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Kupffer Cells Regulate Natural Killer Cells Via the NK group 2, Member D (NKG2D)/Retinoic Acid Early Inducible-1 (RAE-1) Interaction and Cytokines in a Primary Biliary Cholangitis Mouse Model

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Data Interpretation D
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Background: Kupffer cells and natural killer (NK) cells has been identified as contributing factors in the pathogenesis of hepatitis, but the detailed mechanism of these cell types in the pathogenesis of primary biliary cholangitis (PBC) is poorly understood.

Material/Methods: In this study, polyinosinic: polycytidylic acid (poly I: C), 2-octynoic acid-bovine serum albumin (2OA-BSA) and Freund's adjuvant (FA) were injected to establish a murine PBC model, from which NK cells and Kupffer cells were extracted and isolated. The cells were then co-cultivated in a designed culture system, and then NK group 2, member D (NKG2D), retinoic acid early inducible-1 (RAE-1), F4/80, and cytokine expression levels were detected.

Results: The results showed close crosstalk between Kupffer cells and NK cells. PBC mice showed increased surface RAE-1 protein expression and Kupffer cell cytokine secretion, which subsequently activated NK cell-mediated target cell killing via NKG2D/RAE-1 recognition, and increased inflammation. NK cell-derived interferon-γ (IFN-γ) and Kupffer cell-derived tumor necrosis factor α (TNF-α) were found to synergistically regulate inflammation. Moreover, interleukin (IL)-12 and IL-10 improved the crosstalk between NK cells and Kupffer cells.

Conclusions: Our findings in mice are the first to suggest the involvement of the NKG2D/RAE-1 interaction and cytokines in the synergistic effects of NK and Kupffer cells in PBC.

MeSH Keywords: Cytokines • Kupffer Cells • Liver Cirrhosis, Biliary • Receptors, Natural Killer Cell

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Background

Primary biliary cholangitis (PBC) is a chronic progressive intrahepatic cholestatic autoimmune disease of unclear etiology. Biliary epithelial cells (BECs) are targeted for immune attack in PBC, suggesting that immune cells from the hepatic sinus are involved in the pathogenesis of PBC. Kupffer cells and natural killer (NK) cells are the only 2 resident cell types in the hepatic sinusoid. As an important part of the body's immune surveillance system, NK cells may participate in the occurrence and development of PBC. The NK cell population increases in the peripheral blood and livers of PBC patients. In addition, the number of NK cells around the small intrahepatic bile ducts significantly increases, especially in the early stage of disease [1–3]. NK cells express a variety of activating and inhibitory receptors, including the activating receptor NK group 2, member D (NKG2D), major histocompatibility (MHC) class I-related antigen (MIC), which acts as a ligand of NKG2D [4]. The interaction of NKG2D with its ligand MIC can activate NK cells [5], reducing the T cell receptor (TCR) signaling activation threshold and thus contributing to the development of autoimmune diseases. NKG2D ligands in mice include RAE-1, H60, and MULT1 [6]; however, RAE-1 is the primary NKG2D ligand. Kupffer cells are important phagocytic cells in the liver, and they play a key role in the initiation, spread, and regression of liver inflammation [7]. Kupffer cells and NK cells share a close physiological connection, and cell contact and crosstalk between cytokines play a role in their function. Kupffer cells produce the NK cell-activating ligand RAE-1 and secrete inflammatory factors that activate NK cells [8]. Overexpression of cytokines leads to dedifferentiation of cholangiocytes and stress, and induces apoptosis and mitochondrial dysfunction and ultimately leads to PBC immune activation [9]. The CD4/CD8 ratio is used to reflect the functional status of T lymphocytes; activated $CD4^+$ T and $CD8^+$ T can synergistically promote PBC disease progression [10]. Laboratory tests have revealed elevated serum-based alkaline phosphatase (ALP) level and 90–95% positive anti-mitochondrial antibody M2 **(**AMA-M2) in PBC [11]. Herein, we used 2-octynoic acid-bovine serum albumin (2OA-BSA) and polyinosinic: polycytidylic acid (poly I: C) combined with Freund's adjuvant (FA) to establish an animal model of PBC [12] to study the involvement of the NKG2D/RAE-1 interaction and cytokines in the synergistic effects of NK cells and Kupffer cells.

Material and Methods

Mice

Specific-pathogen free (SPF) female C57BL/6 mice (4 to 6 weeks old, weighing 18 to 22 g) were obtained from the Laboratory Animal Science Department of Kunming Medical University and raised in the SPF Laboratory of the Experimental Animal Building of Kunming Medical University. All mice were fed under specific conditions, and the experiments complied with the animal care regulations of Kunming Medical University.

Reagents

The following reagents were used: poly I: C (Merck Millipore, USA), 2OA-BSA (Hapten and Protein Biomedical Institute, China), pertussis toxin (EMD Biosciences, USA), incomplete FA (IFA; Sigma, USA), complete FA (CFA; Sigma, USA), PE-conjugated rat anti-mouse CD4 antibody (BD, USA), FITC-conjugated rat anti-mouse CD8a antibody (BD, USA), a mouse AMA-M2 Ab ELISA kit (Demeditec Diagnostics, Germany), collagenase IV (Sigma, USA), lipopolysaccharide (LPS; Millipore, USA), and a NK Cell Isolation Kit II (Miltenyi, Germany). Anti-NKG2D, anti-F4/80, and anti-RAE-1 antibodies, as well as antibodies against cytokines, antibodies used for flow cytometry, and enzyme-linked immunosorbent assay (ELISA) kits were purchased from Abcam (UK).

Experimental study design

Forty mice were randomly divided into 2 groups, each of which contained 20 mice: the PBC group and the control (CON) group. In the first week, mice in the PBC group were intraperitoneally injected once with 100 μg of 2OA-BSA and an equal volume of complete FACFA, Sigma, USA, F5506-10ML, and 100 ng of pertussis toxin was injected once a day for 3 days. Poly I: C at a dose of 5 mg/kg was injected intraperitoneally on the third and sixth days. Mice in the CON group were not treated. In the second week, mice in the PBC group were intraperitoneally injected once with 100 μg of 2OA-BSA and an equal volume of IFA, and poly I: C at a dose of 5 mg/kg was injected intraperitoneally on the third and sixth days; mice in the CON group were not treated. The same treatments administered in the second week were again administered in the third week, and mice in the CON group were not treated. From the fourth to eighth week, poly I: C at a dose of 5 mg/kg was intraperitoneally injected once every 3 days, and mice in the CON group were not treated. After the first intraperitoneal immunization, vaccination with 2OA-BSA and an equal volume of IFA was used to boost the immune effect; treatment was administered for 8 weeks to establish the animal model.

Isolation of Kupffer cells

Kupffer cells were isolated as described previously [13]. After the model had been established, the liver of each mouse was fully perfused with 5 mL of 0.05% collagenase IV through the portal vein and aseptically removed. Liver tissue was excised in 10 mL of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin)

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at 37°C. Then, the suspension was transferred to a 10 mL glass tube and incubated at 37°C for 30 minutes. The suspension was centrifuged at 300×g for 5 minutes at 4°C, then discarded the supernatant. The cell pellet was resuspended in 10 mL of RPMI 1640 medium, which was then centrifuged at 300×g for 5 minutes at 4°C. The collected pellet was resuspended in 10 mL of RPMI 1640 medium and then centrifuged at 50×g for 5 minutes at 4°C. The collected supernatant was centrifuged at 300×g for 5 minutes at 4°C. The cell pellet was resuspended in RPMI 1640 medium in a humidified 5% CO_2 atmosphere at 37°C (Thermo) for 4 hours. The medium was changed, and the cells were rinsed 3 times with phosphate-buffered saline (PBS); the remaining adherent cells were Kupffer cells, as confirmed by immunofluorescence.

Isolation of NK cells

A liver cell suspension was obtained in the manner aforementioned to isolate Kupffer cells. The NK cells were obtained by density gradient separation of lymphocytes and sorted by magnetic bead sorting. NK cells were identified by flow cytometry detection of CD335.

Coculture

NK cells and Kupffer cells were cocultured in Transwell chambers, with Kupffer cells in the lower chamber and NK cells in the upper chamber, for 48 hours. The upper chamber was removed to block contact with cytokines. The antibodies anti-interleukin (IL)-10, anti-IL-12, anti-tumor necrosis factor α (TNF-α), anti-interferon-γ (IFN-γ), anti-IL-15, and anti-IL-18 at a working concentration of 10 μg/mL were used as blocking antibodies in the cell culture system.

Hematoxylin & eosin (H&E) staining

Fixed mouse liver tissue was dehydrated with an ethanol gradient, cleared with xylene, embedded, and then sectioned, repaired, and heated. The sections were incubated with primary antibodies at 4°C overnight followed by the secondary antibody at room temperature for half an hour. The sections were then subjected to xylene dewaxing and stained with hematoxylin & eosin (H&E). Then, the sections were dehydrated with ethanol and cleared with xylene. A neutral gum seal was affixed, and the sections were imaged under an inverted optical microscope (Olympus CKX53, Tokyo, Japan).

Liver immunohistochemistry to assay NKG2D, F4/80, and RAE-1

The number of positive cells in each group was calculated, and the positive cell staining intensity was evaluated according to quantitative immunohistochemical staining score (IHS) as described previously [14]: IHS=A×B, where A is the proportion of positive cells (<5%, 0 points; 5–25%, 1 point; 26–50%, 2 points; 51–75%, 3 points; and >75%, 4 points) and B is the staining intensity (negative staining, 0 points; weakly positive staining, 1 point; moderate staining, 2 points; and strongly positive staining, 3 points).

