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Activated NK cells kill hepatic stellate cells via p38/PI3K signaling in a TRAIL-involved degranulation manner

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Abstract

NK cells are important in regulating hepatic fibrosis via their cytotoxic killing of hepatic stellate cells (HSCs). NK cells are activated by both cytokines such as IL-12 and IL-18, and innate immune stimuli such as ligation of TLRs. The secretion of IL-18 depends upon activation of the inflammasome, whereas TLRs are stimulated by microbial products. In the case of NK cells, IL-18 acts synergistically with stimulation of TLR3 to cause cell activation and cytotoxic function. In the present study, we activated NK cells to kill HSCs via IL-18 and TLR3 ligand stimulation, and dissected the signaling pathways or molecules critical for such activation or killing. We find that such activation depends on signaling via the p38/PI3K/AKT pathway, and that the activated NK cells mediate HSC death in a TRAIL-involved mechanism. As liver fibrosis is a major global health problem with no good solution, these results emphasize that the p38/PI3K/AKT pathway in NK cells may be a novel drug target to promote fibrosis regression.

KEYWORDS
IL 18, p38 MAPK, TRAIL, TLR3

1 | INTRODUCTION

Liver fibrosis, which result from chronic liver injury of any etiology such as viral infection, alcoholic liver disease, and NASH, is a major global health problem for which there is no effective treatment.[1] As liver fibrosis progresses, liver cirrhosis and liver cancer regularly demand liver transplant.[2] Hepatic stellate cells (HSCs) are essential in the progression of liver fibrosis. HSCs are activated by inflammatory cytokines and mediators to trans-differentiate into myofibroblasts, and become the major source of extracellular matrix including collagens in the liver during the development of fibrosis.[3,4]

As an important component of the innate immune system, NK cells respond rapidly to transformed or virus-infected cells, and kill such target cells without restriction by either major histocompatibility antigen or a need for presensitization.[5] NK cells have been implicated in suppressing the pathogenesis of liver fibrosis,[6] but the exact mechanism remains elusive. It has been reported that NK cells selectively kill early activated (transitional) or senescent-activated HSCs rather than quiescent or fully activated HSCs (myofibroblasts).[7] Activated HSCs express less NK cell inhibitory ligand MHC-1,[8] but strongly express ULBP-2 (UL16 binding protein 2), MICA/B (MHC class I polypeptide-related sequence A/B),[9] and RAE-1 (retinoic acid early inducible 1),[7,10] which activate NK cells through engagement with NKG2D. This leads to killing of activated HSCs by NKG2D-dependent degranulation of NK cells.[7,9] Additionally, NK cells induce HSCs apoptosis through FasL with activated HSCs' intense expression of Fas, as well as TRAIL-mediated killing.[7,9] NK cells also restrain HSCs activation through IFN-γ secretion.[11] However, the effects of NK cells on HSCs through the established mechanisms are attenuated as NK cells are deactivated as liver fibrosis inexorably progresses, and impaired antifibrotic functions of NK cells are associated with accelerated progression of liver fibrosis.[12] Therefore, the restoration and promotion of NK cell activity might promote the regression of liver fibrosis.

IL-18 belongs to the IL-1 family. Once its 24 kDa precursor is cleaved to the 18 kDa form by IL-1β converting enzyme (ICE, also known as caspase-1), IL-18 acquires biologic function to promote proliferation and cytolytic activity of NK and T cells combined with IL-12 or IL-2.[13,14] The immunostimulatory properties of polyinosinic–polycytidylic acid (poly I:C), a surrogate TLR3 ligand, have been verified in various pathologic circumstances in vivo.[15] Furthermore, NK cell activation in response to IL-18/IL-12 or IL-15/IL-12 stimulation is enhanced by TLR3 agonists.[16,17] However, the signaling pathway

Abbreviations: HSC, hepatic stellate cell; poly I:C, polyinosinic–polycytidylic acid

by which IL-18 and poly I:C synergistically activate NK cells is not clearly defined.

In the present study, we investigated the mechanisms by which NK cells are activated by IL-18 and poly I:C to kill HSCs using human NK cells from healthy donors and primary HSCs or LX2 cells. Our results showed that IL-18 and poly I:C synergistically activate NK cells via the p38/PI3K/Akt signaling pathway, and the activated NK cells kill HSC in a TRAIL-involved degranulation manner. We thus propose that p38 MAP/PI3 kinase might be a novel therapeutic target for intervention of human liver fibrosis targeting NK cells.

2 | MATERIALS AND METHODS

2.1 | Human subjects

Buffy coats from healthy donors were provided by the Changchun Blood Center, and informed consent was provided according to the protocols of the Changchun Blood Center. Liver perfusion from cadaver donors were collected as we described previously.[18] All studies were conducted according to the experimental practices and standards that were approved by the Medical Ethics Committee of First Hospital of Jilin University (approval code: 2015–125).

2.2 | Cell isolation and purification

PBMCs were freshly isolated from peripheral blood of healthy individuals by Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) density gradient separation. Liver mononuclear cells were freshly isolated from liver perfusates as we described previously.[18] For magnetic cell sorting, NK cells were purified by NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of NK cells was approximately 95% as determined by flow cytometry (Supplemental Fig. S1A).

2.3 | Cell culture

Purified NK cells were cultured in RPMI 1640 medium (Corning Life Science, Lowell, MA, USA) with 10% FBS and 1% Pen-Strep (GE Healthcare). After stimulated with IL-18 (R&D System, Minneapolis, MN, USA) or poly I:C (Sigma–Aldrich, Saint Louis, MO, USA) for 24 h in 24 wells plate, cells were collected for FACS analysis, the supernatants were harvested for cytokine detection. To inhibit PI3K or p38 MAPK, NK cells were pretreated in the presence of 50 μM LY294002 (Sigma–Aldrich) or 20 μM SB202190 (Sigma–Aldrich) for 1 h and then stimulated with IL-18 and/or poly I:C.

LX2 cells were cultured in DMEM medium (Corning Life Science) supplemented with 10% FBS. Primary human HSCs purchased from ScienCell (San Diego, CA, USA), and were cultured within 3 passages in Stellate Cell Medium (ScienCell). The definition of LX2 cells and primary HSCs were shown as Supplemental Fig. S1B.

2.4 | Cell coculture

NK cells stimulated with IL-18 and/or poly I:C were collected and resuspended in fresh medium, and then cocultured with LX2 or primary HSCs, which were labeled with 1.25 μM CFSE (Lifescience Technology, Carlsbad, CA, USA), in 24 wells plate at $E{:}T = 1{:}1$ for 5 h. For transwell coculture experiments, NK cells were in the upper chamber, whereas LX2 cells or primary HSCs were in the lower chamber using the plates with 0.4 μm pore diameter (Corning Life Science). For the blocking experiments, IL-18/poly I:C-stimulated NK cells were pretreated with 50 μM 3,4-DCI (Sigma–Aldrich) or 1 μg/ml anti-TRAIL (R&D System; Catalog number: AF375) for 1 h, and then cocultured with LX2 or primary HSCs. For IFN-γ-blocking coculture experiments, IL-18/poly I:C-primed NK cells were cocultured with LX2 or primary HSCs with 1 μg/ml anti-IFN-γ supplied in medium. Soluble TRAIL (R&D System; Catalog number: 375-TL-010) was added to HSCs half hour earlier than NK cells in coculture.

