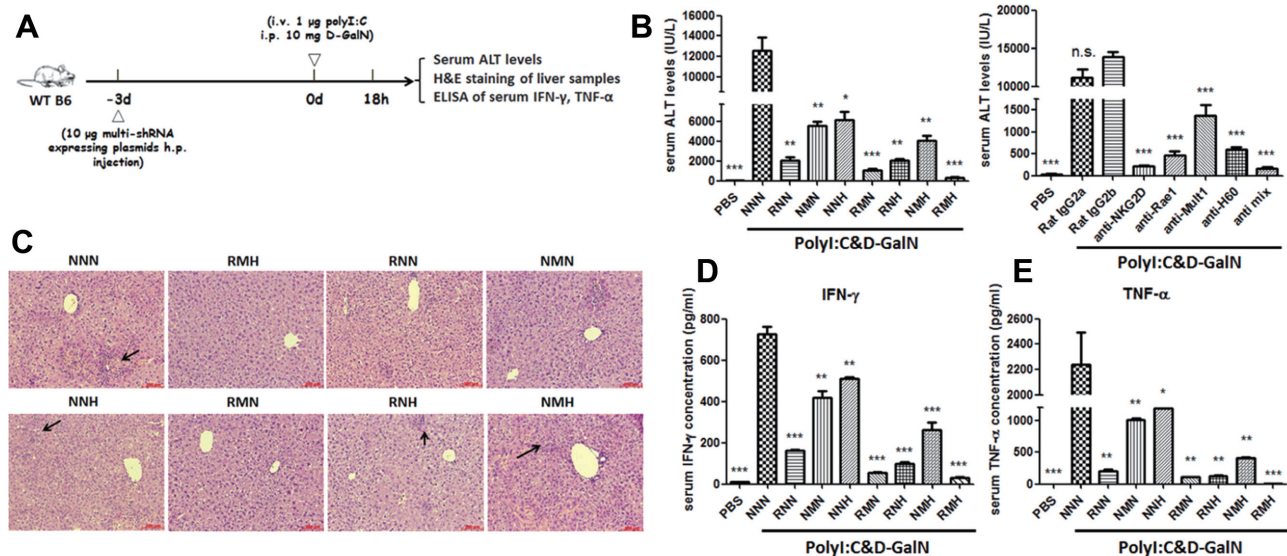


Fig. 3. RNAi against murine NKG2D ligands prevents mice from poly(I:C)/D-GalN-induced liver injury. (A) Procedure for the injection of multiple RNAi vectors 3 days prior to poly(I:C)/D-GalN coadministration. (B) Serum ALT levels were determined 18 hours after poly(I:C)/D-GalN coadministration of multiple RNAi vectors or blocking antibodies in preinjected mice. Data are shown as the mean  $\pm$  SEM ( $n = 3$  mice per group). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus pRNAT-NNN or rat IgG<sub>2b</sub>-preinjected mice. (C) Liver paraffin sections were prepared for hematoxylin and eosin staining from multiple RNAi plasmid-preinjected mice after poly(I:C)/D-GalN coadministration. The arrow indicates the necrosis area (original magnification  $\times 200$ ). (D) Serum IFN- $\gamma$  and (E) TNF- $\alpha$  concentration was determined 18 hours after poly(I:C)/D-GalN coadministration of multiple RNAi vector-preinjected mice. Data are shown as the mean  $\pm$  SEM ( $n = 3$  mice per group). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus pRNAT-NNN-preinjected mice.



liver tissues. Serum IFN- $\gamma$  and TNF- $\alpha$  dramatically increased when poly(I:C)/D-GalN was administered. However, when multiple shRNA-expressing vectors were injected 3 days before poly(I:C)/D-GalN injection, IFN- $\gamma$  and TNF- $\alpha$  decreased to different extents (Fig. 3D,E). Additionally, among three single shRNA-expressing vectors, pRNAT-shRNN (only against Rae1) more greatly alleviated liver inflammation when compared with other two single shRNA-expressing vectors (against Mult1 or H60, respectively), suggesting that Rae1 plays a more important role in poly(I:C)/D-GalN-induced fulminant hepatitis. When one more ligand of NKG2D was simultaneously silenced, the inflammation was further inhibited. For example, pRNAT-shRMN (against both Rae1 and Mult1) and pRNAT-shRNH (against both Rae1 and H60) exerted stronger effects on alleviating liver injury compared with those of the pRNAT-shRNN group. Moreover, the triple shRNA-expressing vector pRNAT-shRMH exhibited the most dramatic alleviation on liver inflammation, and survival rate was increased from 0% to 100% by treatment with pRNAT-shRMH when mice were administered with a lethal dose of poly(I:C)/D-GalN (Table 1).