Flow cytometry assay

Collected peripheral anticoagulant blood was added to red blood cell lysis buffer and incubated for 15 minutes. The prepared peripheral blood and collected cells were stained with fluorescence-labeled monoclonal antibodies (mAbs) against surface antigens based on the manufacturer's instructions. The stained cells were analyzed using a flow cytometer (Partec GmbH, Münster, Germany), and the data were analyzed with Flomax 2.8/3.0 (Partec GmbH, Münster, Germany).

Determination of transaminase, AMA-M2, anti-nuclear antibody (ANA), IL-10, IL-12, TNF-α, IFN-γ, IL-15, and IL-18 expression with ELISA

Blood was taken from the mouse eyeballs, incubated overnight at 4°C and then centrifuged at 1000×g for 20 minutes, following which the supernatant was retained. The design of the culture system is shown in Table 1. During cell culture, the cell culture medium was collected and centrifuged at 1000×g for 20 minutes to remove impurities. The transaminase, AMA-M2, ANA, and cytokine levels in the cell culture medium were detected though ELISA kits (R&D Systems), and the absorbance (OD value) was measured at the corresponding wavelength with a microplate reader (MD SpectraMax 190, USA) to obtain a standard curve. The level of the analyte in the tested serum sample was calculated.

NK cell-killing test

NK cells were plated in 24-well plates at different densities and cultured for 24 hours. Prelabeled YAC-1 cells were collected and added to the wells containing NK cells in the 24-well plates according to cell density. YAC-1 cells alone were added to one well as a control group. The cell suspensions were collected after 4 hours of incubation, and the collected cells were labeled with propidium iodide (PI, Abcam). NK target cell lysis was detected by flow cytometry. The killing rate was calculated as follows: killing rate (%)=(number of CFSE-PI double-positive cells in the PBC group-number of CFSE-PI double-positive cells in the CON group)/number of CFSE-positive cells×100%.

Statistical analysis

Statistical analyses were performed with GraphPad Prison version 8.00 (GraphPad) and SPSS 20.0. ALP and alanine

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Table 1. Culture system design.

Culture system	Group	Culture program
KCs	PBC	Primary culture of PBC Kupffer cells
	CON	Primary culture of CON Kupffer cells
NK	PBC	Primary culture of PBC NK cells
	CON	Primary culture of CON NK cells
KCs+NK	PBC	PBC Kupffer cells+CON NK cells
	CON	CON Kupffer cells+ PBC NK cells
KCs+LPS1	PBC	1 mg/mL LPS+PBC Kupffer cells
	CON	1 mg/mL LPS+ CON Kupffer cells
KCs+LPS10	PBC	10 mg/mL LPS+PBC Kupffer cells
	CON	10 mg/mL LPS+ CON Kupffer cells
KCs+NK+LPS1	PBC	1 mg/mL LPS+PBC Kupffer cells+CON NK cells
	CON	1 mg/mL LPS+CON Kupffer cells+PBC NK cells
KCs+NK+LPS1/B	PBC	1 mg/mL LPS+PBC Kupffer cells+CON NK cells+antibody blocking
	CON	1 mg/mL LPS+CON Kupffer cells+PBC NK cells+antibody blocking
KCs+NK+LPS10	PBC	10 mg/mL LPS+PBC Kupffer cells+CON NK cells
	CON	10 mg/mL LPS+CON Kupffer cells+PBC NK cells
KCs+NK+LPS10/B	PBC	10 mg/mL LPS+ PBC Kupffer cells+CON NK cells+antibody blocking
	CON	10 mg/mL LPS+ CON Kupffer cells+PBC NK cells+antibody blocking

NK – natural killer; LPS – lipopolysaccharide; PBC – primary biliary cholangitis; CON – control.

transaminase (ALT) levels and the proportion of $CD4^+$ and $CD8^+$ cells in the groups treated for 2, 4, 6, and 8 weeks were analyzed with repeated measures ANOVA. Individual effect analyses of the PBC group and CON group were conducted by least significant difference (LSD) *t*-test. Data are shown as the mean±standard deviation expressed as ($\bar{x}\pm s$); differences for which $P<0.05$ were considered statistically significant.

Results

Change in transaminase activity in mice during the modeling period

Differences in ALT and ALP levels between the groups were determined (Figure 1A). There were no obvious differences in ALT levels between the PBC group and CON group at 2, 4, 6, and 8 weeks, suggesting no hepatocyte inflammation due to PBC modeling. ALP levels in the PBC group at 2, 4, 6, and 8 weeks gradually increased over time. In contrast, ALP levels in the CON group did not change significantly. ALP levels in the PBC group were higher than those in the CON group. This gradual rise in ALP level suggests increased capillary bile duct pressure, blocked bile excretion, and cholestasis.

Serum AMA-M2 and ANA levels in mice during the modeling period

AMA-M2 positivity in the PBC group was detected at the fourth week (20%), and the AMA-M2-positive rate gradually increased over time to 80% at the eighth week (Figure 1B). While some PBC mice were positive for ANA at the early stage of PBC, all of the mice were positive for ANA at the eighth week. Mice in the CON group were negative for AMA-M2 and ANA at each stage.

Changes of CD4+ and CD8+ T cells counts in the peripheral circulation of 2 groups of mice during the modeling period

Within mice in the PBC group, the number of $CD4^+$ T cells gradually decreased over time, while the number of $CD8^+$ T cells gradually increased. In contrast, no change in $CD4^+$ or $CD8^+$ T cell number was observed in the CON group (Figure 1C, Supplementary Table 1).

Pathological changes in mouse liver tissue during the modeling period

Liver tissues collected after 2, 4, 6, and 8 weeks of treatment were stained with H&E. In the PBC group, mild inflammation occurred in the second week. As the modeling time progressed,

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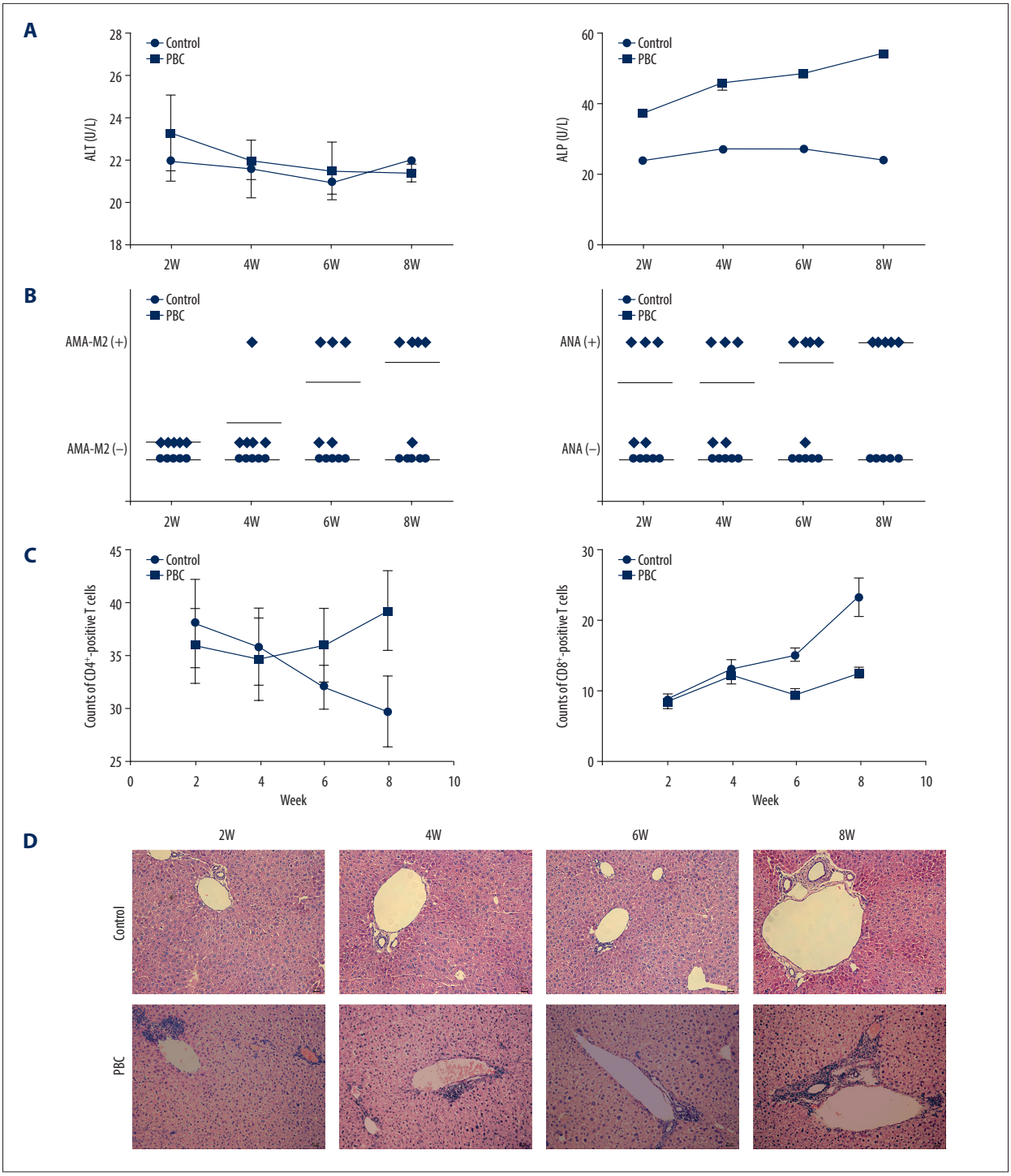

Figure 1. Changes during disease progression in a mouse model of PBC. (**A**) Changes in serum ALT and ALP in the PBC group and CON group at 2, 4, 6, and 8 weeks are expressed as the mean±SEM; n=5 for each group. (**B**) Changes in serum AMA-M2 and ANA in the PBC group and CON group at 2, 4, 6, and 8 weeks. Data are expressed as a count, n=5 for each group. (**C**) Changes in serum $CD4^+$ and $CD8^+$ T cell populations in the PBC and CON groups at 2, 4, 6, and 8 weeks. Data are expressed as the mean±SEM; n=5 for each group. (**D**) The livers of mice in the PBC and CON groups were collected at 2, 4, 6, and 8 weeks and stained with H&E (original magnification, 200×). PBC – primary biliary cholangitis; ALT – alanine transaminase; ALP – alkaline phosphatase; CON – control; SEM – standard error of the mean; AMA-M2 – anti-mitochondrial antibody M2; ANA – anti-nuclear antibody; H&E – hematoxylin & eosin.