2.5 | Flow cytometry

Cell staining and flow cytometry analysis were performed as described.[19] Briefly, the phenotypic and functional markers of NK cell were characterized by staining with the following antibodies: mouse anti-human NKp30-PE (BD Pharmingen; Catalog number: 558407; BD Biosciences, San Jose, CA, USA), mouse anti-human NKp46-APC (BD Pharmingen; Catalog number: 558051), mouse anti-human NKG2D-APC (BD Pharmingen; Catalog number: 558071), mouse anti-human CD94-FITC (BD Pharmingen; Catalog number: 555888), mouse anti-human CD69-V450 (BD Horizon; Catalog number: 560740), mouse anti-human CD253-PE (BD Pharmingen; Catalog number: 550516), mouse anti-human CD16-PE-Cy7 (BD Pharmingen; Catalog number: 557744). Intracellular IFN-γ was stained by mouse anti-human IFN-γ-APC (BD Pharmingen; Catalog number: 551385). Phosphorylation of p38 MAPK or Akt were characterized by staining with mouse anti-Akt-PE-CF594 (pS473; BD Phosflow; Catalog number: 562465) or mouse anti-p38 MAPK-Pacific Blue (pT180/pY182; BD Phosflow; Catalog number: 560313). For CD107a detection, mouse anti-human CD107a-FITC (BD Pharmingen; Catalog number: 555800) was added into medium for 5 h, and Golgi-Plug was involved in the last 4 h, and cells were collected for flow cytometry. For cell apoptosis analysis, LX2 and primary HSCs were stained with Annexin V and PI using Apoptosis Detection Kit (BD Pharmingen; Catalog number: 556547). Flow cytometry was performed using a BD LSRFortessa flow cytometer (BD Biosciences). The data acquired were analyzed with FlowJo (Treestar software, Ashland, OR, USA).

2.6 | ELISA

The cell culture supernatants were collected, and IFN-γ concentrations were measured by Human IFN-gamma ELISA Ready-Set-Go® (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

2.7 | NK cell siRNA transfection

Transfection were performed as described.[20] Briefly, NK cells were seeded in 24-well plates, and transfected with SignalSilence® p38 MAPK siRNA I (Cell Signalling Technology, Danvers, MA, USA) or AllStars Negative Control siRNA (QIAGEN, Germantown, MD, USA) using HiPerFect Transfection Reagent (QIAGEN) before IL-18/poly I:C stimulation.

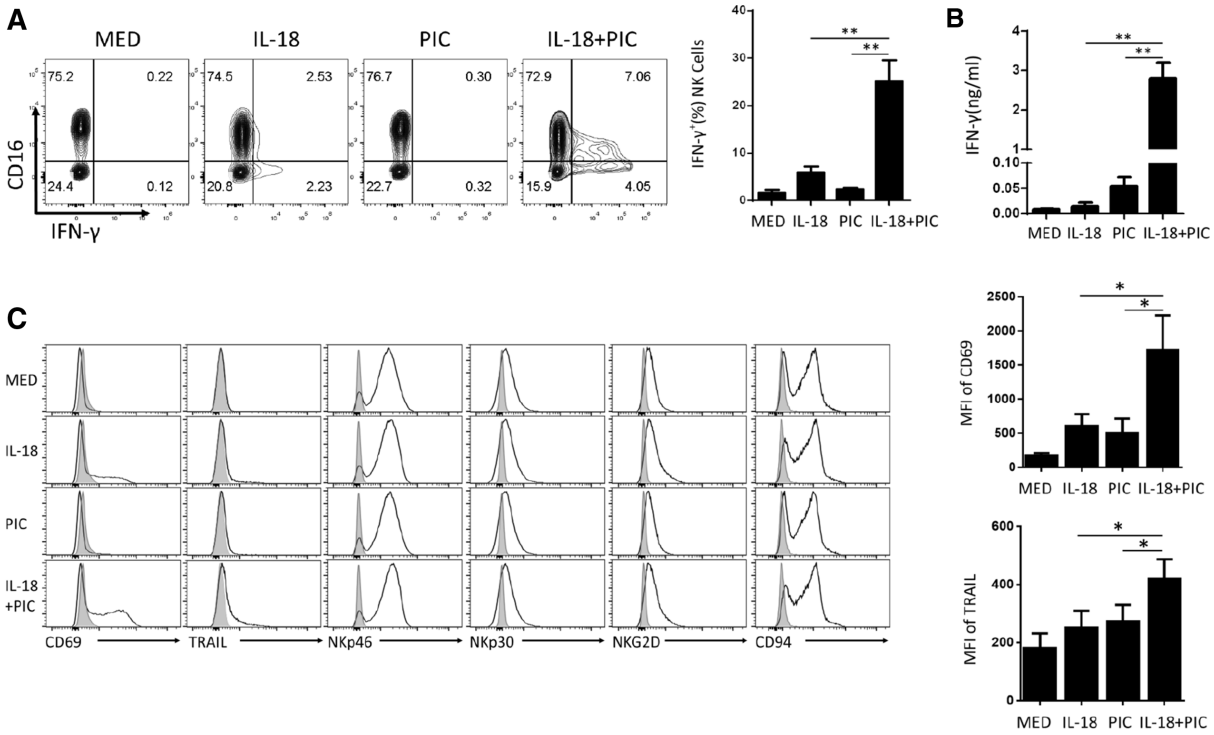

FIGURE 1 **IL-18 and poly I:C synergistically activate NK cells.** Purified NK cells were treated with IL-18 or/and poly I:C at indicated concentration for 24 h. IFN-γ expression was measured by intracellular stain (A). IFN-γ secretion in the supernatant was detected by ELISA (B). CD69/TRAIL NKp46/NKp30/NKG2D/CD94 expression on NK cells was analyzed by flow cytometry (C). Results were shown as mean ± SEM of 6 independent experiments performed with 6 different donor samples. $^{**}P < 0.01$ and $^{***}P < 0.001$, paired *t*-test

2.8 | Western blot analysis

Purified NK cells stimulated with IL-18 and/or poly I:C were collected and lysed for Western blotting analysis as described previously.[21] Briefly, cells were collected by centrifugation and resuspended in RIPA buffer (Cell Signalling Technology) to extract protein. Protein extracts were resolved on a 10% SDS-PAGE under constant Voltage condition. After electrophoresis, protein was transferred to a transfer membrane (Merck Millipore, Darmstadt, Germany). Blots were incubated at room temperature for 1 h and at 4 °C overnight with anti-p38 (Cell Signalling Technology; Catalog number: 9212), anti-Akt (Cell Signalling Technology; Catalog number: 9272), anti-MAPKAPK-2 (Cell Signalling Technology; Catalog number: 3042), anti-phosphorylated p38 (Cell Signalling Technology; Catalog number: 4511), anti-phosphorylated Akt (Cell Signalling Technology; Catalog number: 4060), anti-phosphorylated MAPKAPK-2 (Cell Signalling Technology; Catalog number: 3041), and anti-beta-actin (Cell Signalling Technology; Catalog number: 4970). After washed 3 times by 0.1% tween 20 (Amresco, Solon, OH, USA) PBS, peroxidase-conjugated anti-rabbit IgG (Proteintech, Rosemont, IL, USA; Catalog number: 10545-2-AP) was involved for another 1 h at room temperature. Immunoreactivity was determined by using ECL kit (PerkinElmer, Waltham, MA, USA) by XRS+ (BIO-RAD, Hercules, USA). Results were analyzed by Image Lab (BIO-RAD).