The protective effects of simultaneous knockdown of multiple ligands of NKG2D in liver injury were almost the same as blocking antibody against NKG2D or multiple NKG2D ligands. NKG2D antibody (250  $\mu$ g/mouse, Supporting Fig. 4) or Rae1, Mult1, or H60 antibody treatment alleviated liver injury induced by poly(I:C)/D-GalN injection, showing the decrease in serum ALT, IFN- $\gamma$ , and TNF- $\alpha$  levels and histopathological score of liver tissue (Fig. 3B, Supporting Fig. 5). Though each antibody of NKG2D ligands, including Rae1, Mult1, and H60 antibody, alleviated liver inflammation to varying degrees (Rae1 > H60 > Mult1) and did not reach the level of anti-NKG2D antibody, the mixture of Rae1, Mult1, and H60 antibodies reached the therapeutic level of anti-NKG2D antibody, further implying that all NKG2D ligands participate in NK cell-mediated NKG2D-dependent hepatitis. Noticeably, blocking antibody kept its protective effect for less than 24 hours for its protein property, whereas RNAi therapy, by using shRNA-expressing vector, could last for 7 days (Table 1), indicating that RNAi gene therapy has advantages over blocking antibody in long-lasting *in vivo* efficiency.

After verification of the highly efficient protective effect of simultaneous knockdown of multiple NKG2D ligands on liver immune injury via a hydrodynamics-based procedure, we packaged the shRNA-expressing plasmids with clinical grad recombinant ad-

**Table 1. Survival Rate of Multiple RNAi Plasmid-Preinjected Mice After Poly(I:C)/D-GalN Coadministration**

| RNAi Plasmid | Survival Rate |        |
|--------------|---------------|--------|
|              | 3 Days        | 7 Days |
| pRNAT-NNN    | 0/25          | 0/15   |
| pRNAT-MNM    | 16/25         | 7/15   |
| pRNAT-RMN    | 24/25         | 13/15  |
| pRNAT-NMH    | 12/25         | 7/15   |
| pRNAT-RNN    | 12/25         | 8/15   |
| pRNAT-NNH    | 9/25          | 6/15   |
| pRNAT-RNH    | 18/25         | 11/15  |
| pRNAT-RMH    | 25/25         | 14/15  |

enovirus for its hepatotropism and high infection efficiency.<sup>29</sup> The triple shRNA-expressing vector pRNAT-shRMH and control vector pRNAT-shNNN were further reconstructed with the Adeno-X Expression System, forming Adeno-shRMH (known as Ad-RMH) and Adeno-shNNN (known as Ad-NNN) (Supporting Fig. 6). Similarly, the recombinant adenovirus Ad-RMH prevented mice from fulminant hepatitis either by hydrodynamic injection or intravenous infection when administered 3 days prior to poly(I:C)/D-GalN injection (Fig. 4 A,B). Ad-RMH-treated mice showed significant alleviation of liver immune injury, showing a decrease in serum ALT levels (Fig. 4B,C). Surprisingly, it appears this protective effect could last more than 10 days, because when mice were infected intravenously with Ad-RMH at 10, 7, or 3 days before poly(I:C)/D-GalN injection, protection was sustained (Fig. 4C).

Although there was no difference in recruitment of MNCs and NK cells to liver and NKG2D expression on NK cells after poly(I:C)/D-GalN injection among pRNAT-shNNN-, pRNAT-shRNN-, or pRNAT-shRMH-treated mice (Fig. 5A,B), we found that intracellular IFN- $\gamma$ , one of the critical cytokines produced by activated NK cells,<sup>30</sup> decreased in pRNAT-shRMH-treated mice via flow cytometry, from 18.28% to 2.81% (Fig. 5C), indicating that NKG2D-mediated NK cell activation was inhibited after knockdown of its ligands on hepatocytes.