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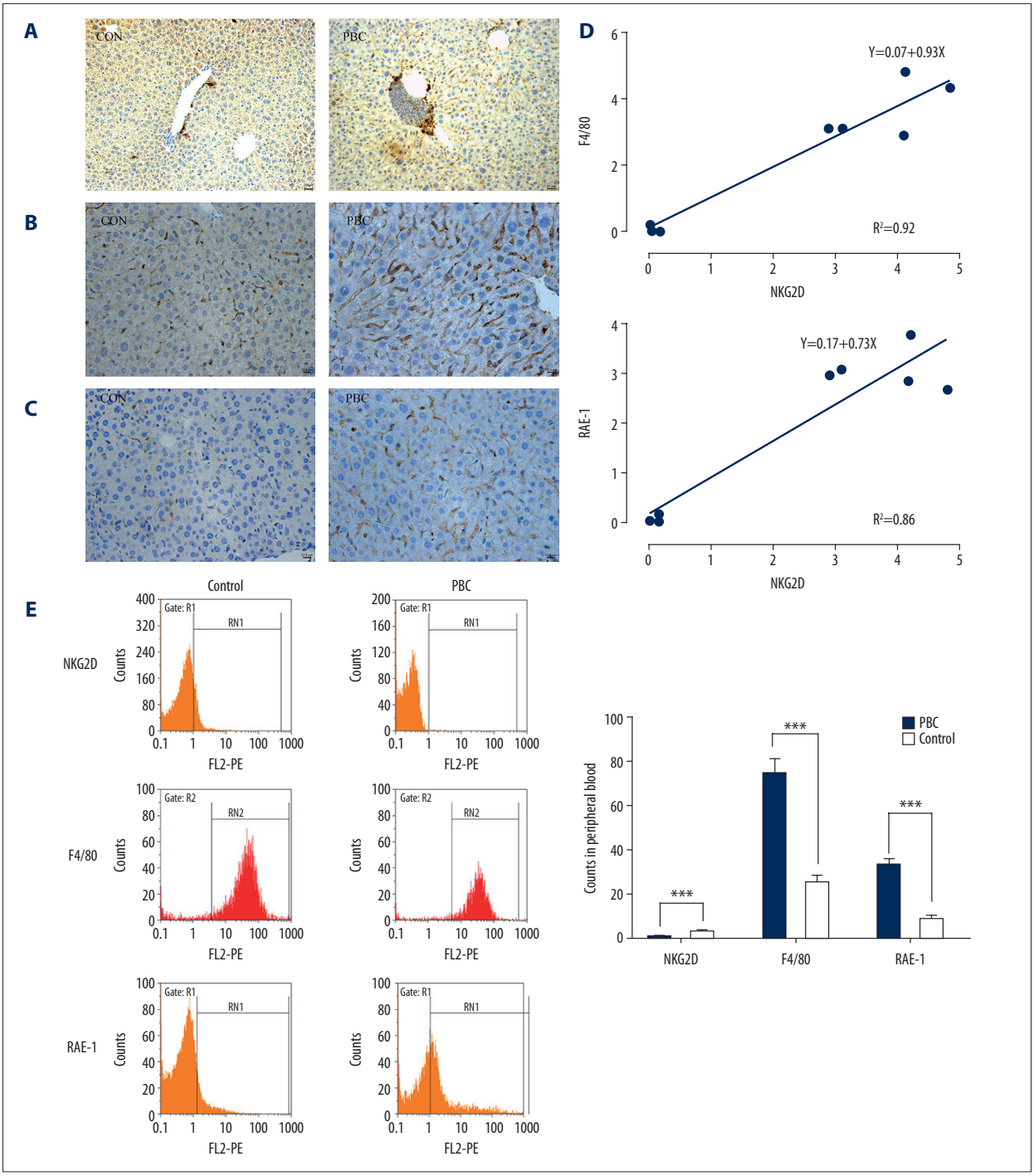

Figure 2. Expression of NKG2D and its ligand in the livers and peripheral blood of mice. (**A**) Expression of NKG2D in the livers of mice in the PBC and CON groups at 8 weeks was determined by immunohistochemistry. (**B**) Expression of F4/80 in the livers of mice in the PBC and CON groups at 8 weeks was determined by immunohistochemistry. (**C**) Expression of RAE-1 in the livers of mice in the PBC and CON groups at 8 weeks was determined by immunohistochemistry. (**D**) Correlation curves for NKG2D and F4/80 expression and NKG2D and RAE-1 expression in the peripheral blood from PBC mice; r=–0.754 and r=–0.866, respectively, n=6. (**E**) Flow cytometry showing the expression of NKG2D, F4/80 and RAE-1 in the peripheral blood of mice in the PBC and CON groups. Data are expressed as a count; n=5 for each group. Specific data are displayed as a histogram, *** $P<0.001$. NKG2D – natural killer group 2, member D; PBC – primary biliary cholangitis; CON – control; RAE-1 – retinoic acid early inducible-1.

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inflammation increased and was aggravated at the fourth week, and obvious inflammatory cell infiltration around the bile duct was observed at the sixth week. Intense inflammatory cell infiltration was observed at the eighth week, and bile duct injury was obvious (Figure 1D). Through determining the levels of transaminase, the specific antibody AMA-M2, and ANA and observing changes in T cell numbers and pathological changes, the model was confirmed to be successful, and the data could then be appropriately evaluated.

Expression of NKG2D, F4/80 and RAE-1 in the 2 groups of mice after modeling

Expression of NKG2D, F4/80 and RAE-1 in mouse liver tissue

In the PBC group, NKG2D was mainly expressed in lymphocytes around the portal area and bile duct, the nucleus and the cytoplasm, and on the cell membrane. Obvious inflammatory cell infiltration around the bile duct was observed. In contrast, NKG2D staining in the CON group was negative (Figure 2A).

F4/80 was mainly expressed on the surface of liver Kupffer cells in the PBC group. The number and area of F4/80-positive Kupffer cells were observed to be increased by microscopy, and these cells aggregated in areas of inflammatory necrosis in the liver tissue. F4/80 was expressed on the membrane and mainly in the cytoplasm. Compared to the PBC group, the CON group showed reduced F4/80 expression (Figure 2B).

RAE-1 was mainly expressed on the surface of liver Kupffer cells in the PBC group and accumulated in areas of inflammatory necrosis in the liver tissue. RAE-1 was expressed on the membrane and in the nucleus. RAE-1 expression was reduced in the CON group compared to the PBC group (Figure 2C).

According to the IHS values, NKG2D expression was positively correlated with the levels of F4/80 and RAE-1 in the PBC groups, with correlation coefficients of r=0.962 (Supplementary Figure 1) and r=0.930 (Supplementary Figure 2), respectively.

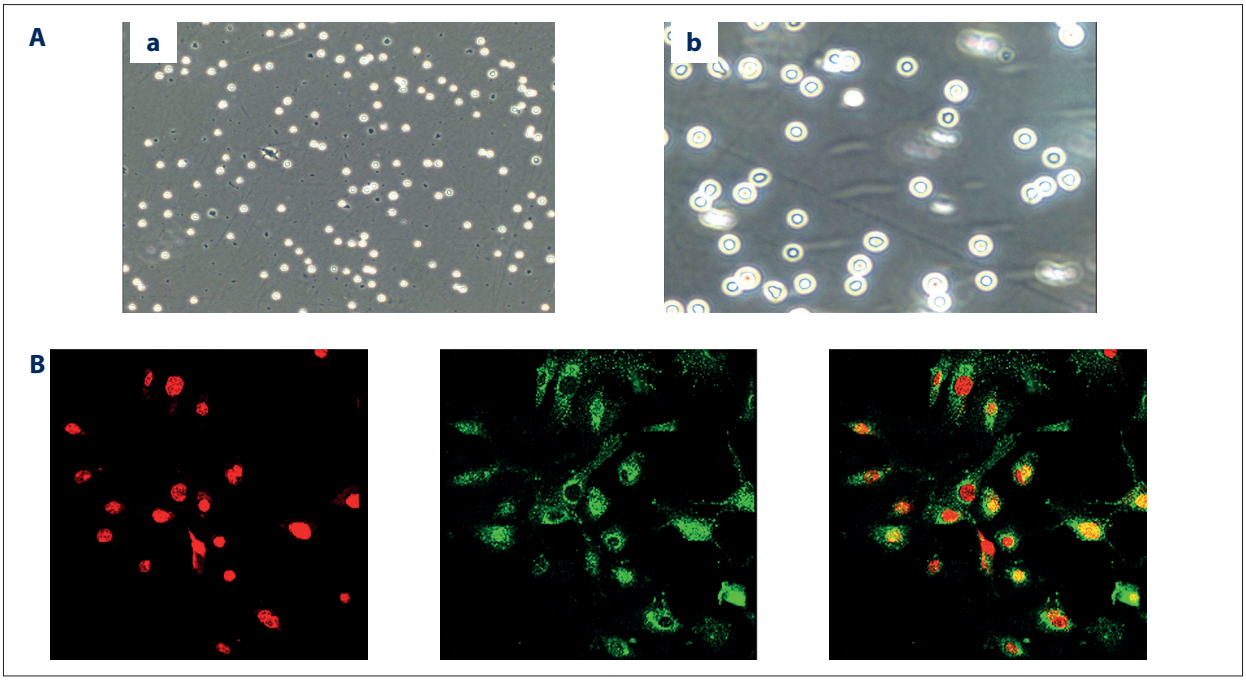
Expression levels of NKG2D, F4/80, and RAE-1 in the peripheral circulation of mice

The NKG2D level in the peripheral blood of mice in the PBC group was significantly negatively correlated with the F4/80 and RAE-1 levels, with correlation coefficients of r=–0.754 and r=–0.866 (Figure 2D), respectively.