2.9 | Live cell imaging

Live cell imaging was performed as described.[22] LX2 cells were seeded in glass bottom dish (In Vitro Scientific, Mountain View, CA, USA) and cultured for 30 min. Then prestimulated NK cells as described above were added at NK: HSC ratio of 2:1 and immediately coincubated by using DeltaVision Elite (GE Healthcare, Buckinghamshire, UK). Cells were maintained in a 37 °C, 5% CO_2 chamber while videos were recorded.

2.10 | Statistical analysis

All data were analyzed using the D'Agostino and Pearson omnibus normality test. *P* values of <0.05 were considered statistically significant. Mean values were compared using either a paired *t*-test (2 groups) or ANOVA (more than 2 groups), followed by a Bonferroni correction for multiple comparisons test. All statistical tests were performed using GraphPad Prism software (San Diego, CA, USA).

2.11 | Online supplemental material

Supplemental Figs. S1–S3 and Supplemental Videos S1–S3 were included as Online Supplemental Material.

3 | RESULTS

3.1 | IL-18 and poly I:C synergistically activate NK cells to express IFN-γ, TRAIL, and CD69

To investigate whether IL-18 and poly I:C activate NK cells, we first purified NK cells from peripheral blood of healthy donors, and the distribution of $CD56^{dim}CD16^{+}$/ $CD56^{bright}CD16^{-}$ subsets was shown as Supplemental Fig. S2B. Then purified NK cells were stimulated

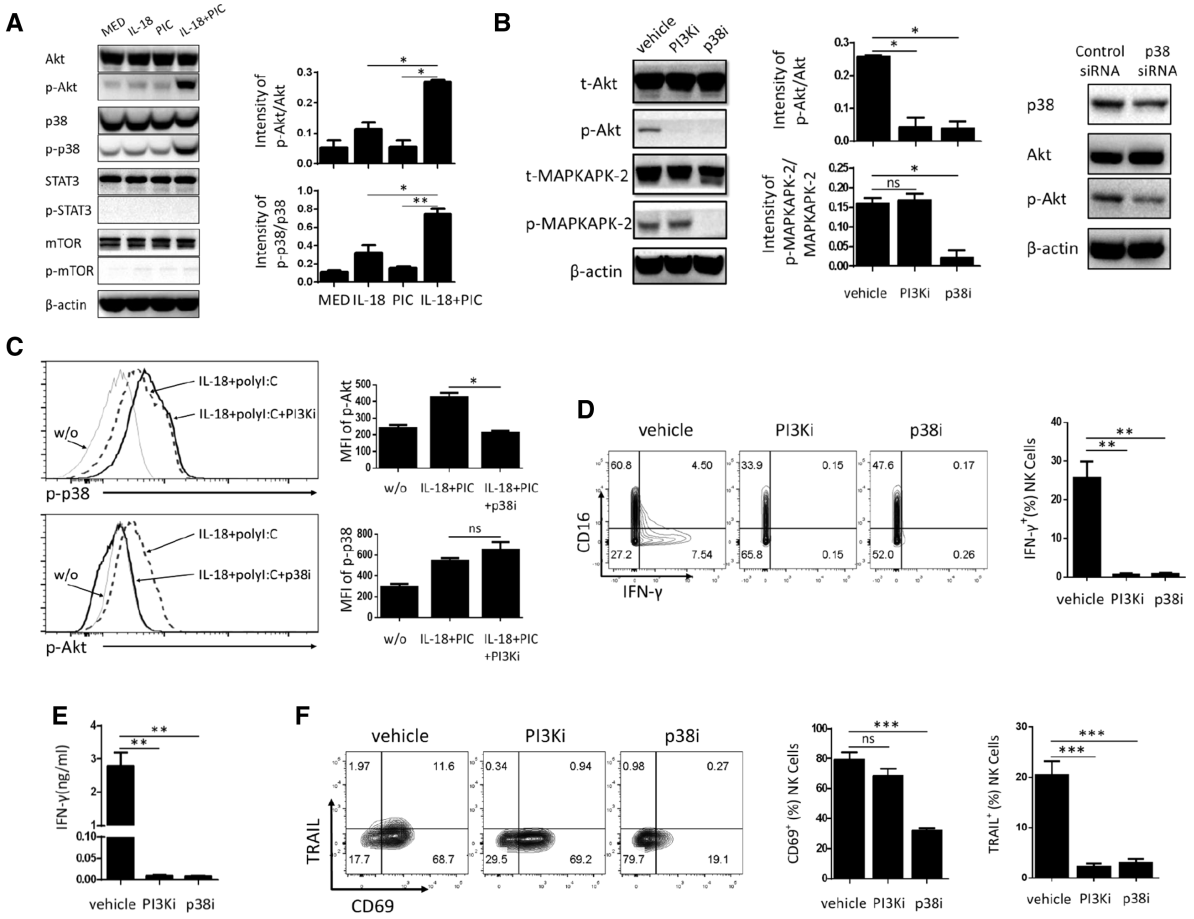

FIGURE 2 **IL-18 and poly I:C synergistically activate p38 MAPK/PI3K/Akt to induce NK cell activation.** Purified NK cells ($n = 3$) were stimulated with IL-18 or/and poly I:C for 30 min, the expression of p38 MAPK, Akt, STAT3, mTOR, and the phosphorylation of p38 MAPK, Akt, STAT3, mTOR was determined by Western blot. β-Actin was used as loading control (A). NK cells were pretreated with PI3Ki (LY294002) or p38i (SB202190) for 1 h, and then stimulated with IL-18 and/or poly I:C, the expression and phosphorylation of Akt, MAPKAPK-2, p38 MAPK was measured by Western blot ($n = 3$, B) or flow cytometry ($n = 3$, C). NK cells were transfected with control siRNA or p38 siRNA before stimulated by IL-18 and poly I:C, the expression of p38 MAPK, Akt, β-actin, and the phosphorylation of Akt were detected by Western blot (B). The expression of IFN-γ in NK cells ($n = 6$) was measured by intracellular cytokine staining (D) and ELISA (E). CD69 and TRAIL expression on NK cells ($n = 6$) were detected by using flow cytometer analysis (F). Results are shown as mean ± SEM of the indicated numbers of independent experiments performed with different donor samples. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, paired t-test; ns, not significant

with the optimal concentration of IL-18 (100 ng/ml) and/or poly I:C (50 μg/ml) as determined in Supplemental Fig. S2C. IFN-γ production was analyzed by intracellular staining and ELISA, the activating receptor and inhibitory receptor expressed on NK cells were examined by flow cytometry. Consistent with liver NK cells,[23] IL-18 and poly I:C synergistically induce IFN-γ expression ($P < 0.01$; Fig. 1A) and production ($P < 0.01$; Fig. 1B) as well as TRAIL expression ($P < 0.01$; Fig. 1C) of peripheral NK cells, compared with IL-18 or poly I:C alone. Moreover, we observed that IL-18 and poly I:C synergistically up-regulate CD69 expression ($P < 0.01$), but fail to affect Nkp30/ Nkp46/ NKG2D/ CD94 expression on NK cells (Fig. 1C).