In addition to the poly(I:C)/D-GalN-induced hepatitis, we wondered whether the multiple shRNA-expressing vector is also effective in other inflammatory models. It has been reported that NKG2D recognition of hepatocytes by NK cells plays a critical role in over-sensitive liver injury during chronic HBV infection.<sup>20</sup> HBsAg-Tg mice were more sensitive to acute liver injury after ConA stimulation compared with C57BL/6 mice, shown by the levels of serum ALT (Fig. 6). It was found that NKG2D ligands Rae1 and Mult1 were

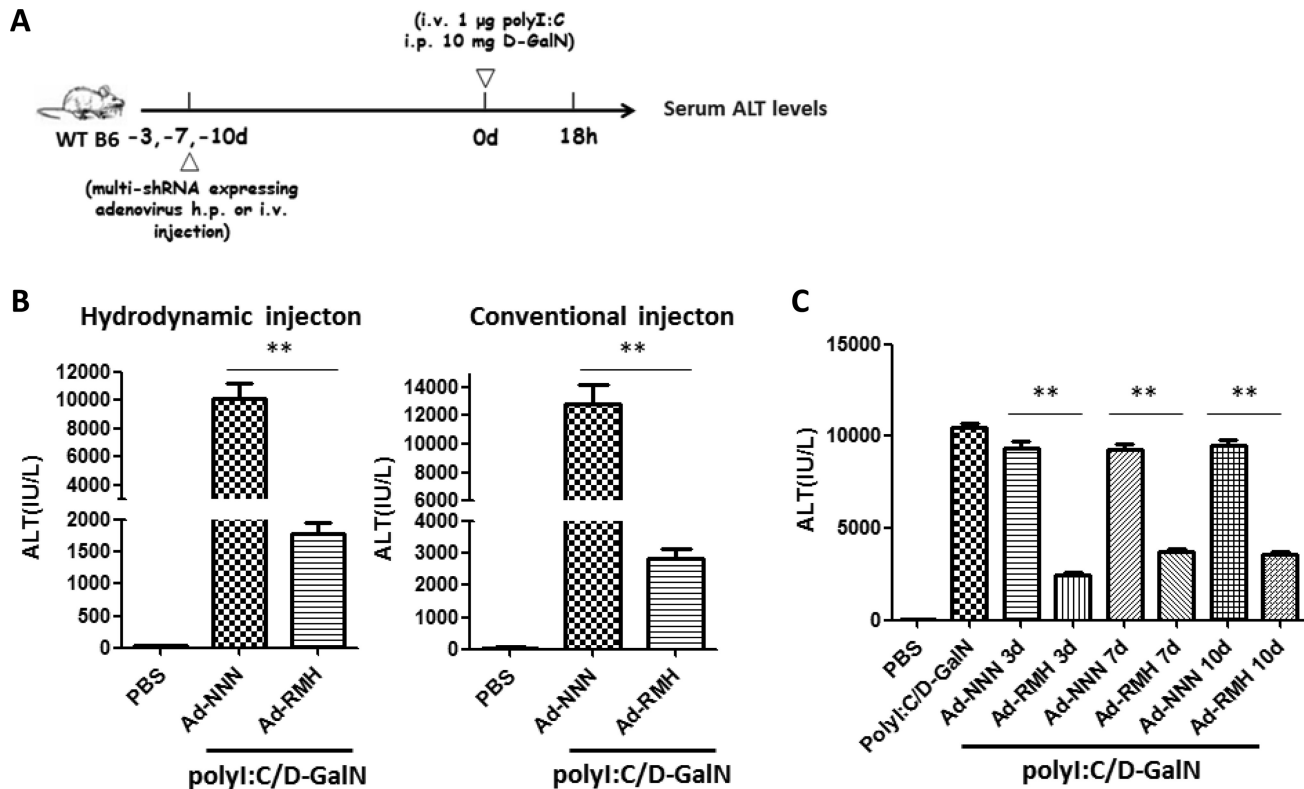


Fig. 4. Recombinant adenovirus-expressing multiple shRNAs against murine NKG2D ligands prevents mice from poly(I:C)/D-GalN-induced fulminant hepatitis. (A) Procedure for the treatment of poly(I:C)/D-GalN-induced liver injury by injection of multiple RNAi adenovirus 3, 7, or 10 days before. (B) Ad-RMH and Ad-NNN were preinjected 3 days before poly(I:C)/D-GalN injection hydrodynamically (left,  $4 \times 10^9$  ifu/mouse) or intravenously (right,  $1 \times 10^{11}$  ifu/mouse), and serum was harvested 18 hours after poly(I:C)/D-GalN injection. ALT levels were determined and data are shown as the mean  $\pm$  SEM ( $n = 3$  mice per group). (C) Ad-RMH and Ad-NNN ( $1 \times 10^{11}$  ifu/mouse) were preinjected 3, 7, or 10 days before poly(I:C)/D-GalN injection intravenously, and serum were harvested 18 hours after poly(I:C)/D-GalN injection. ALT levels were determined and data are shown as the mean  $\pm$  SEM ( $n = 3$  mice per group). \*\* $P < 0.01$  versus Ad-NNN preinjected mice.