The expression of NKG2D in the PBC group was lower than that in the CON group. In contrast, the PBC group expressed more F4/80 and RAE-1 than the CON group (Figure 2E).

Expression of the molecular markers NKG2D and RAE-1 on the surface of NK and Kupffer cells

NK cells cultured *in vitro* for 36 hours (Figure 3A) and Kupffer cells cultured for 72 hours (Figure 3B) were collected. The expression levels of NKG2D and RAE-1 in the PBC group were significantly higher than those in the CON group, as shown by flow cytometry (Figure 3C, Supplementary Table 1).



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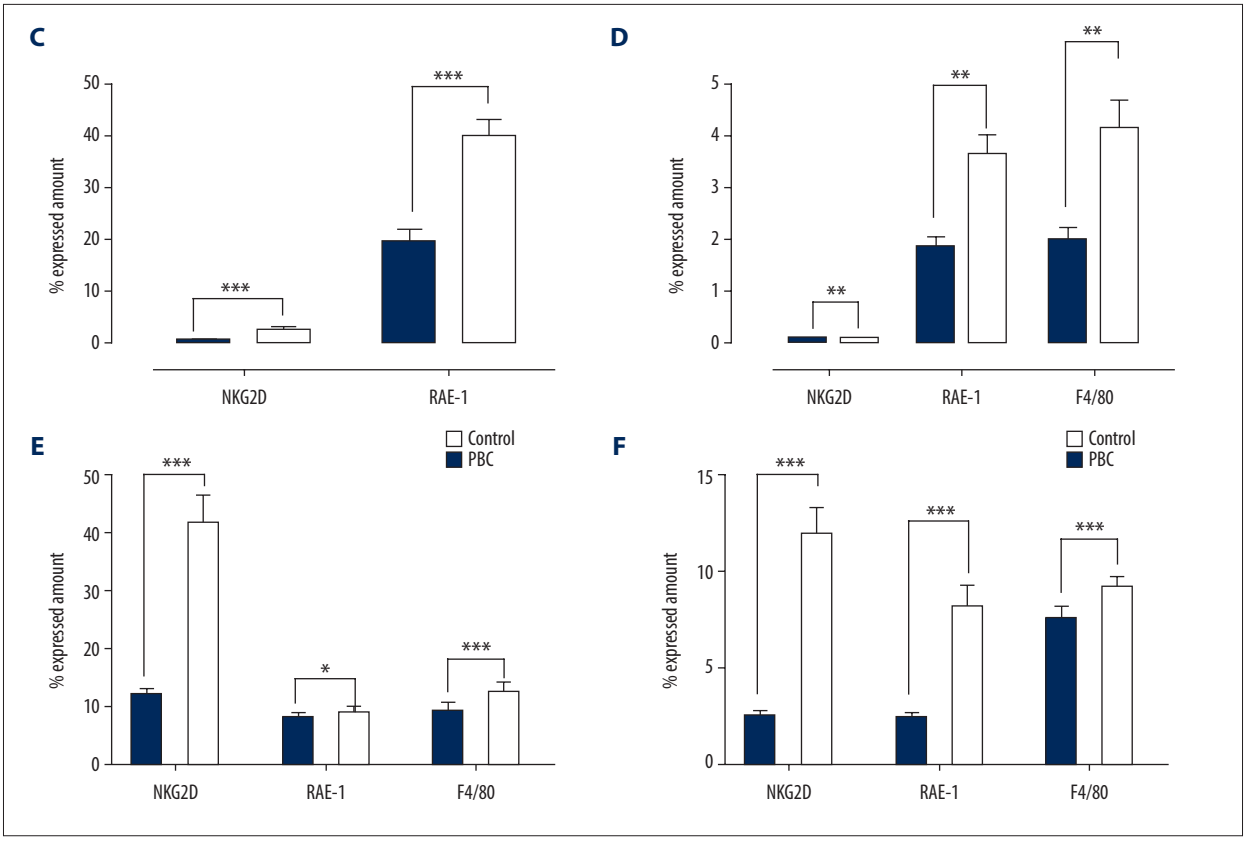

Figure 3. Mechanism of Kupffer cell-mediated regulation of NK cells via a receptor-ligand pathway in PBC mice. (**A**) NK cells were sorted with immunomagnetic beads. After 1 hour, the morphology of the cells was observed under a microscope. **a**) 100×, cells were round, **b**) cells at 200×. (**B**) Immunofluorescence. F4/80 (green)-labeled Kupffer cells and PI (red)-labeled nuclei were observed with laser confocal microscopy. (**C**) Flow cytometry detection of NKG2D and RAE-1 expression in NK cells cultured for 36 hours and Kupffer cells cultured for 72 hours. Data are expressed as the mean±SEM, n=3 for each group, *** $P<0.001$. (**D**) The upper chamber was withdrawn, and NK-Kupffer cell contact was eliminated. Flow cytometry was applied to measure the expression of NKG2D, RAE-1, and F4/80 after the coculture of NK cells from mice in the PBC and CON groups in Transwell chambers with unstimulated Kupffer cells. Data are expressed as the mean±SEM, n=3 for each group, ** $P<0.01$. (**E**). NK cells from mice in the PBC and CON groups were cocultured in Transwell chambers with Kupffer cells stimulated with 10 μg/mL LPS, and the expression levels of NKG2D, RAE-1 and F4/80 were detected by flow cytometry. The data are expressed as the mean±SEM, n=3 for each group, *** $P<0.001$, * $P<0.05$, ** $P<0.01$. (**F**) The upper chamber was withdrawn, eliminating NK cell-Kupffer cell contact, following which the NK cells in the PBC and CON groups were cocultured with Kupffer cells stimulated with 10 μg/mL LPS in Transwell chambers, and the expression levels of NKG2D, RAE-1 and F4/80 were detected by flow cytometry. The data are expressed as the mean±SEM, n=3 for each group, *** $P<0.001$. NK – natural killer; PBC – primary biliary cholangitis; NKG2D – natural killer group 2, member D; PI – propidium iodide; RAE-1 – retinoic acid early inducible-1; SEM – standard error of the mean; CON – control; LPS – lipopolysaccharide.

Expression of RAE-1 in Kupffer cells stimulated with LPS *in vitro*

LPS is a well-known immune system activator of macrophages which is the main structural component on the surface of gram-negative bacteria and also an important pathogen-related molecular model [15]. Therefore, in this study, LPS was chosen to stimulate Kupffer cells. Flow cytometry analysis of Kupffer cells stimulated with LPS at different concentrations (0 μg/mL, 1 μg/mL, and 10 μg/mL) showed that the expression of RAE-1 following stimulation with LPS at all concentrations tested was higher in the PBC group than in the CON group. The expression of F4/80 following 0 μg/mL, 1 μg/mL, and 10 μg/mL LPS stimulation was also higher in the PBC group than in the CON group. Moreover, the expression levels of RAE-1 and F4/80 in the 2 groups increased with increasing LPS concentration (Supplementary Figures 3, 4 and Supplementary Table 2).

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Expression of NKG2D and RAE-1 after the coculture of NK cells with Kupffer cells

Among NK cells cocultured with Kupffer cells without LPS stimulation, the expression of NKG2D and RAE-1 was significantly higher in the PBC group than in the CON group (Supplementary Figure 5, Supplementary Table 3).

NK cells were cocultured with Kupffer cells stimulated with 10 μg/mL LPS. Flow cytometry was conducted to detect the expression of receptors in the 2 groups, which showed that NKG2D, RAE-1, and F4/80 levels were higher in the PBC group than in the CON group. These differences were statistically significant and are shown in a histogram (Figure 3E, Supplementary Table 4).

NKG2D and RAE-1 expression after the contact between NK cells and Kupffer cells was blocked

NK cells were cocultured with Kupffer cells in Transwell chambers without LPS stimulation. The upper chamber was then withdrawn, terminating NK cell-Kupffer cell contact. Flow cytometry detection showed that the expression of NKG2D, RAE-1, and F4/80 was higher in the PBC group than in the CON group. These differences in expression were statistically significant and are shown in a histogram (Figure 3D, Supplementary Table 5). When NK cells were cocultured with 10 μg/mL LPS-stimulated Kupffer cells in Transwell chambers, the expression of NKG2D, RAE-1, and F4/80 was higher in the PBC group than in the CON group, as shown by flow cytometry detection. A histogram shows the statistically significant differences in expression (Figure 3F, Supplementary Table 6).

Cytokine expression levels in each culture system

IL-10

The secretion of IL-10 in the peripheral blood of the PBC group was lower than that in the CON group when Kupffer cells and NK cells isolated from the mouse liver were individually cultured. There was no obvious difference in IL-19 secretion between the 2 groups when the cell types were cultured alone, cocultured, stimulated with LPS or blocked with antibody (Figure 4A, Supplementary Table 7).

