



Natural Killer Cells Regulate the Maturation of Liver Sinusoidal Endothelial Cells Thereby Promoting Intrahepatic T-Cell Responses in a Mouse Model

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Functional maturation of liver sinusoidal endothelial cells (LSECs) plays an important role in intrahepatic T-cell activation and control of viral infections. Natural killer (NK) cells have been reported to prompt the maturation of antigen-presenting cells (APCs), especially for dendritic cells (DCs), but the interaction between NK cells and LSECs is elusive. Here, we investigated whether and how NK cells are involved in regulating LSEC maturation and if this has a role in controlling hepatitis B virus (HBV) infection in a mouse model. A chronic HBV replication mouse model was established by hydrodynamic injection (HI) of 6 μ g adeno-associated virus plasmid (pAAV)/HBV 1.2. The nucleotide-binding oligomerization domain-containing protein 1 (NOD1) ligand diaminopemelic acid (DAP) was imported into liver by HI at day 14 after plasmid injection. We found that HI of DAP recruited conventional NK cells (cNK) into the liver and promoted tumor necrosis factor alpha (TNF- α) and interferon-gamma (IFN- γ) production of NK cells in a chemokine (C-X-C motif) receptor 3 (CXCR3)-dependent manner. Importantly, the maturation of LSECs and the anti-HBV effects of DAP were impaired in CXCR3^{-/-} mice; this possibly was associated with the decreased number of intrahepatic cNK cells. Consistently, depleting cNK cells but not liver-resident NK cells also impaired the maturation and antigen-presenting function of LSECs, which reduced intrahepatic HBV-specific T-cell responses and thus inhibited HBV clearance both in wild-type and in Rag1^{-/-} mice. Moreover, TNF- α or IFN- γ stimulation as well as coculture with intrahepatic NK cells partly promoted LSEC phenotypic and functional maturation *in vitro*. **Conclusion:** NOD1-triggered NK cell activation may lead to the enhancement of intrahepatic T-cell responses by promoting maturation of LSECs through soluble cytokines and cell-cell contact, thereby controlling HBV replication and expression. (*Hepatology Communications* 2021;5:865-881).

Hepatitis B virus (HBV) chronically infects around 250 million people worldwide and can lead to severe liver disease, including liver cirrhosis, liver failure, and hepatocellular carcinoma.⁽¹⁾ Increasing evidence suggests that HBV persistence is

related to the hepatic tolerogenic immune microenvironment.⁽²⁾ Liver sinusoidal endothelial cells (LSECs) comprise the most prominent nonparenchymal cell populations in liver that strategically line the liver sinusoids and interact with passenger leucocytes. Due

Abbreviations: ANOVA, analysis of variance; APC, antigen-presenting cell; AsGM1, asialo ganglio-N-tetraosylceramide; CD, cluster of differentiation; cNK, conventional natural killer; CXCL, chemokine (C-X-C motif) ligand; CXCR, chemokine (C-X-C motif) receptor; DAP, diaminopemelic acid; DC, dendritic cell; dpi, days postinjection; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HI, hydrodynamic injection; ICS, intracellular cytokine staining; IFN, interferon; IL, interleukin; LIL, liver infiltrating lymphocyte; lrNK, liver-resident natural killer; LSEC, liver sinusoidal endothelial cell; MFI, mean fluorescence intensity; MHC-II, major histocompatibility complex class II; NK, natural killer; NOD1, nucleotide-binding oligomerization domain1; NS, normal saline; pAAV, adeno-associated virus plasmid; PD-L1, programmed death-ligand 1; pSM2, plasmid SM2; r, recombinant; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

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to their extraordinary scavenger function, LSECs are particularly competent in antigen cross-presentation to cluster of differentiation (CD)8⁺ T cells.⁽³⁾ However, antigen-specific interaction of LSECs with CD8⁺ T cells generally leads to nonresponsiveness toward T-cell receptor-mediated stimulation,⁽⁴⁾ which inhibits virus-specific immune responses and thus promotes virus persistence.