3.2 | IL-18 and poly I:C synergistically activate NK cells via p38 MAPK/PI3K signaling pathway

We further investigated the mechanism by which IL-18 and poly I:C synergistically activate NK cells. Purified NK cells were stimulated with IL-18 and/or poly I:C, the protein expression and phosphorylation levels of Akt, p38, STAT3, and mTOR were determined by Western blots. The results showed that the phosphorylation of p38 and Akt, but not STAT3 and mTOR, are activated by the synergy of IL-18 and poly I:C, whereas neither IL-18 nor poly I:C alone did ($P < 0.05$; Fig 2A). To define the signaling pathway of IL-18/poly I:C-induced NK cell activation, the inhibitors of p38 (SB202190) and PI3K (LY294002) were added to NK cells before stimulation with IL-18 and poly I:C. The phosphorylation levels of Akt, p38, and MAPKAPK-2 (directly phosphorylated by p-p38) were examined by Western blot and flow cytometry, IFN-γ production by intracellular staining and ELISA, and the expression of CD69 and TRAIL by flow cytometry. We observed that either SB202190 or LY294002 inhibit IL-18/poly I:C-induced Akt phosphorylation ($P < 0.05$), but LY294002 fail to inhibit IL-18/poly I:C-induced activation of p38/MAPKAPK-2 (Figs. 2B and C). Phosphorylation levels of Akt were reduced when NK cells were transfected with p38 siRNA (Fig. 2B), suggesting that PI3K/Akt is downstream signaling of p38 in IL-18/poly I:C-activated NK cells. Moreover, both LY294002 and SB202190 inhibit IL-18/poly I:C-induced IFN-γ expression ($P < 0.01$;

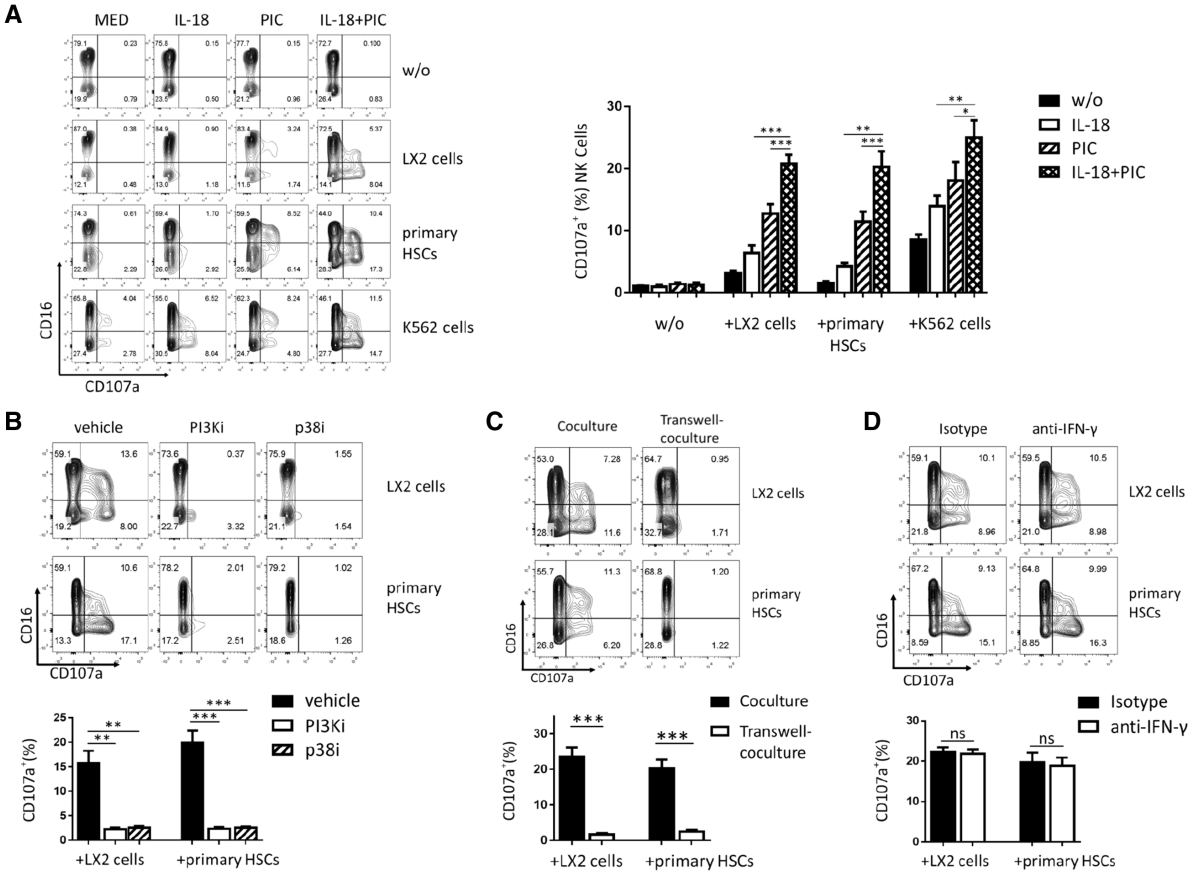

FIGURE 3 **HSCs activate IL-18/poly I:C-primed NK cell degranulation.** NK cells stimulated with IL-18 and/or poly I:C were collected and resuspended in fresh medium, and then cocultured with LX2, primary hepatic stellate or K562 cells at E: $T = 1{:}1$, the expression of CD16 and CD107a was analyzed by flow cytometry (A). NK cells were pretreated with SB202190 or LY294002 before IL-18 and poly I:C stimulation, and then cocultured with LX2 or primary HSCs, CD107a expression was shown (B). CD107a expression was shown when transwell-coculture was used to separate NK cells from LX2 or primary HSCs (C). Anti-IFN-γ or isotype antibody were added in coculture, and CD107a expression of NK cells were detected (D). Results were shown as mean ± SEM of 6 independent experiments performed with 6 different donor NK cells. $^{**}P < 0.01$, $^{***}P < 0.001$, paired *t*-test

Fig. 2D) and production ($P < 0.01$; Fig. 2E) as well as TRAIL expression ($P < 0.001$; Fig. 2F). The CD69 expression induced by IL-18/poly I:C, however, is only inhibited by SB202190 ($P < 0.001$) but not LY294002 (Fig. 2F). It indicates that IL-18/poly I:C-activated p38/PI3K/AKT signaling pathway is involved in IFN-γ and TRAIL expression, and an alternative p38 signaling pathway in CD69 expression on NK cells.