upregulated in hepatocytes, but not H60 (Supporting Fig. 7). We hydrodynamically injected multiple shRNA-expressing vectors 3 days before ConA stimulation and found that the multiple shRNA-expressing vectors had a similar effect in this inflammatory model. HBsAg-Tg mice showed decreased serum ALT levels and alleviation of liver injury when Rael and Mult1 were both inhibited (Fig. 6A,B), suggesting *in vivo* application potential of the multiple shRNA-expressing vector.

**Simultaneous Knockdown of Multiple Ligands of Human NKG2D Prevents NK Cell Cytolysis Against Human Hepatocytes.** In order to verify whether knockdown of multiple ligands of human NKG2D prevents NK cell-mediated liver inflammation in human liver diseases, we tried to construct multiple shRNA-expressing vectors against human NKG2D ligands, including MICA/B, ULBP2, and ULBP3 molecules. Triple (called shMica-shUlbp2/3) and single shRNA-expressing vectors against human NKG2D ligands were constructed (Fig. 7A) and transfected into hepatocyte L-02 cells that constitutively expressed sur-

face MICA/B, ULBP2, and ULBP3. Stable shRNA-expressing L-02 cells were generated and showed downregulated expression of the NKG2D ligands MICA/B, ULBP2, and ULBP3 (Fig. 7B). For example, triple knockdown of these NKG2D ligands led to decreased expression of MICA/B, ULBP2, and ULBP3, whereas single knockdown of NKG2D ligands led the silence of one corresponding ligand. Furthermore, when incubated with NKG cells, which highly express human NKG2D (Supporting Fig. 8), triple shRNA-treated L-02 cells became less sensitive to cytolysis of NKG cells than control shRNA-treated L-02 cells (Fig. 7C). And, NKG cells expressed lower CD107a when cocultured with NKG2D ligand-silenced L-02 cells, indicating inactivation of NK cells by knockdown of NKG2D ligand on hepatocytes, may protect against immune injury of human liver.

## Discussion

NK cells, as the major innate immune cells, play a critical role in innate immune response against viral