On the other hand, T-cell tolerance induced by LSECs can be disrupted by several regulatory mechanisms, such as toll-like receptor 2 agonist⁽⁵⁾ and the combinatorial stimulation of CD28 and interleukin (IL)-12.⁽⁶⁾ Previously, our group found that hydrodynamic injection (HI) of the nucleotide-binding oligomerization domain-containing protein 1 (NOD1) ligand diaminopimelic acid (DAP) promoted the functional maturation of LSECs, characterized by up-regulation of major histocompatibility complex (MHC)-II, costimulatory molecules CD80,

CD86, and CD40, adhesion molecules CD54 and CD106 and down-regulation of programmed death-ligand 1 (PD-L1); and enhanced ability to promote T-cell activation, which consequently induced enhanced HBV-specific T-cell responses.⁽⁷⁾ However, the mechanism of LSEC maturation behind this is unknown because DAP cannot induce the phenotypic maturation of LSECs *in vitro*.

Natural killer (NK) cells are innate effector cells that play an important part in host defense against various viruses and account for approximately 5%-10% and 30%-40% of total lymphocytes in mice and human liver, respectively.⁽⁸⁾ Hepatic NK cells contain both conventional NK (cNK) cells, also known as circulating NK cells (CD49a⁻DX5⁺ in mice), and liver-resident NK (lrNK) cells (CD49a⁺DX5⁻ in mice).⁽⁸⁾ These two subsets display a different phenotype and functionality. Accumulating evidence suggests that NK cells could regulate the maturation

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of antigen-presenting cells (APCs), especially for dendritic cells (DCs). NK cells have been shown to induce DCs to up-regulate human leukocyte antigen molecule expression and to secrete IL-18 and IL-12p70 through up-regulation of CD86 molecules on DCs.^(9,10) In addition, activated NK cells release profound amounts of interferon-gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α), which promote DC maturation⁽¹¹⁾ and modify the phenotype of microvascular endothelial cells.⁽¹²⁾ However, little is known regarding the interaction between NK cells and LSECs.

In the current study, we investigated whether NK cells participate in the maturation of LSECs after NOD1 activation and the mechanisms behind it. By using a chronic HBV replication mouse model by HI of the adeno-associated virus plasmid (pAAV)/HBV1.2, we found that local stimulation of DAP by HI promoted the migration and activation of cNK cells in a chemokine (C-X-C motif) receptor 3 (CXCR3)-dependent manner and that cNK cells were essential for the activation and antigen-presenting function of LSECs. The possible mechanism by which NK cells regulate the maturation of LSECs involved the soluble cytokines TNF- α and IFN- γ and cell-cell contact.

Materials and Methods

ANIMAL MODELS AND EXPERIMENTS

Male C57BL/6 mice (6 weeks old) were purchased from Hunan Slack Scene of Laboratory animal co (Hunan, China). CXCR3^{-/-} mice were produced in the Jackson Laboratory, and embryo recovery was performed by the Beijing Experimental Animal Research Center. Rag1^{-/-} mice were kindly provided by Professor Hong Tang from the Wuhan Institute of Virology, Chinese Academy of Sciences. All mice were maintained according to the Guidelines of the National Institutes of Health for Animal Care and Use. The protocols and procedures employed were ethically reviewed and approved by the institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology (Permit Number S196).

CHRONIC HBV REPLICATION MOUSE MODEL AND DAP TREATMENT

The chronic HBV replication mouse model was established by HI of 6 μ g of pAAV/HBV 1.2, as reported.⁽¹³⁾ For DAP treatment, 20 μ g C12-iE-DAP (InvivoGen, San Diego, CA) or an equal volume of normal saline (NS) was applied to the mice by HI at day 14 after HBV plasmid injection. Serum hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were detected by enzyme-linked immunosorbent assay (KHB, Shanghai, China) according to the manufacturer's instructions. Serum HBV DNA level was detected by real-time polymerase chain reaction using a commercial reagent (Sansure, Hunan, China).

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed as described.⁽¹⁴⁾ In brief, mice liver tissues were fixed in 4% paraformaldehyde and embedded in paraffin and were cut into 4- μ m sections. The expression of hepatitis B core antigen (HBcAg) was determined by staining with rabbit anti-hepatitis B core antibody (Dako, Carpinteria, CA) and calculated by quick scores according to the methods described by Detre et al.⁽¹⁵⁾

CELL ISOLATION

Liver-infiltrating lymphocytes (LILs) were isolated as described.⁽¹⁶⁾ Liver nonparenchymal cells were obtained by digestion in a medium containing 0.1% Liberase (Roche, Switzerland), as described.⁽¹⁷⁾ LSECs were isolated by positive isolation with CD146 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Intrahepatic NK cells were isolated by the NK Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions. The purity of isolated LSECs and NK cells was >90% after isolation.