IL-12

Excessive IL-12 secretion of up to 8265.14 pg/mL was observed in the peripheral blood of the PBC group. In primary cultures of Kupffer cells and NK cells alone, the IL-12 level was lower than the minimum detectable level; however, when the cells were cocultured, IL-12 was secreted at a low level. After LPS was added, IL-12 levels increased significantly, especially those in Kupffer cells stimulated with 10 mg/mL LPS in the PBC group and cocultured NK cells and Kupffer cells in the CON group, in which the secretion of IL-12 was greatest and IL-12 levels were higher than the limit of detection of 2000 pg/mL. However, IL-12 levels decreased when the cells were cultured with a cytokine-blocking antibody (Figure 4B, Supplementary Table 8).

TNF-α

The secretion of TNF-α in the peripheral blood of the PBC group was 9.38 pg/mL. When TNF-α secretion in all culture systems was examined, Kupffer cells secreted more TNF-α than NK cells, and the TNF-α level in the PBC group was higher than that in the CON group. The secretion of TNF-α by Kupffer cells gradually increased as the LPS concentration increased from 1 mg/mL to 10 mg/mL, showing that TNF-α secretion is LPS concentration-dependent. TNF-α secretion was further increased with increasing LPS concentration in NK cells, although the cells secreted TNF-α at low levels. In addition, the secretion of TNF-α decreased after TNF-α-blocking antibody treatment (Figure 4C, Supplementary Table 9).

IFN-γ

A small amount of IFN-γ was secreted in the peripheral blood of mice in the PBC group, and in the culture system, IFN-γ was mainly secreted by NK cells. In addition, the ability of NK cells to secrete IFN-γ was greater in the PBC group than in the CON group. After NK cells were activated by coculture with Kupffer cells, the secretion of IFN-γ was enhanced, especially when the Kupffer cells were stimulated with LPS. Reduced secretion of IFN-γ was observed after the inclusion of a cytokine-blocking antibody (Figure 4D, Supplementary Table 10).

IL-15 and IL-18

The levels of secreted IL-15 and IL-18 in each culture system were low.

Ability of NK cells to kill YAC-1 target cells

NK cells isolated from the liver tissue of mice in the PBC and CON groups were similarly able to kill target YAC-1 cells. The killing ability of the NK cells in both groups was low and increased slightly when the NK cells were cocultured with Kupffer cells. When the Kupffer cells were stimulated with LPS, the killing ability of the cocultured NK cells increased with increasing LPS concentration. The killing ability of the PBC group was always greater than that of the CON group, and a histogram shows the statistically significant differences (Figure 4E).

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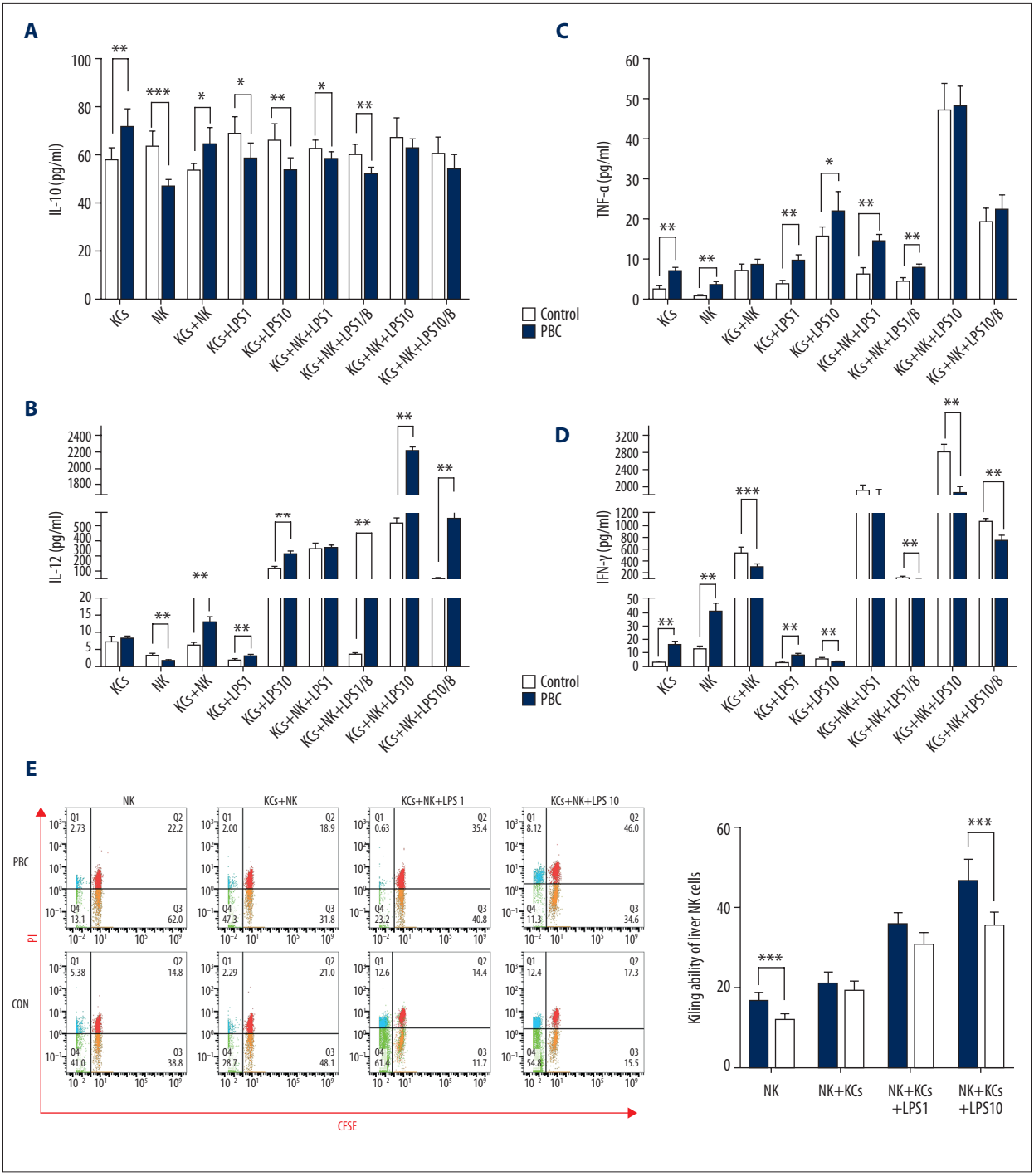

Figure 4. The expression of cytokines in each group of cultured cells and the NK cell-killing ability. (**A**) The expression of IL-10 in each culture system is expressed as the mean±SEM, and the experiment was repeated 3 times; *** $P<0.001$, * $P<0.05$, ** $P<0.01$. (**B**) Expression of IL-12 in each culture system. Data are expressed as the mean±SEM, and the experiment was repeated three times; ** $P<0.01$. (**C**) Expression of TNF-α in each culture system. Data are expressed as the mean±SEM, and the experiment was repeated 3 times; ** $P<0.01$. (**D**) Expression of IFN-γ in each culture system. Data are expressed as the mean±SEM, and the experiment was repeated 3 times; *** $P<0.001$, ** $P<0.01$. (**E**) YAC-1 cell-killing ability of NK cells isolated from liver tissue from mice in the PBC and CON groups. The experiment was repeated 3 times, and a histogram shows statistically significant differences; *** $P<0.001$. NK – natural killer; IL – interleukin; SEM – standard error of the mean; TNF – tumor necrosis factor; IFN-γ – interferon-γ.

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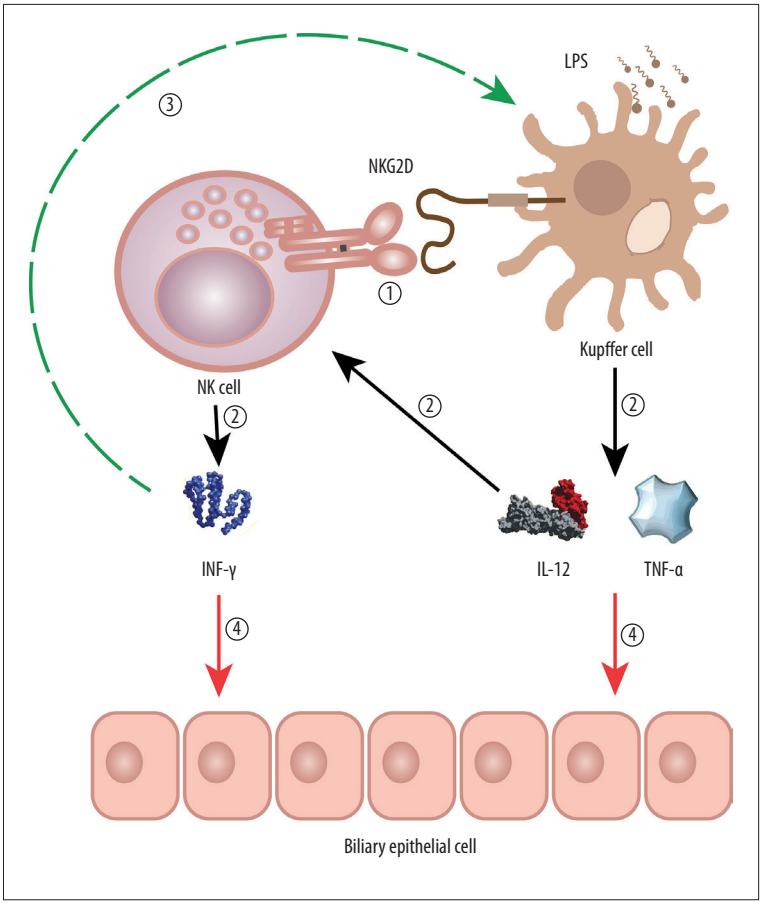

Figure 5. Model of the crosstalk between Kupffer cells and NK cells in PBC mice. 1) RAE-l expressed on Kupffer cells directly activates NK cells by its interaction with NKG2D. 2) Kupffer cell-derived IL-12 and TNF-α help to indirectly stimulate the production of IFN-γ by NK cells. 3) NK cell-derived IFN-γ stimulates the increased production of IL-12 and TNF-α by Kupffer cells. 4) Kupffer cell-derived IL-12 and TNF-α and NK cell-derived IFN-γ synergistically induce biliary epithelial cell damage. NK – natural killer; PBC – primary biliary cholangitis; RAE-1 – retinoic acid early inducible-1 – NKG2D, natural killer group 2, member D; IL – interleukin; TNF – tumor necrosis factor; IFN-γ – interferon-γ.