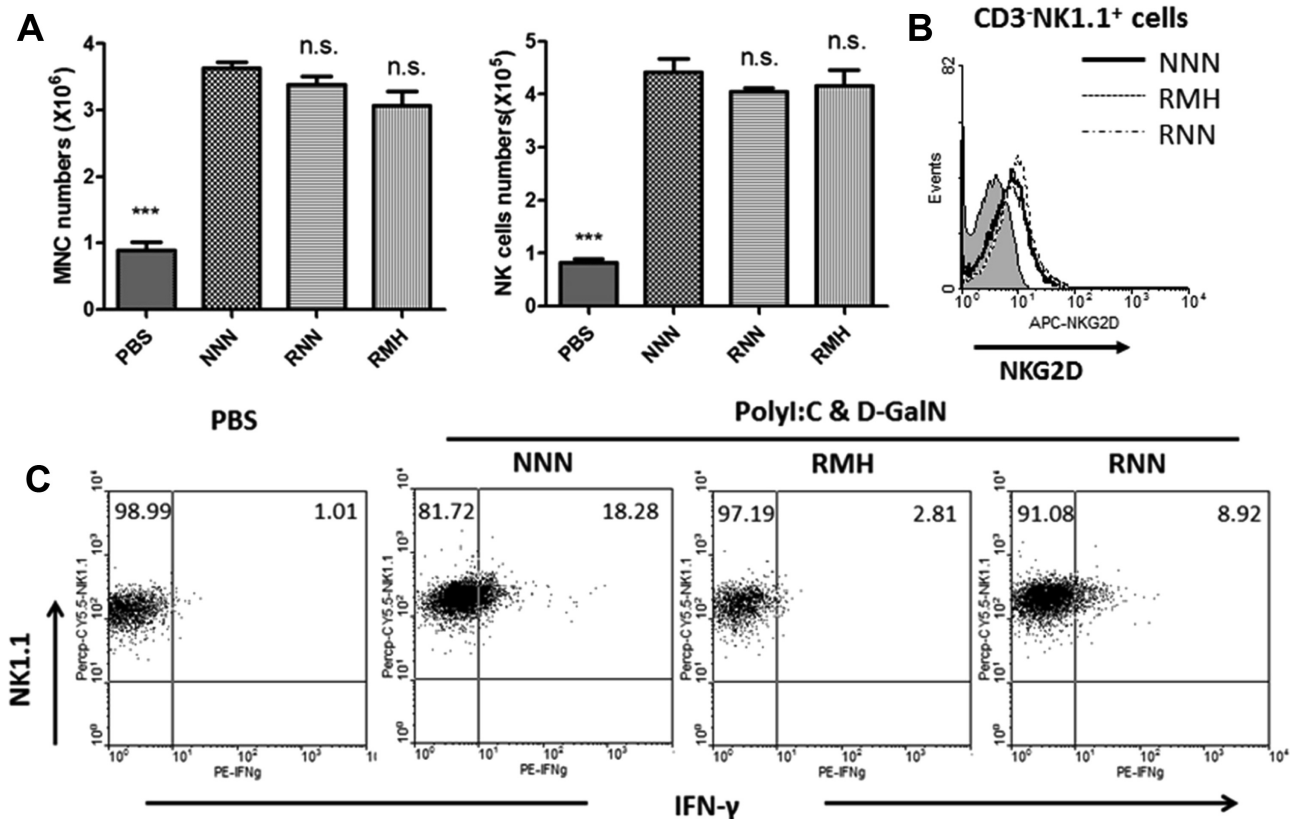


Fig. 5. NKG2D-mediated NK cell activation was inhibited after knockdown of its ligands. (A) Absolute numbers of MNCs and NK cells per liver were counted at 18 hours after poly(I:C)/D-GalN coadministration of multiple RNAi-preinjected mice or phosphate-buffered saline (PBS) injection ( $n = 3$  mice per group). (B) Liver NK cells were harvested for flow cytometry analysis of NKG2D expression and (C) intercellular staining of IFN- $\gamma$  at 18 hours after poly(I:C)/D-GalN coadministration of multiple RNAi-preinjected mice.

infections and tumor growth.<sup>31</sup> Unlike T and B cells, NK cells lack variable receptors that recognize foreign antigens, but they possess conserved inhibitory and

activating receptors to balance their cytotoxicity.<sup>32</sup> NKG2D, one of activating receptors, recognizes a variety of inducible self-proteins, including MICA/B,