NK CELL DEPLETION

NK cell depletion was performed by intraperitoneal injection of 30 μ g anti-asialo ganglio-N-tetraosylceramide (AsGM1) antibody or an equal amount of isotype (eBioscience) 1 day before the HI of DAP. Mice were injected with the antibodies every 3 days (3 times total).

ADOPTIVE CD8⁺ T-CELL TRANSFER

CD8⁺ T cells were isolated from the spleen of naive C57BL/6 mice by magnetic bead sorting (Miltenyi Biotec). The purity of CD8⁺ T cells was above 95% after isolation. Rag1^{-/-} mice were injected intravenously with 5×10^6 CD8⁺ T cells suspended in 500 μ L sterile phosphate-buffered saline per mouse 1 day before DAP treatment.

ABILITY OF LSECS TO ACTIVATE T CELLS

The acute HBV replication mouse model was established by HI with 10 μ g of HBV plasmid SM2 (pSM2) to C57BL/6 mice. Splenocytes were isolated from mice at day 21 after HI of pSM2. LSECs were isolated from DAP-treated mice at indicated time points and precultured for 24 hours in collagenized 48-well cell culture plates (5×10^5 /well). LSECs were then loaded with 2 μ g/mL recombinant (r)HBsAg or rHBcAg (PeproTech, Rocky Hill, NJ) overnight and then cocultured with the splenocytes in the presence of 1 μ g/mL anti-CD28 antibody (BD Bioscience) for 3 days. The ratio of splenocytes/LSECs was 2:1. Intracellular cytokine staining (ICS) was performed to determine the percentage of T cells producing IFN- γ , IL-2, and TNF- α after 3 days coculture.

HBV-SPECIFIC T-CELL RESPONSE

Splenocytes and LILs were stimulated with 10 μ g/mL of HBcAg-derived CD8⁺ epitope peptide core 93-100 (MGLKFRQL) or 10 μ g/mL HBsAg-derived CD8⁺ epitope peptide env208-216 (ILSPFLPLL) in the presence of 1 μ g/mL anti-CD28 (eBioscience) and 1 μ g/mL Brefeldin A (eBioscience) at 37°C in 5% CO₂ for 5 hours. Cells stimulated with 1 μ g/mL ionomycin and 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) served as a positive control. The CD8⁺ T cells producing IFN- γ , IL-2, and TNF- α were measured by ICS after 5 hours incubation.

STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS version 18.0 and GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). Differences between two groups were compared using the Student *t* test, and differences

among multiple groups were analyzed by one-way analysis of variance (ANOVA). Data were expressed as mean \pm SEM, and *P* < 0.05 was determined as statistically significant. Details of the material and experimental protocols of *in vitro* experiments and flow cytometry are provided in the Supporting Information.

Results

DAP APPLICATION INDUCED INTRAHEPATIC NK CELL ACCUMULATION AND ACTIVATION

Our previous study identified that HI of the NOD1 ligand DAP enhanced HBV-specific T-cell responses in an LSEC-dependent manner in a chronic HBV replication mouse model.⁽⁷⁾ However, the mechanism of LSEC maturation was unclear. To determine which immune subset regulates the maturation of LSECs, different immune cells were detected following HI of 20 μ g DAP or NS at 14 days after HI of pAAV/HBV 1.2. Because LSEC maturation and T-cell activation had been found at days 10 and 20 after DAP HI,⁽⁷⁾ we selected these two time points for further analysis. Compared to the NS control, DAP application significantly increased MHC-II⁺CD11c⁺ DCs and CD4⁺ T cells at 10 days postinjection (dpi) and NK cells at 20 dpi in liver but not in spleen or peripheral blood (Supporting Fig. S1A).