Discussion

Our previous study showed positive regulation of the killing function of liver NK cells in patients with PBC [16], suggesting the presence of regulatory mechanisms upstream of NK cells. However, how NK cells are actually activated is not yet clear. Kupffer cells, which reside in the hepatic sinusoid, are important phagocytic cells involved in liver inflammation [17]. As the largest group of mononuclear macrophages, Kupffer cells are believed to transmit regulatory signals that activate NK cells. Herein, we established a mouse model that was then confirmed through our experiments. Kupffer cells recognize targets through surface RAE-1, activating NK cells via NKG2D/RAE-1 recognition and promoting the production of IL-12, TNF-α, and IFN-γ, which are involved in synergistically mediating inflammation in PBC (Figure 5).

In vitro studies have demonstrated that NK cells are cytotoxic to BECs at high NK cell/BEC ratios [3]. Patients with PBC exhibit a marked increase in the frequency and absolute number of blood and liver NK cells. The increased presence of scattered NK cells near disrupted small bile ducts was observed at an increased frequency in liver tissues from PBC patients by immunohistochemical observation [2]. A similar phenomenon was observed in our animal model. The level of NKG2D in the liver tissue of mice in the PBC group was higher than that of mice in the CON group, and most NKG2D was scattered around the portal area and bile duct (Figure 2A). Pathological examination showed inflammation around the small bile duct, suggesting that NK cells are activated during PBC and cause inflammatory cell infiltration around the bile duct.

Different subgroups of Kupffer cells, which are at the center of the immune response, exhibit heterogeneous functions that are affected by bile acids. Based on their activation method, macrophages can be divided into 2 main phenotypes, the M1 (classically activated macrophages) and M2 (alternatively activated macrophages) phenotypes. M1 macrophages produce proinflammatory TNF-α, IFN-γ, IL-1, and IL-12, which mediate tissue damage. In contrast, M2 macrophages secrete IL-10, IL-4, IL-13, TGF-β, and vascular endothelial growth factor (VEGF), which are involved in the maintenance of tissue homeostasis and downregulation of inflammation and repair [18]. In cholestatic liver injury, activated Kupffer cells secrete a variety of cytokines, triggering liver inflammation and host immune responses [19]. In PBC, Kupffer cells are unable to effectively remove damaged cells, resulting in exposure to unmodified mitochondrial antigen and the accumulation of secondary necrotic

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substances, which are involved in the occurrence and expansion of inflammation [3].

Kupffer cells and NK cells have regulatory mechanisms. In an experiment it was found that the NK cells derived from PBC group had significantly greater potential of killing YAC-1 cells compared to the NK cells from the CON group. Moreover, after by stimulating with LPS-treated Kupffer cells, the killing activity of NK cells was enhanced (Figure 4E). This enhanced killing function was also confirmed in our culture system. IFN-γ is mainly produced by activated NK cells. When Kupffer cells were cocultured with NK cells, the secretion of IFN-γ in the CON group was higher than that in the PBC group (Figure 4D). Notably, an increase in the NK cell ratio results in damage to bile duct epithelial cells, and activated NK cells also aggravate damage to target cells. Unfortunately, these 2 regulatory factors are present in PBC. Many studies suggest that inappropriate expression of NKG2D or its ligand can cause inflammation and promote autoimmune responses, including those in diseases such as rheumatoid arthritis [20], colitis [21], Crohn's disease [22], type 1 diabetes [23], and chronic obstructive pulmonary disease [24]. We wondered whether RAE-1, an NKG2D ligand in mice [25], would have a similar effect in PBC mice. We used immunohistochemistry to calculate the expression levels of NKG2D, RAE-1, and F4/80 and found that NKG2D expression was positively correlated with the expression of RAE-1 and F4/80 in PBC mice, a finding that we do not believe is coincidental. Then, we detected the expression levels of these 3 proteins in the peripheral blood of mice by flow cytometry. The expression of NKG2D in the peripheral blood of mice in the PBC group was significantly lower than that of mice in the CON group. Furthermore, the expression levels of F4/80 and RAE-1 were increased, which is consistent with the findings of Cerwenka et al. [26]. These studies support the hypothesis that NKG2D/RAE-1 involved in the innate immune response of PBC mice.

LPS levels have been associated with PBC disease progression [27]. On the cell surface, macrophages (even Kupffer cells) express LPS receptors and various cytokines, which are involved in recognition, phagocytosis and activation. We isolated and cultured Kupffer cells from mouse livers and detected the expression of RAE-1 after stimulation with LPS at different concentrations. The expression of RAE-1 was increased in both the PBC group and CON group but higher in the PBC group than in the CON group. The expression of RAE-1 in both groups increased with increasing LPS concentration. The results of other studies [28] are consistent, showing that the extent of Kupffer cell activation in PBC mice is dependent on the LPS concentration to a certain extent. This concentration-dependent phenomenon was also found when NK cells and LPS-stimulated Kupffer cells were cocultured and when Kupffer cells without LPS stimulation were cocultured with NK cells. Although the expression levels of NKG2D and RAE-1 were higher in the PBC group than in the CON group, both were reduced (Supplementary Figure 5). When Kupffer cells were stimulated with 10 μg/mL LPS, the expression levels of NKG2D and RAE-1 increased significantly (Figure 3E), but their expression was still higher in the PBC group than in the CON group. After contact between NK cells and Kupffer cells was blocked with a Transwell chamber, the expression of NKG2D and RAE-1 decreased, especially after 10 ng/mL LPS stimulation (Figure 3F).

Pattern recognition by NK cells and macrophages (even Kupffer cells) is widespread in the immune response [13]. In addition to the NKG2D/RAE-1 interaction, cytokines participate in the crosstalk between Kupffer cells and NK cells in mice. The level of IL-10, which has anti-inflammatory effects, was found to be significantly decreased in PBC mice, while the levels of IL-12 and TNF-α, which have proinflammatory effects, were elevated in PBC mice; however, although IL-18 is also a proinflammatory factor, PBC group and CON group were no significant increase in IL-18 levels. *In vitro* studies have shown that [29] crosstalk between human NK cells and monocytes is regulated by NKp80-AICL, which can promote the secretion of IFN by NK cells and the secretion of TNF by monocytes. IFN-γ, which is mainly produced by activated NK cells, activates macrophages. We found that in cocultured Kupffer cells derived from the CON group and NK cells derived from the PBC group, the IFN-γ level was significantly elevated after Kupffer cell activation. We suggest that NK and Kupffer cells positively regulate the secretion of IFN-γ. Elevated IFN-γ activates the release of IL-12 from Kupffer cells, in turn activating the production of IFN-γ and TNF-α by NK cells, promoting macrophage activation and forming a closed loop (Figure 5). However, IL-10 can inhibit IFN-γ and TNF-α production by NK cells and inhibit the proliferation of Th1 cells, thereby inhibiting antigen presentation and reducing T cell proliferation and cytokine production. However, IL-10 can inhibit IFN-γ and TNF-α in NK cells, which consequently results in the inhibition of cytokine production and the reduction of Th1 cells proliferation [30]. In the culture system, IL-10 expression in the PBC group was significantly reduced. We speculate that dysregulated IL-10 secretion in PBC mice results in the inability to inhibit NK cells, which in turn activates NK cells and promotes the development of PBC. In contrast, IL-12 promotes the Th1 response, stimulates the production of IFN-γ by NK and T cells, and regulates lymphocyte proliferation [30]. In a mouse model of hepatitis B virus infection, IL-12 and IFN-γ were overexpressed, which induced liver inflammation; however, IL-10 inhibited inflammation and protected the liver [30].

IL-18 is an IFN-γ-inducible factor that can upregulate the expression of membrane-type IL-15 on monocytes, maintaining lymphocyte homeostasis, promoting NK cell differentiation, and inducing the expression of activated NKG2D receptors.

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IL-1 [31], IL-18 [32], and IL-12 levels were found to be increased in the peripheral circulation of patients with PBC. However, in the cell culture system, IL-12 was highly expressed, IL-18 was expressed at low levels, and IL-15 was not expressed. IL-15 is a key factor that promotes early NK cell differentiation. IL-15, IL-12, and IL-18 enhanced the killing of target cells by NK cells. Moreover, IL-12/Th1 cells are important in the persistent local inflammatory response in PBC [33]. When we blocked cytokines with antibodies, a small amount of IL-10, IL-12, TNF-α, and IFN-γ was still detected in the coculture system. In our view, in addition to cytokine crosstalk between NK cells and Kupffer cells, direct contact may be activated by NKG2D/RAE-1.

Imbalance in the proportions of $CD4^+/CD8^+$ T cells causes immune dysfunction, so the $CD4^+/CD8^+$ T cell ratio is indicative of the balance in the immune system and affects overall health. Autoreactive $CD4^+$ and $CD8^+$ T cells, including overlapping $CD4^+$ helper T cells and $CD8^+$ cytotoxic T lymphocytes (CTLs), are involved in the pathogenesis of PBC [34]. The ratio of $CD4^+$ CD25-high natural regulatory T cells (Tregs) is lower in PBC patients than in healthy study participants. The proportion of liver $CD8^+$ cells was lower in PBC patients than hepatitis C virus (HCV) patients, and autoimmune hepatitis patients [34]. In our experiments, we found that the plasma $CD4^+$ T cell proportion in PBC mice gradually decreased as the modeling time progressed, while the proportion of $CD8^+$ T cells gradually increased (Figure 1C); thus, the $CD4^+/CD8^+$ ratio was imbalanced. These results show that the immune balance and immune environment are disordered in PBC mice.