3.3 | HSCs activate IL-18/poly I:C-primed NK cell degranulation

During liver injury, the activation of HSCs in response to hepatocyte damage results in the increased NK cell stimulation and decreased NK cell inhibition.[4] We further determined whether HSCs activate IL-18/poly I:C-primed NK cell degranulation. IL-18 and/or poly I:C-pretreated NK cells were cocultured with LX2 cells, primary HSCs, or K562 cells. CD107a expression level of NK cells was used to evaluate the primed NK cell degranulation using flow cytometer analysis. As the results shown in Fig. 3A, IL-18/poly I:C-pretreated NK cells, which were cocultured with HSCs or K562 cells, show significantly higher CD107a expression than that of IL-18 or poly I:C alone pretreated NK cells ($P < 0.01$), whereas both LX2 cells and primary HSCs induce slightly CD107a expression of IL-18 or poly I:C pretreated NK cells, but not rest NK cells. As control, IL-18 and/or poly I:C could not directly induce the CD107a expression of NK cells without target cell stimulus, K562 cells induce the CD107a expression of rest NK cell. We also observed that CD107a expression is also suppressed when NK cells were treated with inhibitors of PI3K or p38 before stimulated by IL-18 and poly I:C ($P < 0.01$; Fig. 3B). CD107a expression is completely blocked using transwell to separate IL-18/poly I:C-pretreated NK cells from LX2 cells or primary HSCs ($P < 0.001$; Fig. 3C). But neutralization of IFN-γ in coculture did not affect CD107a expression (Fig. 3D). This suggests that HSCs induce the degranulation of IL-18/poly I:C-primed NK cells in a cell–cell contact-dependent manner, and that IL-18/poly I:C-activated p38/PI3K/AKT signaling is required for HSC-induced NK cell degranulation.

3.4 | IL-18/poly I:C-primed NK cells reciprocally induce HSCs death through degranulation

Next, to investigate IL-18/poly I:C-primed NK cell cytotoxicity to HSCs, CFSE-labeled LX2 cells or primary HSCs were cocultured with IL-18 and/or poly I:C-primed NK cells. Cell gating strategy was shown

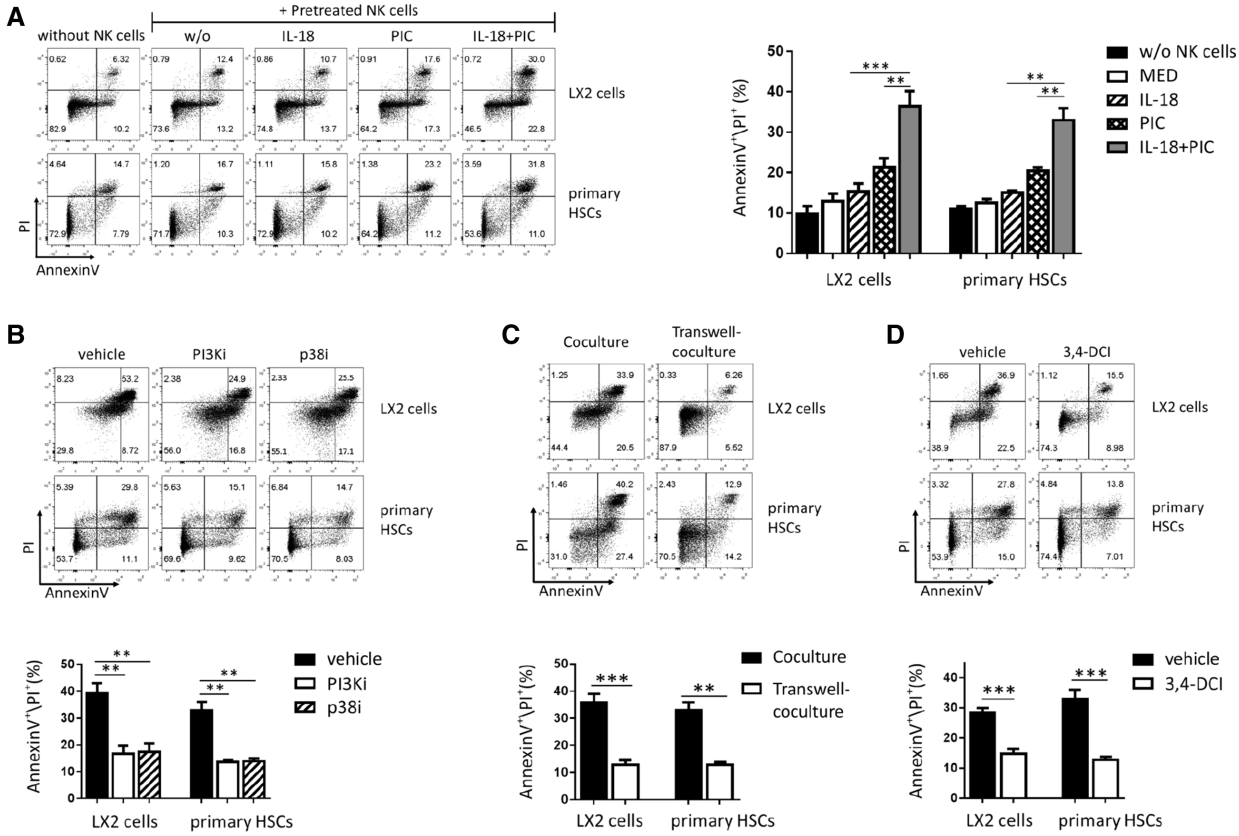

FIGURE 4 **IL-18/poly I:C-primed NK cells reciprocally induce HSC death through degranulation.** Primary HSCs or LX2 cells were cocultured with IL-18- and/or poly I:C-pretreated NK cells, the cell death was evaluated by AnnexinV and PI staining. The cell death of primary HSCs and LX2 cells was shown (A). NK cells were pretreated with PI3Ki (LY294002) or p38i (SB202190) before IL-18 and poly I:C stimulation, and then cocultured with LX2 or primary HSCs, the cell death of HSCs was shown (B). Primary HSC and LX2 cell deaths were shown when transwell-coculture was used to separate LX2 or primary HSCs from NK cells (C). IL-18 and poly I:C-stimulated NK cells were pretreated with 3,4-DCI before cocultured with LX2 or primary HSCs, and the cell death of LX2 and primary HSCs was shown (D). Results were shown as mean ± SEM of 6 independent experiments performed with 6 different donor NK cells. $^{**}P < 0.01$, $^{***}P < 0.001$, paired *t*-test; ns, not significant