Studies have demonstrated that activated NK cells may induce the phenotypic and functional maturation of APCs by secreting cytokines.⁽¹¹⁾ To determine the activation status of NK cells, subsets and different activation markers of NK cells were detected. HI of DAP significantly increased the percentage of CD49a⁻DX5⁺ cNK cells both in total lymphocytes and in total NK cells at 20 dpi but not CD49a⁺DX5⁻ lrNK cells (Fig. 1B,C), indicating that the increased intrahepatic NK cells were mainly cNK cells instead of lrNK cells. Importantly, DAP application also enhanced the secretion of IFN- γ , TNF- α , and CD107a on intrahepatic cNK cells at 20 dpi but not on spleen NK cells (Fig. 1D). In contrast, DAP had no impact on the expression of CD69 and TNF-related apoptosis-inducing ligand (TRAIL) on intrahepatic NK cells at either time point. (Fig. 1D). Taken together, these findings indicated that HI of DAP specifically induced

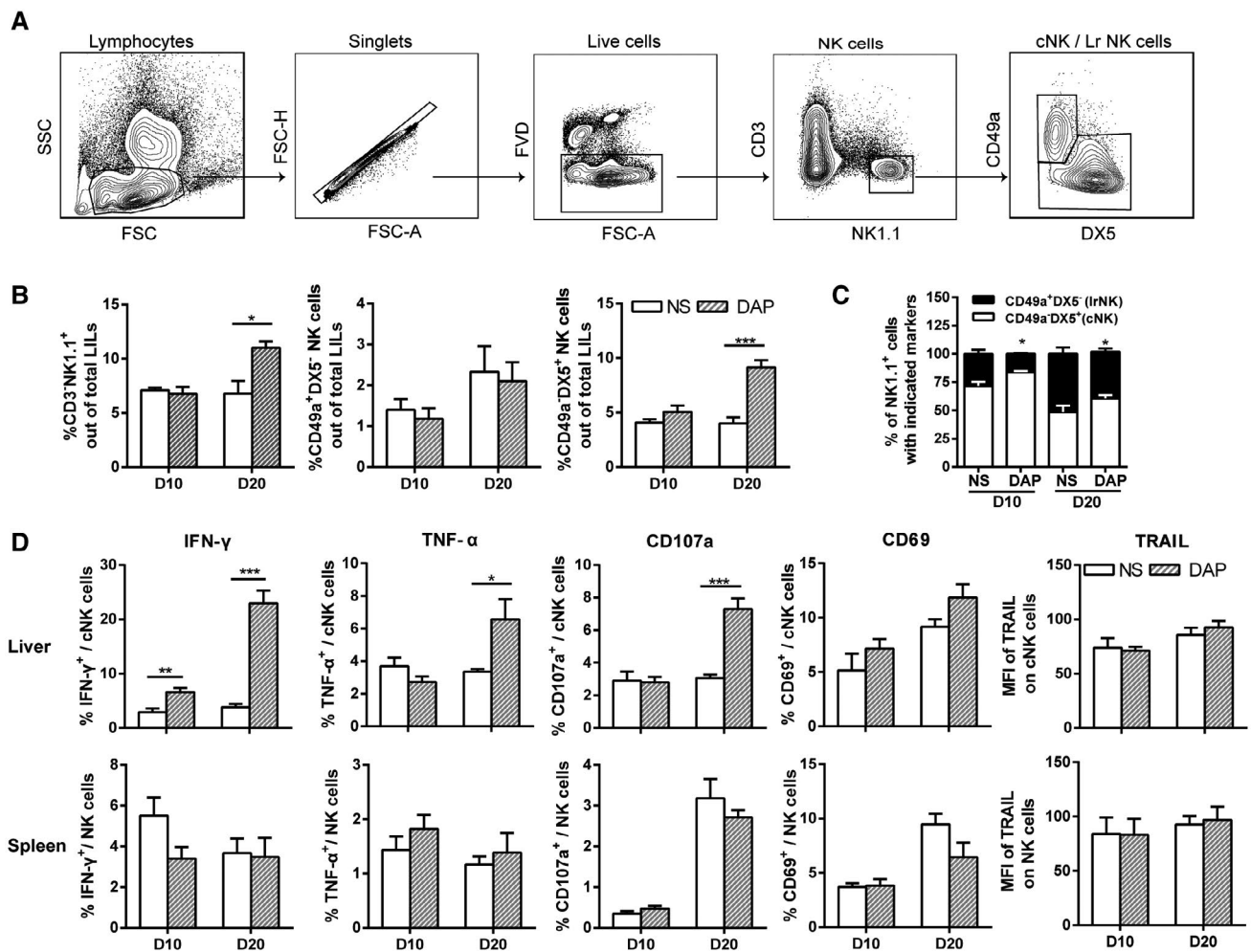


FIG. 1. Number and phenotypic changes of NK cells after HI of NOD1 ligand. C57BL/6 mice were treated with DAP or NS at day 14 after HI of pAAV/HBV 1.2. Mice were killed at days 10 and 20 after DAP HI, and LILs and splenocytes were isolated. (A) Gating strategy to identify total NK, cNK, and lrNK cells. (B,C) The percentage of cNK and lrNK cells in (B) total lymphocytes and (C) total liver NK cells were determined. (D) Expression