Notably, scholars found in a mouse model of colitis that the use of an NKG2D-blocking antibody in cells in contact with each other effectively reduced the inflammatory response [35]. In nonobese diabetic diseases, blockade of the NKG2D/MIC interaction effectively prevented the occurrence of disease [36]. These studies suggest that NKG2D blockade can alleviate the clinical symptoms of autoimmune disease. Some scholars have also confirmed that endogenous IL-12 derived from Kupffer cells can neutralize the accumulation of hepatic NK cells and reduce liver inflammation [37].

Conclusions

Our research demonstrates that blocking or modulating the NKG2D/MIC pathway and cytokine pathway may be important for the treatment of PBC.

Ethics statement

Experiments were systemized to the China Experiments on Animals Act and approved by the Animal Care and Use Committee of Kunming Medical University (Permit number: KMMH2019052). Mice were handled in strict accordance with good animal practice.

Acknowledgements

We wish to thank Dr. Xiaojia Yi, and Professor Lin Wang for analyzing the results of hematoxylin & eosin staining.

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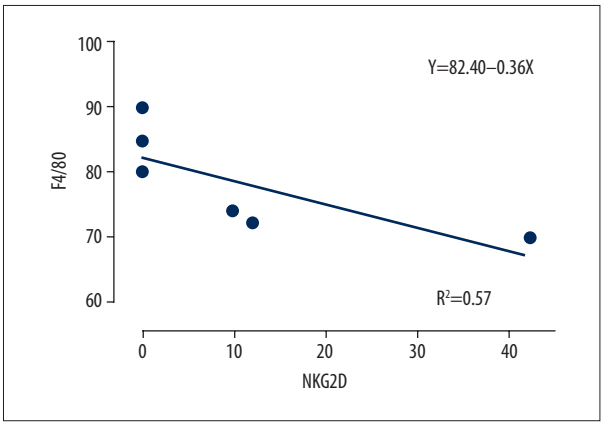
Supplementary Data

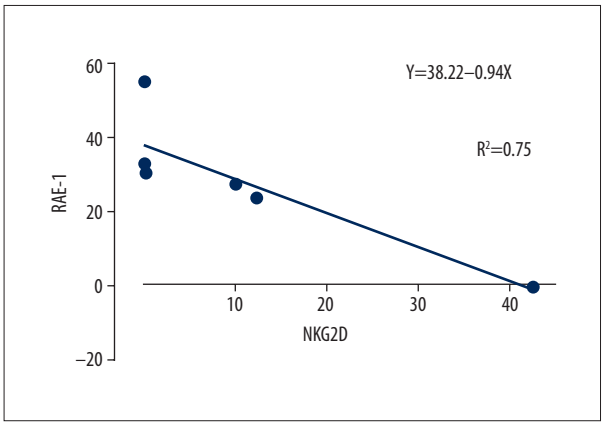
Supplementary Table 1. Expression of NKG2D and RAE-1 in NK cells and Kupffer cells co-cultured *in vitro*.

	PBC	CON	*t*	*P*
NKG2D	2.83±0.25	0.63±0.08	26.5041	<0.001
RAE-1	39.92±3.16	19.62±2.08	16.9686	<0.001

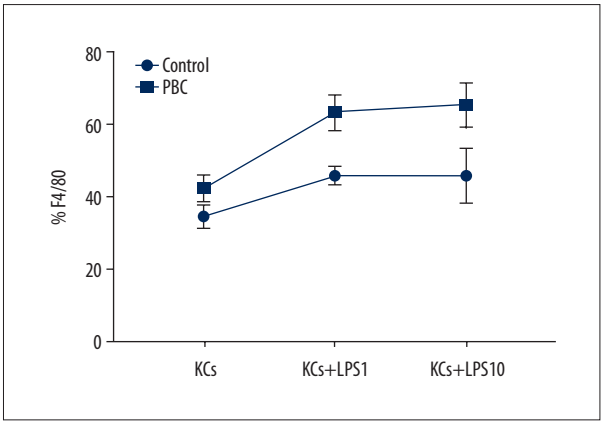
NKG2D – natural killer group 2, member D; RAE-1 – retinoic acid early inducible-1; NK – natural killer; PBC – primary biliary cholangitis; CON – control.



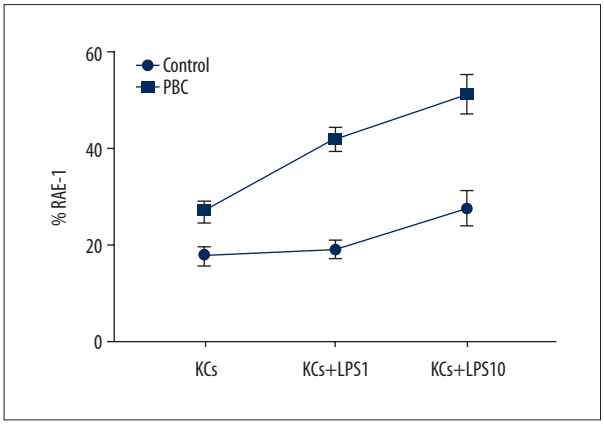
Supplementary Figure 1. Correlation curve between NKG2D and F4/80 expression in peripheral blood from PBC mice, r=–0.754, n=6. NKG2D – natural killer group 2, member D; PBC – primary biliary cholangitis.



Supplementary Figure 2. Correlation curve between NKG2D and RAE-1 expression in peripheral blood from PBC mice, r=–0.866, n=6. NKG2D – natural killer group 2, member D; PBC – primary biliary cholangitis; RAE-1 – retinoic acid early inducible-1.



Supplementary Figure 3. Flow cytometry-detected expression of F4/80 in Kupffer cells without LPS stimulation, with 1 μg/mL LPS stimulation, and with 10 μg/mL LPS stimulation. F4/80 expression showed LPS concentration dependence, which was more obvious in the PBC group than CON group. The data are expressed as the mean SEM, n=3 for each group. LPS – lipopolysaccharide; PBC – primary biliary cholangitis; CON – control; SEM – standard error of the mean.



Supplementary Figure 4. Flow cytometry-detected expression of RAE-1 in Kupffer cells without LPS stimulation, with 1 μg/mL LPS stimulation, and with 10 μg/mL LPS stimulation. The expression of both RAE-1 and NKG2D showed LPS concentration dependence, which was more obvious in the PBC group than CON group. RAE-1 – retinoic acid early inducible-1; LPS – lipopolysaccharide; NKG2D – natural killer group 2, member D; PBC – primary biliary cholangitis; CON – control; SEM – standard error of the mean.

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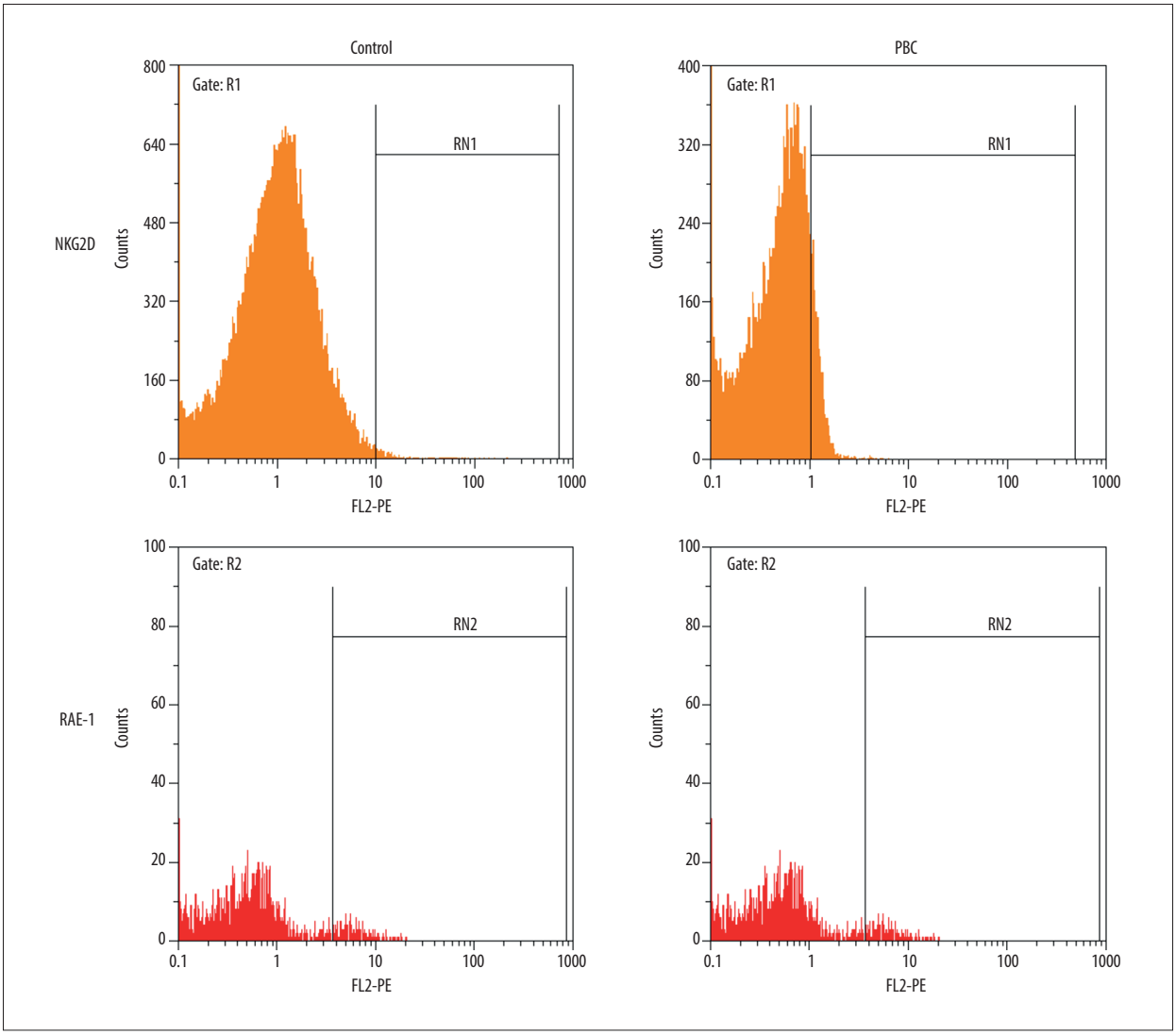
Supplementary Table 2. Expression of RAE-1 and F4/80 after stimulation of Kupffer with different concentrations of LPS.