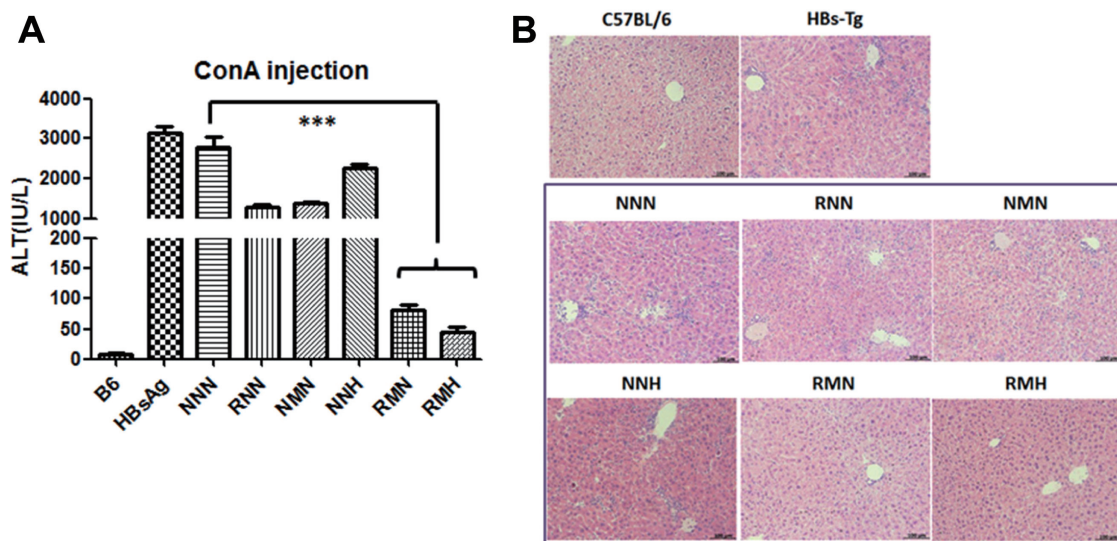


Fig. 6. RNAi against murine NKG2D ligands prevents HBsAg-Tg mice from ConA-induced liver injury. (A) Serum ALT levels were determined 18 hours after ConA administration of multiple RNAi vector-preinjected HBsAg-Tg mice or nontreated HBsAg-Tg mice and littermate C57BL/6 mice. Data are shown as the mean  $\pm$  SEM ( $n = 3$  mice per group). \*\*\* $P < 0.001$  versus pRNAT-NNN preinjected mice. (B) Liver paraffin sections were prepared for hematoxylin and eosin staining from multiple RNAi plasmid-preinjected mice after ConA administration (original magnification  $\times 200$ ).

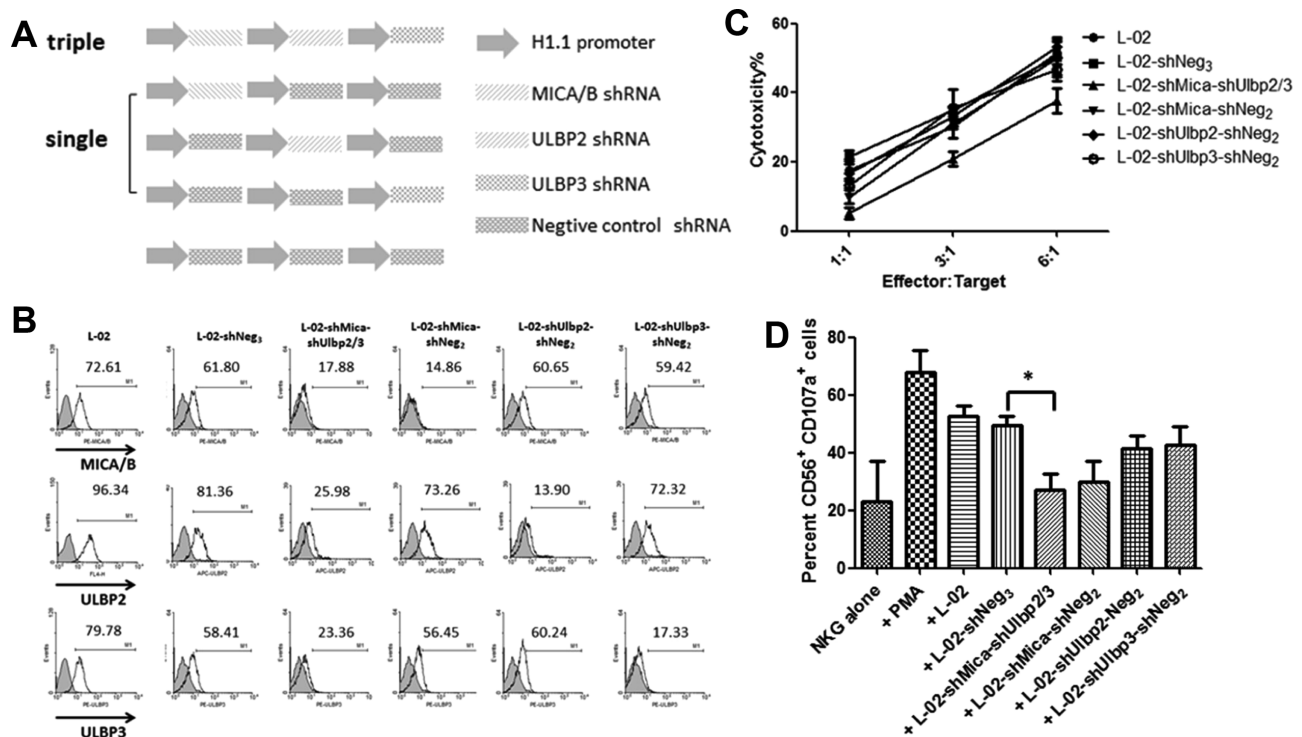


Fig. 7. RNAi against human NKG2D ligands confers protection from lysis by NK cells. (A) Triple and single shRNA-expressing vectors against human NKG2D ligands and negative shRNA-expressing vectors were constructed. (B) L-O2 cells were transfected with multiple RNAi vectors and control vector pRNAT-shNNN. Stable cell lines were generated and harvested for flow cytometry analysis of the human NKG2D ligands MICA/B, ULBP2, and ULBP3. (C) The  $^{51}\text{Cr}$  release assay was performed to test cytotoxicity of NKG cells against stable cell lines or L-O2 cells. Data are shown as the mean  $\pm$  SD and are representative of three experiments. (D) CD107a expression was analyzed on the surface of NKG cells following stimulation with stable cell lines or L-O2 cells.