of CD69, TRAIL, CD107a, IFN- γ , and TNF- α on liver cNK or spleen NK cells is shown. Five to six mice were analyzed per group, and at least two independent experiments were performed. Data represent mean \pm SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, using unpaired Student t test. Abbreviations: D, days; FSC-A, forward scatter-area; FSC-H, forward scatter-height; FVD, fixable viability dye; SSC, side scatter.

the infiltration and activation of intrahepatic cNK cells.

RECRUITMENT AND ACTIVATION OF NK CELLS DEPEND ON THE CXCR3 AXIS AFTER NOD1 ACTIVATION

Migration of NK cells to inflammatory stimuli reportedly depends on the CXCR3 axis.⁽¹⁸⁾ Previously, we identified that the CXCR3 ligands CXCL9 and CXCL10 were elevated in liver tissue at 20 dpi in

DAP-treated mice.⁽⁷⁾ Consistently, the expression of CXCR3 on intrahepatic NK cells was also up-regulated at 20 dpi (Supporting Fig. S1B). Therefore, we assumed that cNK cells were recruited into liver through the CXCR3 axis. To further investigate this hypothesis, we compared the number and functionality of NK cells between CXCR3^{-/-} and wild-type mice that received 20 μ g DAP at 14 days after HI of pAAV/HBV 1.2. Compared to the wild-type mice, NK cell frequency was significantly decreased in the liver of CXCR3^{-/-} mice (Fig. 2A,B); however, other immune cells in the liver were hardly affected by the