in Supplemental Fig. S2D, the cell death of LX2 and primary HSCs were evaluated with PI^+/$AnnexinV^+$. We observed that the activated NK cell-induced HSC death is dependent on the NK cells/HSCs ratio (Supplemental Fig. S2E), and E/T ratio = 1:1 was used in the following NK cells/HSCs coculture experiments. IL-18/poly I:C-pretreated NK cells significantly induce the cell death of primary HSCs and LX2, compared with IL-18 or poly I:C alone pretreated NK cells ($P < 0.01$; Fig. 4A), whereas pretreated NK cells with medium did not do that. The cell death of primary HSCs and LX2 was suppressed by PI3K or p38 inhibitors, which were added to NK cells before stimulated with IL-18 and poly I:C ($P < 0.01$; Fig. 4B). Transwell, which was used to separate primary HSCs or LX2 cells from IL-18/poly I:C-activated NK cells in coculture, completely inhibits primary HSCs and LX2 cell death ($P < 0.01$; Fig. 4C). Furthermore, when IL-18/poly I:C-activated NK cells were pretreated with GranzymeB inhibitor (3,4-dichloroisocoumarin) before cocultured with LX2 cells or primary HSCs, the cell death of primary HSCs and LX2 is entirely inhibited as well ($P < 0.001$; Fig. 4D). However, neutralization of IFN-γ by anti-IFN-γ antibody in cell coculture did not affect the cell death of LX2 and primary HSCs (Supplemental Fig. S3A). These data indicate that NK cell cytotoxicity to HSCs occurs through degranulation in a cell–cell contact-dependent manner, but not directly through IFN-γ production.

3.5 | IL-18/poly I:C-primed NK cells kill HSCs in a TRAIL-involved degranulation manner

Previous studies reported that NK cells induce a rapid apoptosis of HSCs through TRAIL[7,9] or degranulation.[8,20] We thus investigated the role of TRAIL in NK cells killing HSCs. Primary HSCs and LX2 cells expressed TRAIL-R2 and TRAIL-R4 (Supplemental Fig. S3B). Using anti-TRAIL antibody or soluble TRAIL (sTRAIL) in HSC/NK cell coculture for the blockades of TRAIL or TRAIL receptor, which are expressed on NK cells or HSCs, respectively, the expression levels of CD107a or PI/AnnexinV were used to evaluate NK cell degranulation or HSC cell death. We observed that the blockades of TRAIL and TRAIL receptor with anti-TRAIL and sTRAIL inhibit the cell death of LX2 and primary HSCs ($P < 0.01$; Fig. 5A and B), as well as NK cell degranulation ($P < 0.05$; Fig. 5C and D).

To further analyze the immune synapse of HSC/NK cell interaction, we visualized LX2 cells and PKH26-labeled NK cells coculture by live cell imaging for 150 min. As shown in Fig. 5E and Supplemental Video, IL-18/poly I:C-activated NK cells rapidly induce LX2 cell budding after adhesion (Supplemental Video S2) contrast to resting NK cells (Supplemental Video S1). Consistently, the blockade of TRAIL inhibited LX2 cell budding (Supplemental Video S3). Besides, PKH26-labeled

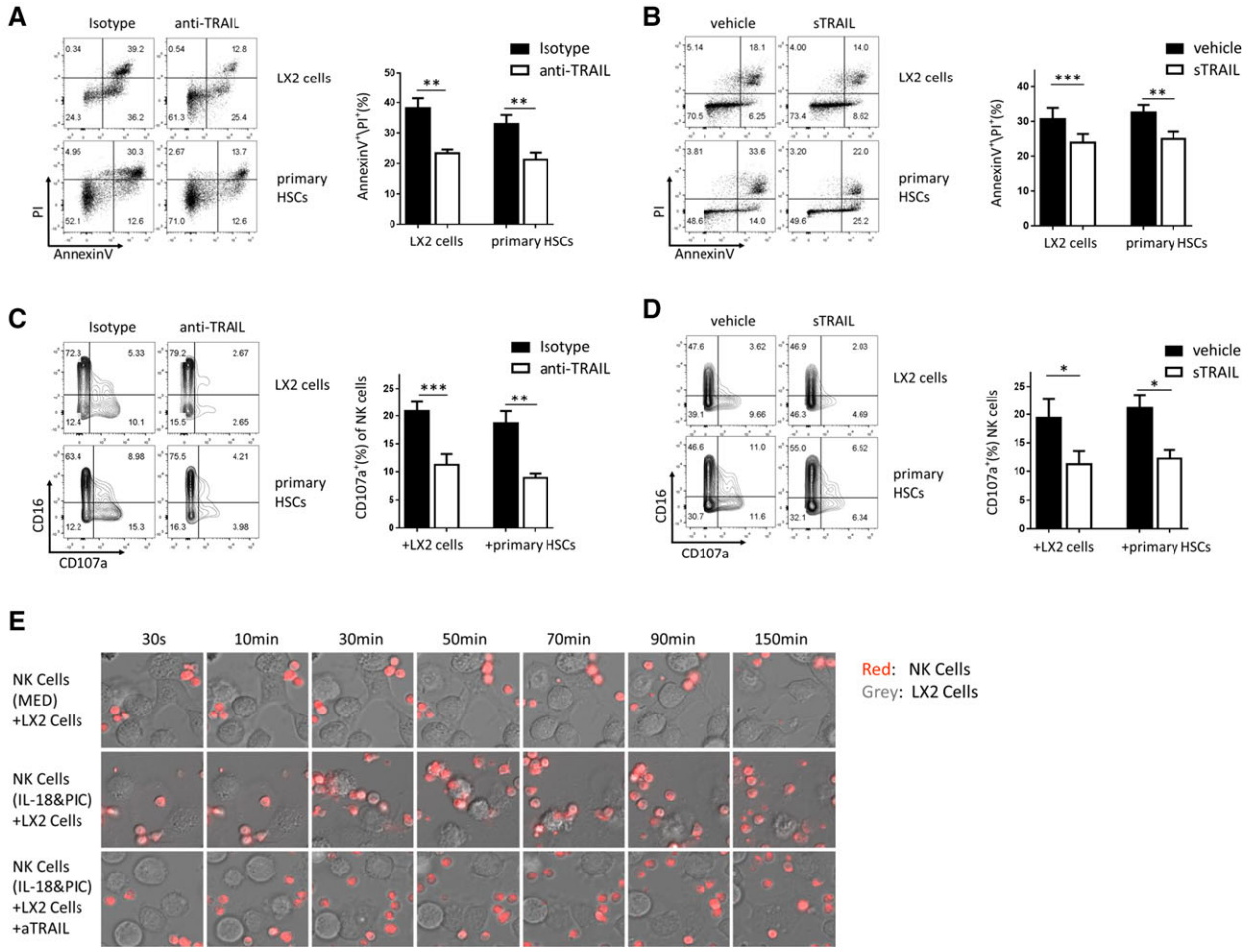

FIGURE 5 **IL-18/poly I:C-primed NK cells kill hepatic stellate cells in a TRAIL- involved degranulation manner.** IL-18 and poly I:C-primed NK cells were treated by anti-TRAIL or isotype antibody before coculture. HSC death was measured (A), and NK cell degranulation was evaluated by CD107a expression (C). Soluble TRAIL was added to cell coculture, the cell death of HSCs (B) and CD107a expression of NK cells (D) was shown. Results were shown as mean ± SEM of 6 independent experiments performed with 6 different donor NK cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, paired *t*-test. The kinetics of the activated NK cell-mediated LX2 cell apoptosis were observed by using live-cell imaging (E)

NK cells were cocultured transiently with CFSE-labeled LX2 cells and washed gently to remove unattached NK cells. The results showed that anti-TRAIL decrease the number of adhesive NK cells on LX2 cells (Supplemental Fig. S3C).