ULBP (1-5) in humans and Rae1 ( $\alpha$ - $\epsilon$ ), Mult1, and H60 (a-c) in mice. These ligands were inducible by stress stimuli, such as viral infections and DNA damage. The role of NK cells and NKG2D ligand recognition in inflammation is already well known. Anti-NKG2D, one of the effective methods for blocking NKG2D ligand interaction, had been used often to intervene in NKG2D-dependent inflammation because of its higher target specificity. It was reported that anti-NKG2D antibody could delay NK cell-mediated airway allergy<sup>15</sup> and colitis<sup>17,18</sup> and ameliorate collagen-induced arthritis.<sup>19</sup> In addition, anti-NKG2D antibody was also applied to attenuate NKG2D-mediated acute hepatitis in subjects infected with hepatitis B virus.<sup>3,20</sup> However, this therapeutic antibody also has its disadvantages: a short half-life, a limited route for administration, and an economic burden placed on patients.

To circumvent these problems, we chose RNAi, one of the most promising and rapidly advancing frontiers in gene therapy, which works by operating upstream of protein synthesis by eliminating the mRNAs coding for such proteins.<sup>33</sup> There are few reports of NKG2D or its ligands RNAi, which may because of the widespread use of NKG2D antibody and diversity of

NKG2D ligands. Compared with NKG2D receptor on circulating immune cells, NKG2D ligands are more ideal intervention molecular targets than NKG2D itself in NKG2D-dependent autoimmunity and tissue injury. Considering the various ligands of NKG2D, we constructed multiple shRNA-expressing vectors against all NKG2D ligands. In our study, shRNA-expressing vectors that block synthesis of NKG2D ligands are similarly effective as blocking antibody, but last longer. Recombinant adenovirus that contains multiple shRNA cassettes could persistently express the shRNA cassettes for 10 days in mice by intravenous injection.

In our study, we found that the murine NKG2D ligands were absent in hepatocytes in C57BL/6 mice but were induced after poly(I:C)/D-GalN injection, which mimics a double-stranded RNA virus infection. In ConA-injected HBsAg-Tg mice, NKG2D ligands were upregulated in hepatocytes compared with C57BL/6 mice. pRNAT-shRMH treatment not only alleviated poly(I:C)/D-GalN-induced hepatitis in C57BL/6 mice, but also decreased susceptibility to ConA-induced liver injury in HBsAg-Tg mice. These findings indicate that increased expression of NKG2D

ligands on hepatocytes is related to immune surveillance and indicate a promising potential of the multiple shRNA-expressing vectors in *in vivo* application and clinical intervention.

Although NKG2D ligand interaction appears to be an important pathway of hepatocyte killing mediated by NK cells, the severe liver injury suggests that the other receptors on NK cells—including the CD69, DNAM-1, CD94, and Ly49 families—may also participate in immune injury. For example, DNAM-1 has been shown to play a role together with NKG2D in NK cell killing of Ewing's sarcoma cells.<sup>34</sup> And the activated NK cells may bridge innate and adoptive immune responses through the secretion of a variety of cytokines and chemokines to aggravate the liver injury. In addition to hepatocytes, Kupffer cells have a major role in the poly(I:C)/D-GalN model. We also reported that the interaction between Kupffer cells and NK cells mediated by NKG2D-Rae1 recognition plays a critical role in fulminant hepatitis.<sup>23</sup> Except for IFN- $\gamma$ , there may also be other factors involved, including TNF- $\alpha$  secreted by Kupffer cells, and other cytokines such as interleukin-12 and interleukin-18.

The *in vitro* results of human NKG2D ligand knockdown further encourage us to apply this multi-target RNA interference technology to clinical practice. Although the cellular and molecular mechanism underlying the therapeutic efficiency of multiple shRNA-expressing vector against murine NKG2D ligands in fulminant hepatitis requires further investigation, design and construction of multifunctional shRNA vectors against other innate immune receptors are worthy to practice.

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