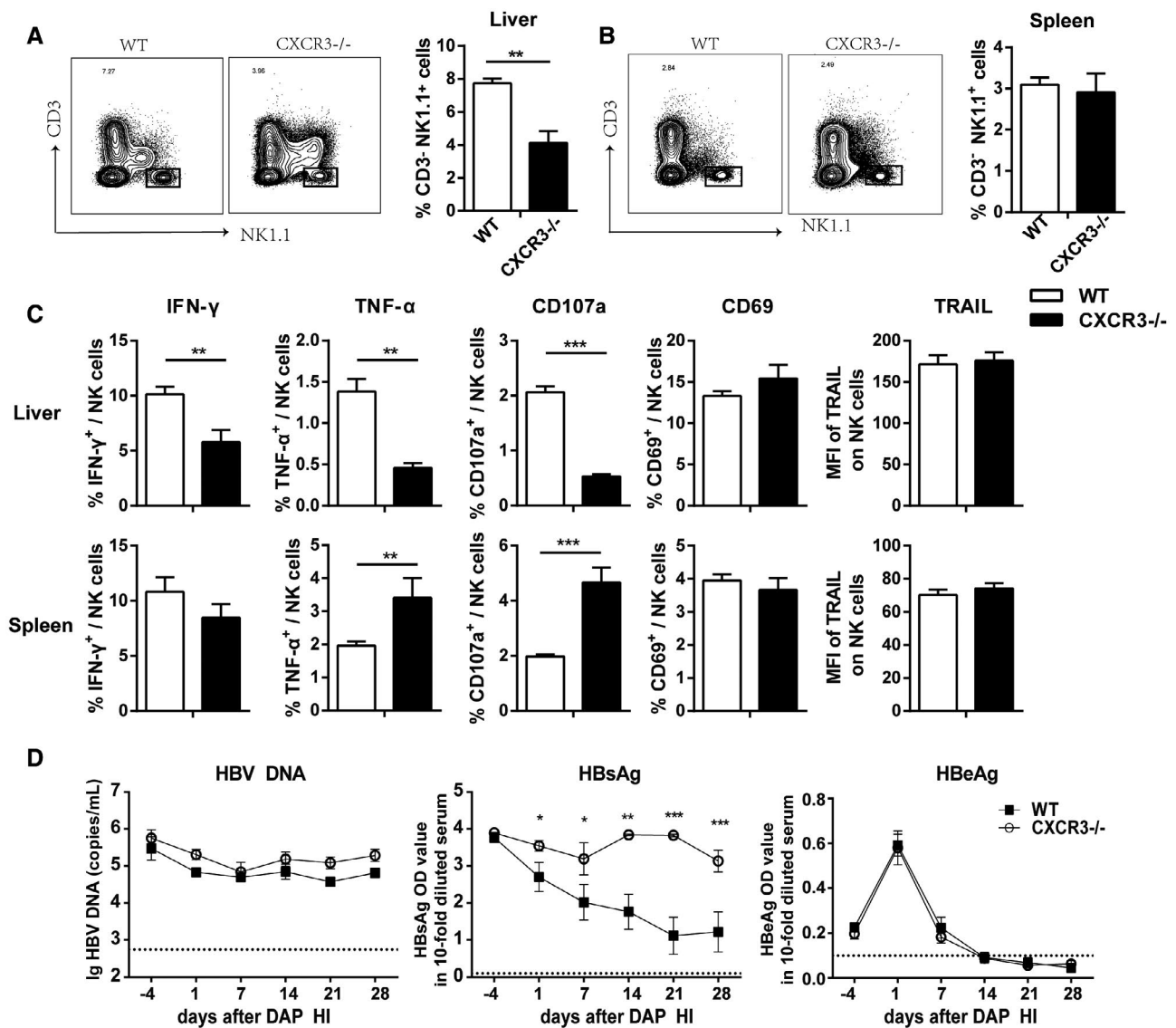


FIG. 2. Recruitment and activation of intrahepatic NK cells is dependent on the CXCR3 axis. C57BL/6 or CXCR3^{-/-} mice were treated with DAP administered through HI at day14 after HI of pAAV/HBV 1.2. LILs and splenocytes were isolated at 20 days after DAP HI. (A,B) Frequency of NK cells in (A) liver and (B) spleen was calculated. (C) Activation of NK cells was analyzed by expression of CD69, TRAIL, CD107a, IFN- γ , and TNF- α . (D) Kinetics of serum HBsAg, HBeAg, and HBV DNA levels are shown. Five to seven mice were analyzed per group, and at least two independent experiments were performed. Data represent mean \pm SEM, * P < 0.05, ** P < 0.01, *** P < 0.001, using unpaired Student t test or repeated measures analysis. Abbreviations: Ig, log10; OD, optical density; WT, wild type.

CXCR3 axis (Supporting Fig. S2A). More interestingly, production of IFN- γ , TNF- α , and CD107a on intrahepatic NK cells was also significantly reduced in CXCR3^{-/-} mice, whereas TNF- α and CD107a were conversely increased on spleen NK cells in CXCR3^{-/-} mice (Fig. 2C). Altogether, these results indicated that the CXCR3 axis is essential for intrahepatic NK cell trafficking and activation.

MATURATION OF LSECs AND ANTI-HBV EFFECTS OF DAP WERE IMPAIRED IN CXCR3^{-/-} MICE COMPARED TO WILD-TYPE MICE

To explore effects of decreased numbers of NK cells on LSEC maturation, we further examined the

phenotype and the antigen-presenting function of LSECs at 14 days after HI of DAP. Down-regulation of CD40, CD54, and MHC-II and up-regulation of PD-L1 on LSECs in CXCR3^{-/-} mice were observed compared to wild-type mice (Supporting Fig. S2B). To determine the antigen-presenting function of LSECs, the corresponding peptide-loaded LSECs were cocultured with splenocytes from pSM2-immunized mice. We found that LSECs from CXCR3^{-/-} mice induced a significant decrease in TNF- α and IFN- γ production of CD8⁺ T cells (Supporting Fig. S2C). These findings indicated that both phenotypic and functional maturation of LSECs were impaired in CXCR3^{-/-} mice. Due to the anti-HBV effects of DAP relying on LSECs, the expression of HBsAg and HBV DNA was elevated in CXCR3^{-/-} mice compared to wild-type mice (Fig. 2D). Taken together, these data suggest that the impairment of LSEC function might associate with decreased intrahepatic NK cells in CXCR3-deficient mice.