Taken together, these data suggest that the engagement of TRAIL expressed on activated NK cells with TRAIL receptors expressed on HSCs play a role in the immune synapse formation, which initiates NK cell degranulation to kill HSCs.

3.6 | IL-18/poly I:C-primed hepatic NK cells kill HSCs in a TRAIL-involved degranulation manner

Finally, to testify whether IL-18 and poly I:C increase hepatic NK cell cytotoxicity to HSCs as peripheral NK cells did, we isolated hepatic NK cells from liver perfusate of cadaver donors to establish hepatic NK cells and primary HSCs coculture as we did in peripheral NK cells. As expected, primary HSCs activate IL-18/poly I:C-primed hepatic NK cell degranulation ($P < 0.05$; Fig. 6A). IL-18/poly I:C-primed hepatic NK cells induce the cell death of primary HSCs ($P < 0.01$; Fig. 6B). Moreover, blockade of TRAIL with anti-TRAIL antibody inhibit hepatic NK cell degranulation ($P < 0.05$; Fig. 6C) and the cell death of primary HSCs ($P < 0.01$; Fig. 6D).

4 | DISCUSSION

We have previously reported that poly I:C induces IL-18 expression in Kupffer cells to activate liver NK cells.[19] Liver NK cells are also synergistically activated by IL-18 and poly I:C to produce IFN-γ and express TRAIL on their surface.[23] But the mechanism by which IL-18 and poly I:C synergistically activate NK cells remains to be defined. In the present study, our results showed that consistent with hepatic NK cells, the synergy of IL-18 and poly I:C activate NK cells (Fig. 1). We further found that IL-18 and poly I:C synergistically induce NK cell activation through P38/PI3K/AKT signaling pathway (Fig. 2). IL-18 signaling is MyD88 (Myeloid differentiation primary response gene 88) dependent,[13] whereas poly I:C/TLR3 signaling is TIR-domain-containing adapter-inducing interferon-β (TRIF) dependent and MyD88 independent.[16] Downstream of TRIF and MyD88, signaling as ERK,[24] NF-κB,[25] IFN regulatory factor 5 (IRF5),[26]

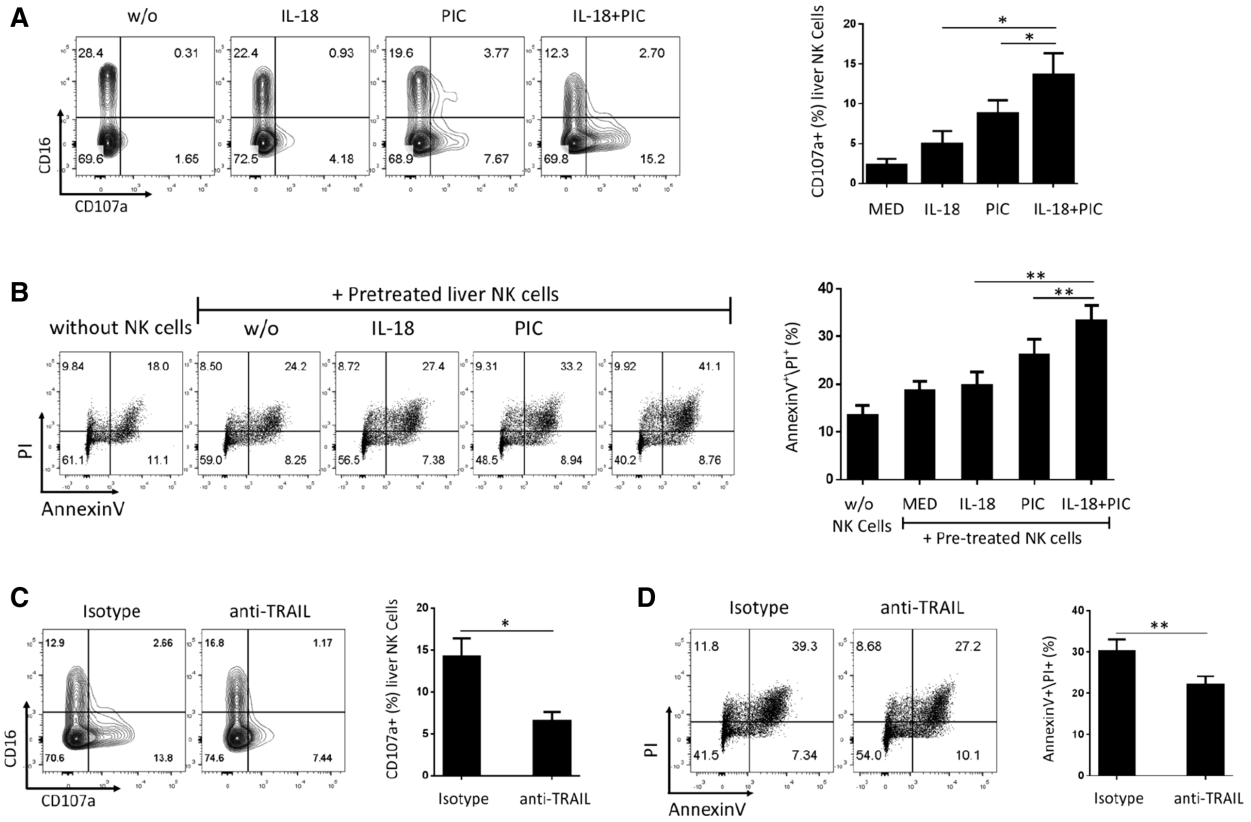

FIGURE 6 **IL-18/poly I:C-primed hepatic NK cells kill HSCs in a TRAIL-involved degranulation manner.** Liver NK cells were purified from specimen, then primed by IL-18 and/or poly I:C before cocultured with primary HSCs as above. CD107a expression of liver NK cells (A) and PI/AnnexinV level of primary HSCs (B) were detected. Anti-TRAIL or isotype antibody were used to treat IL-18/poly I:C-primed liver NK cells before cocultured with primary HSCs, then CD107a expression of liver NK cells (C) and the cell death of primary HSCs (D) was measured. Results were shown as mean ± SEM of 5 independent experiments performed with 5 different donor NK cells. $^{*}P < 0.05$, $^{**}P < 0.01$, paired *t*-test