	Group	KCs	KCs+LPS1	KCs+LPS10	F	P
RAE-1	PBC	26.89±2.26	42.05±6.03	51.23±4.17	61.6088	<0.001
	CON	17.67±1.77	19.09±1.68	27.65±3.40	39.9400	<0.001
	t	9.0845	10.3745	12.3957		
	P	<0.001	<0.001	<0.001		
F4/80	PBC	42.68±4.26	63.52±4.85	65.49±6.05	48.9818	<0.001
	CON	34.88±4.81	46.18±2.41	50.99±7.68	18.6682	<0.001
	t	3.43360	9.05594	4.19487		
	P	0.002	<0.001	0.0004		

RAE-1 – retinoic acid early inducible-1; LPS – lipopolysaccharide; PBC – primary biliary cholangitis; CON – control.



Supplementary Figure 5. Flow cytometry-detected expression of NKG2D and RAE-1 in PBC and CON group NK cells cocultured without LPS-stimulated Kupffer cells. NKG2D – natural killer group 2, member D; RAE-1 – retinoic acid early inducible-1; PBC – primary biliary cholangitis; CON – control; LPS – lipopolysaccharide.

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Supplementary Table 3. Expression of NKG2D and RAE-1 in NK cells co-cultured with Kupffer cells without LPS.

	PBC	CON	*t*	*P*
NKG2D	0.08±0.01	0.04±0.003	12.1157	<0.001
RAE-1	4.88±0.26	2.29±0.31	20.2430	<0.001

NKG2D – natural killer group 2, member D; RAE-1 – retinoic acid early inducible-1; NK – natural killer; LPS – lipopolysaccharide; PBC – primary biliary cholangitis; CON – control.

Supplementary Table 4. Expression of NKG2D and RAE-1 and F4/80 in NK cells co-cultured with 10 ug/mL LPS-stimulated Kupffer cells.

	PBC	CON	*t*	*P*
NKG2D	41.65±4.81	12.00±1.00	19.0850	<0.001
RAE-1	9.02±0.74	8.20±0.60	2.7219	0.0140
F4/80	12.47±1.73	9.23±1.32	4.7084	0.0002

NKG2D – natural killer group 2, member D; RAE-1 – retinoic acid early inducible-1; NK – natural killer; LPS – lipopolysaccharide; PBC – primary biliary cholangitis; CON – control.

Supplementary Table 5. Co-culture of NK cells and Kupffer cells without LPS stimulation in transwell chamber.

	PBC	CON	*t*	*P*
NKG2D	0.02±0.003	0.03±0.002	–8.7706	<0.001
RAE-1	3.65±0.34	1.86±0.15	15.2320	<0.001
F4/80	4.15±0.53	2.01±0.20	11.9642	<0.001

NK – natural killer; LPS – lipopolysaccharide; NKG2D – natural killer group 2, member D; RAE-1 – retinoic acid early inducible-1; PBC – primary biliary cholangitis; CON – control.

Supplementary Table 6. NK cells were co-cultured with 10 ug/mL LPS-stimulated Kupffer cells in transwell chamber.

	PBC	CON	*t*	*P*
NKG2D	12.00±1.29	2.44±0.25	23.0071	<0.001
RAE-1	8.20±0.98	2.44±0.21	18.1739	<0.001
F4/80	9.23±0.49	7.60±0.59	6.7209	<0.001

NK – natural killer; LPS – lipopolysaccharide; NKG2D – natural killer group 2, member D; RAE-1 – retinoic acid early inducible-1; PBC – primary biliary cholangitis; CON – control.

Supplementary Table 7. The amount of IL-10 secreted by each culture system (unit: pg/mL).

PBC	Mean	Std	CON	Mean	Std	*t*	*P*
K1	71.78	7.43	K2	58.11	5.12	3.3876	0.0095
N1	47.29	2.40	N2	63.70	6.27	–5.4656	0.0006
K1+N2	64.61	6.95	K2+N1	53.76	2.5	3.2848	0.0111
K1+L1	58.39	6.76	K2+L1	68.93	7.17	–2.3917	0.0437
K1+L10	53.79	5.07	K2+L10	66.43	6.41	–3.4583	0.0086
K1+N2+L1	58.59	2.64	K2+N1+L1	62.83	3.07	–2.3415	0.0473

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Supplementary Table 7 continued. The amount of IL-10 secreted by each culture system (unit: pg/mL).

PBC	Mean	Std	CON	Mean	Std	*t*	*P*
K1+N2+L1/T	52.20	2.47	K2+N1+L1/T	60.23	4.11	–3.7446	0.0057
K1+N2+L10	63.00	3.30	K2+N1+L10	67.30	8.13	–1.0958	0.305
K1+N2+L10/T	54.03	5.99	K2+N1+L10/T	60.69	6.76	–1.6488	0.1378

1 indicates the cells are derived from the PBC group, 2 indicates the cells are derived from the CON group. IL – interleukin; PBC – primary biliary cholangitis; CON – control.

Supplementary Table 8. The amount of IL-12 secreted by each culture system (unit: pg/mL).

PBC	Mean	Std	CON	Mean	Std	*t*	*P*
K1	8.045	0.419	K2	7.003	1.256	1.7597	0.1165
N1	1.202	0.1124	N2	3.164	0.1778	–20.8566	<0.001
K1+N2	12.778	1.391	K2+N1	5.9485	0.499	10.3333	<0.001
K1+L1	2.9695	0.311	K2+L1	1.612	0.0683	9.5331	<0.001
K1+L10	218.852	18.55	K2+L10	123.282	9.74	10.1997	<0.001
K1+N2+L1	263.9345	12.79	K2+N1+L1	253.4485	33.9	0.6471	0.5357
K1+N2+L1/T	22.7775	2.27	K2+N1+L1/T	3.2215	0.408	18.9599	<0.001
K1+N2+L10	2218.3217	27.5	K2+N1+L10	428.737	33.5	92.3276	<0.001
K1+N2+L10/T	461.027	50.2	K2+N1+L10/T	47.928	3.42	18.3582	<.001

1 indicates the cells are derived from the PBC group, 2 indicates the cells are derived from the CON group. IL – interleukin; PBC – primary biliary cholangitis; CON – control.

Supplementary Table 9. The amount of TNF-α secreted by each culture system (unit: pg/mL).

PBC	Mean	Std	CON	Mean	Std	*t*	*P*
K1	7.03	0.852	K2	2.63	0.544	9.733	<0.001
N1	3.39	0.609	N2	0.53	0.349	9.111	<0.001
K1+N2	8.56	1.404	K2+N1	6.97	1.62	1.6585	0.1358
K1+L1	9.65	1.23	K2+L1	3.67	0.762	9.2416	<0.001
K1+L10	22.24	4.65	K2+L10	15.69	2.197	2.8479	0.0215
K1+N2+L1	14.49	1.636	K2+N1+L1	6.06	1.465	8.5835	<0.001
K1+N2+L1/T	7.96	0.631	K2+N1+L1/T	4.46	0.503	9.6985	<0.001
K1+N2+L10	48.78	4.67	K2+N1+L10	47.69	6.5	0.3045	0.7685
K1+N2+L10/T	22.54	3.61	K2+N1+L10/T	19.28	3.47	1.4558	0.1835

1 indicates the cells are derived from the PBC group, 2 indicates the cells are derived from the CON group. TNF – tumor necrosis factor; PBC – primary biliary cholangitis; CON – control.

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Supplementary Table 10. The amount of IFN-γ secreted by each culture system (unit: pg/mL).

PBC	Mean	Std	CON	Mean	Std	*t*	*P*
K1	16.80	1.631	K2	3.64	0.397	17.5303	<0.001
N1	41.28	5.39	N2	13.94	1.22	11.0623	<0.001
K1+N2	315.35	31.8	K2+N1	545.89	89.8	–5.4113	0.0006
K1+L1	9.33	0.721	K2+L1	3.47	0.606	13.9124	<0.001
K1+L10	3.83	0.526	K2+L10	6.27	0.357	–8.5826	<0.001
K1+N2+L1	1818.07	143	K2+N1+L1	1928.78	137.4	–1.2483	0.2472
K1+N2+L1/T	66.35	5.29	K2+N1+L1/T	132.94	13.05	–10.5742	<0.001
K1+N2+L10	1906.59	118.8	K2+N1+L10	2846.04	186.6	–9.4964	<0.001
K1+N2+L10/T	768.80	79.9	K2+N1+L10/T	1077.30	53.4	–7.1781	0.0001

1 indicates the cells are derived from the PBC group, 2 indicates the cells are derived from the CON group. IFN-γ – interferon-γ; PBC – primary biliary cholangitis; CON – control.

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