NK CELL DEPLETION IMPAIRED THE MATURATION AND FUNCTION OF LSECs AND IMPAIRED THE ANTI-HBV EFFECTS OF DAP

To further confirm whether the impaired LSEC function correlated with reduced intrahepatic cNK cells in wild-type mice, anti-AsGM1 or isotype was injected intraperitoneally into C57BL/6 wild-type mice 1 day before DAP HI (Fig. 3A). Approximately 95% of CD3⁻CD49b⁺ cNK cells were depleted at 1 day after injection, while CD3⁻CD49a⁺ IrNK cells were hardly affected (Supporting Fig. S3). The phenotype and the antigen-presenting function of LSECs at 10 and 20 days after HI of DAP were then detected again. As expected, NK cell depletion remarkably changed the LSEC phenotype, with down-regulation of CD40, CD54, and CD106 and up-regulation of PD-L1 at 10 days after HI (Fig. 3B,C). Moreover, LSECs from NK cell-depleted mice induced significant decreases in IL-2, TNF- α , and IFN- γ production in CD4 and CD8⁺ T cells 10 days after HI. This finding illustrated that NK cell depletion suppressed the phenotypic and functional maturation of LSECs (Fig. 3D). However, a similar pattern of change was not observed at 20 dpi, which coincides with

our previous study that phenotypic maturation of LSECs was mainly found at 10 days after DAP HI.

It is well defined that HBV-specific CD8⁺ T-cell responses determine the outcome of HBV infection. We subsequently examined HBV-specific CD8⁺ T-cell responses and kinetics of HBV infection markers after NK cell depletion. Compared to the control group, NK cell depletion significantly reduced the percentage of activated CD8⁺ T cells (CD69⁺CD8⁺) and effector CD8⁺ T cells (CD62L⁻CD44⁺) (Fig. 4A). Consistently and despite increased frequency (Supporting Fig. S4A), the absolute number of HBsAg- or HBcAg-specific CD8⁺ T cells producing IFN- γ or IL-2 was significantly reduced in the liver of NK cell-depleted mice (Fig. 4B). In fact, liver CD8⁺ T cells were also partly depleted by anti-AsGM1 (Fig. 4B), which may partly explain the phenomenon. Altogether, NK cell depletion suppressed activation of CD8⁺ T cells and HBV-specific CD8⁺ T-cell responses. Consequently, HBV DNA, HBsAg, and HBeAg in serum (Fig. 4C,D) and HBcAg (Fig. 4E) in liver was much higher in NK-depleted mice than in isotype-treated mice.

NK CELL DEPLETION IMPAIRED T-CELL FUNCTION INDUCED BY LSECs IN Rag1^{-/-} MICE

To minimize the effects of anti-AsGM1 on CD8⁺ T cells, we used an adoptive transfer model to confirm the effect of NK cells on the maturation of LSECs as well as HBV-specific T-cell responses (Fig. 5A). Anti-AsGM1 had no impact on transferred CD8⁺ T cells in Rag1^{-/-} mice (Fig. 5D), which was consistent with a previous study.⁽¹⁹⁾ We further analyzed the phenotype and antigen-presenting function of LSECs at day14 after DAP HI. NK cell depletion up-regulated the expression of PD-L1 on LSECs in Rag1^{-/-} mice but hardly affected other surface markers (Fig. 5B,C). Importantly, in NK cell-depleted mice, LSECs induced a substantial reduction of IL-2 production in CD4⁺ T cells and TNF- α production in both CD4⁺ T and CD8⁺ T cells (Fig. 5E). Consistently, NK cell depletion significantly lowered TNF- α producing HBcAg-specific CD8⁺ T cells in liver, and slightly reduced the IL-2 and TNF production on CD8 T cells without significant difference (Fig. 5F). Taken together, NK cell depletion impaired both the antigen-presenting function of LSECs and the intrahepatic HBV-specific