and p38[27] could be the coordinates of these 2 pathways. IL-12 plus IL-18 stimulate greater IFN-γ secretion by resting NK cells through stabilization of IFN-γ mRNA via p38 MAPK,[28] and highly augment human NK cell cytotoxicity and degranulation in vitro.[29] p38 MAPK activation controls poly I:C-enhanced cytotoxicity and poly I:C/ IL-12-costimulated IFN-γ secretion in human NK cells.[30] There is emerging evidence about cross-talk between p38 MAPK and PI3K/Akt signaling. TLR2-mediated interplay between MAPK and PI3K signaling axis controls ESAT-6 (early secreted antigenic target protein 6) induced expression of cyclooxygenase-2 in macrophages.[31] PI3K/Akt/eNOS inhibit p38 MAPK and maintain the integrity of vasculature in mouse lung.[32] P38/PI3K/Akt signaling activates heat shock protein 27 to antagonize melatonin-induced apoptosis of gastric cancer cells.[33] The synergy between Poly I:C/TLR3/TRIF and IL-18/MyD88 signaling pathways may be mediated via IRF5 to activate the NF-κB transcription factor through the canonical pathway,[26] which we identified it as p38/PI3K/Akt signaling pathway. To our knowledge, this is the first description of NK cell activation via the p38 MAPK/PI3K signaling pathway.

NK cells play an important role in inhibition of liver fibrosis by killing HSCs.[4,11] The present study provided several lines of evidence to support a novel mechanism by which IL-18/poly I:C activated NK cells via p38/PI3K/AKT signaling kill HSCs. First, HSCs activate IL-18/poly I:C-pretreated NK cell degranulation (Fig. 3), and IL-18/poly I:C-activated NK cells reciprocally induce HSCs death through degranulation (Fig. 4). The interaction between NK cell and HSCs is dependent on cell-cell contact and IL-18/poly I:C-activated p38/PI3K/AKT signaling in NK cells, but independent on IFN-γ production. The direct cytotoxic degranulation of NK cells killing HSCs depends on the engagement between NKG2D of NK cells with ULBP-2, MICA/B, and RAE-1 of HSCs and NK activation via p38/PI3K/AKT signaling pathway to release perforin and Granzyme.[4,8,11,20] Although IFN-γ enhances NK cells TRAIL expression[23] and cytotoxicity to HSCs,[7,34] it has no direct cytotoxic effect against HSCs in 5 h coculture (Supplemental Fig. S3A).

It has been demonstrated that activated NK cells killing HSC occurred 2 separate pathways: direct cytotoxic degranulation of NK cells[4,8,11,20] and TRAIL-induced apoptosis.[7,9] However, the present study showed that blockades of engagement between TRAIL with TRAIL receptor inhibit NK cell-induced HSC apoptosis as well as NK cell degranulation (Figs. 5A–D). Using the live-cell image we found that IL-18/poly I:C-activated NK cells rapidly induce LX2 cells death, and the blockade of TRAIL obviously inhibit the interaction between NK cells and LX2 cells (Fig. 5E). NK cells' natural cytotoxicity is activated by interactions between different ligand–receptor pairs, and requires composed signals of adhesion, granule polarization and degranulation.[35] As a prerequisite for NK cell effector functions, the engagement of TRAIL and TRAIL receptor is suggested supplying the basis for the formation of an immune synapse by rendering a

firm connection with HSCs (Supplemental Fig. S3C). Previous study indicated that ligation of membrane TRAIL and its receptors transduced a costimulation signal and acted as a coreceptor of TCR in T cells.[36] Ligation of the TRAIL by its soluble receptor, DR4-Fc, alone induced phosphorylation of Lck and ZAP70, resulting in activation of the downstream NF-kB pathway. Integration of the TRAIL with TCR signaling is via enhanced lipid raft recruitment of Lck, which integrates mitogenic NF-kB-dependent signals from the TCR and TRAIL in T lymphocytes.[37] In activated NK cells, membrane TRAIL supplements the perforin/granzyme cytotoxic pathway, contributing to TRAIL-resistant neuroblastoma cell lysis.[38] These findings suggest that in addition to directly induce HSC apoptosis, NK cell membrane TRAIL binding to its receptors is involved in NK cell cytotoxic degranulation to HSCs. But the mechanism needs to be further investigated.

On the basis of CD56 and CD16 expression, human NK cells can be divided into 2 functional subsets of cytokine secretion and cytotoxic effector. Overall, approximately 90% of peripheral blood NK cells are $CD56^{dim}$ $CD16^{+}$ cells, which efficiently kill target cells with low secretion of cytokines. In contrast, $CD56^{bright}$ $CD16^{-}$ NK cells, which produce large amounts of cytokines with low cytolytic activity, compose of <10% peripheral blood NK cells, but represent up to 70% of total NK cells in the liver.[39] NK cells traffic between liver and peripheral blood,[39,40] also redistribute in liver and spleen during TLR3-ligand-induced inflammation of liver.[41] NK cells are widely distributed in both lymphoid (bone marrow and liver) and non-lymphoid organs (peripheral blood, lung, and uterus), and peripheral NK cells could accumulate into liver and attenuate hepatic fibrosis.[41,42] Although the main subset of human blood NK cells is represented by $NK56^{dim}$ cells that are phenotypically and functionally different from liver resident NK cells that are mainly represented by $NK56^{bright}$ subset, hepatic NK cells are also synergistically activated by IL-18 and poly I:C to produce IFN-γ and express TRAIL on their surface.[23] In this study, we showed the signaling pathway through which this occurs, and reproduced that IL-18 and poly I:C synergistically induce the degranulation in hepatic NK cells to kill HSCs in TRAIL-involved manner as peripheral NK cells did (Fig. 6). It makes more relevant in pathophysiologic context of liver fibrosis.

NK cells play a paradoxical role in the development of liver fibrosis. On one hand, NK cells can enhance liver injury by killing stressed hepatocytes via engagement of NKG2D, NKp30, and/or TRAIL, leading to the development of fibrosis.[43] On other hand, the cytotoxic activity of NK cells plays an important role in inducing HSC apoptosis and thus curtailing the progression of fibrosis.[7] However, NK cells are deactivated in chronic liver injury of some etiologies such as viral infection, and incapable of killing the activated HSCs.[12] The better understanding of the contributions of NK cells to liver fibrosis will benefit for developing therapeutics that target the restoration and promotion of NK cells. Targeting of p38 MAP/PI3 kinase in NK cell activity may be as an intervention strategy against liver fibrosis in the clinic setting.

AUTHORSHIP

T.L. and Z.T. conceptualized the study. T.L., Y.Y., H.S., H.L., A.C., and Y.L. investigated for the study. T.L., Y.Y., and Z.T. did the data curation-formal analysis. Project administration/oversight was carried out by T.L. and Z.T. T.L., Y.Y., and Z.T. wrote the manuscript and prepared its original draft. I.N.C., L.S., and Z.T. wrote, reviewed, and edited the manuscript. T.L. and Y.Y. contributed equally to this work.

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DISCLOSURES

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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