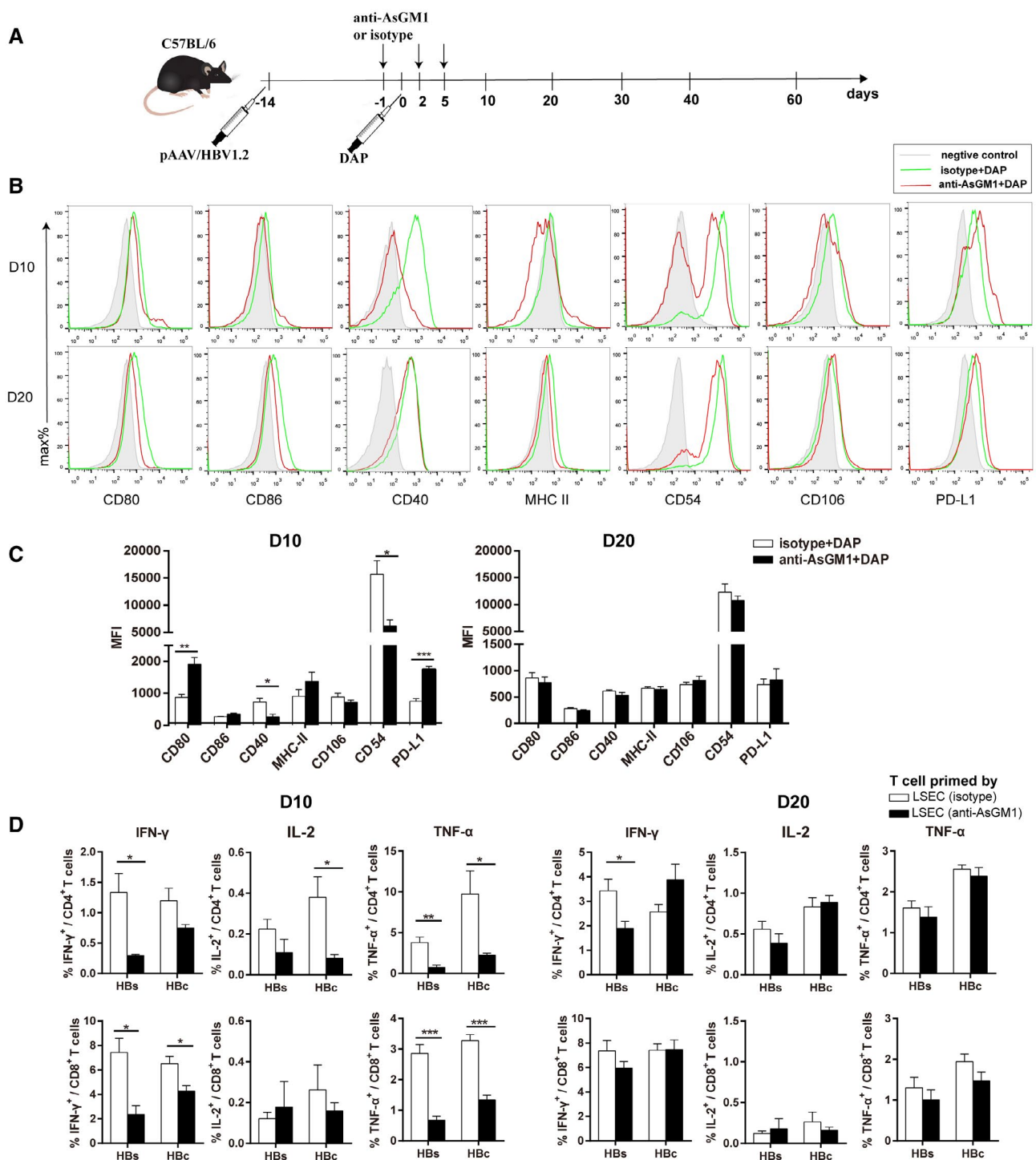


FIG. 3. NK cell depletion impaired the functional maturation of LSECs in C57BL/6 mice. (A) Study design: NK cell depletion antibodies (anti-AsGM1) or isotype control administered to mice intraperitoneally 1 day before DAP HI. Anti-AsGM1 or isotype was injected into mice every 3 days (3 times in total). LSECs were isolated at 10 and 20 days after DAP HI. (B) Representative histogram and (C) MFI of costimulatory molecules (CD40, CD80, and CD86), adhesion molecules (CD54 and CD106), and coinhibitory molecules (PD-L1) on LSECs are shown. (D) Antigen-presenting function of LSECs was determined by coculturing the corresponding peptide-loaded LSECs with splenocytes from pSM2-immunized mice for 3 days. IFN- γ , IL-2, or TNF- α production by T cells was measured through ICS. Four to five mice were analyzed per group, and at least two independent experiments were performed. Data represent mean \pm SEM, * P < 0.05, ** P < 0.01, *** P < 0.001, using unpaired Student t test. Abbreviation: D, days.

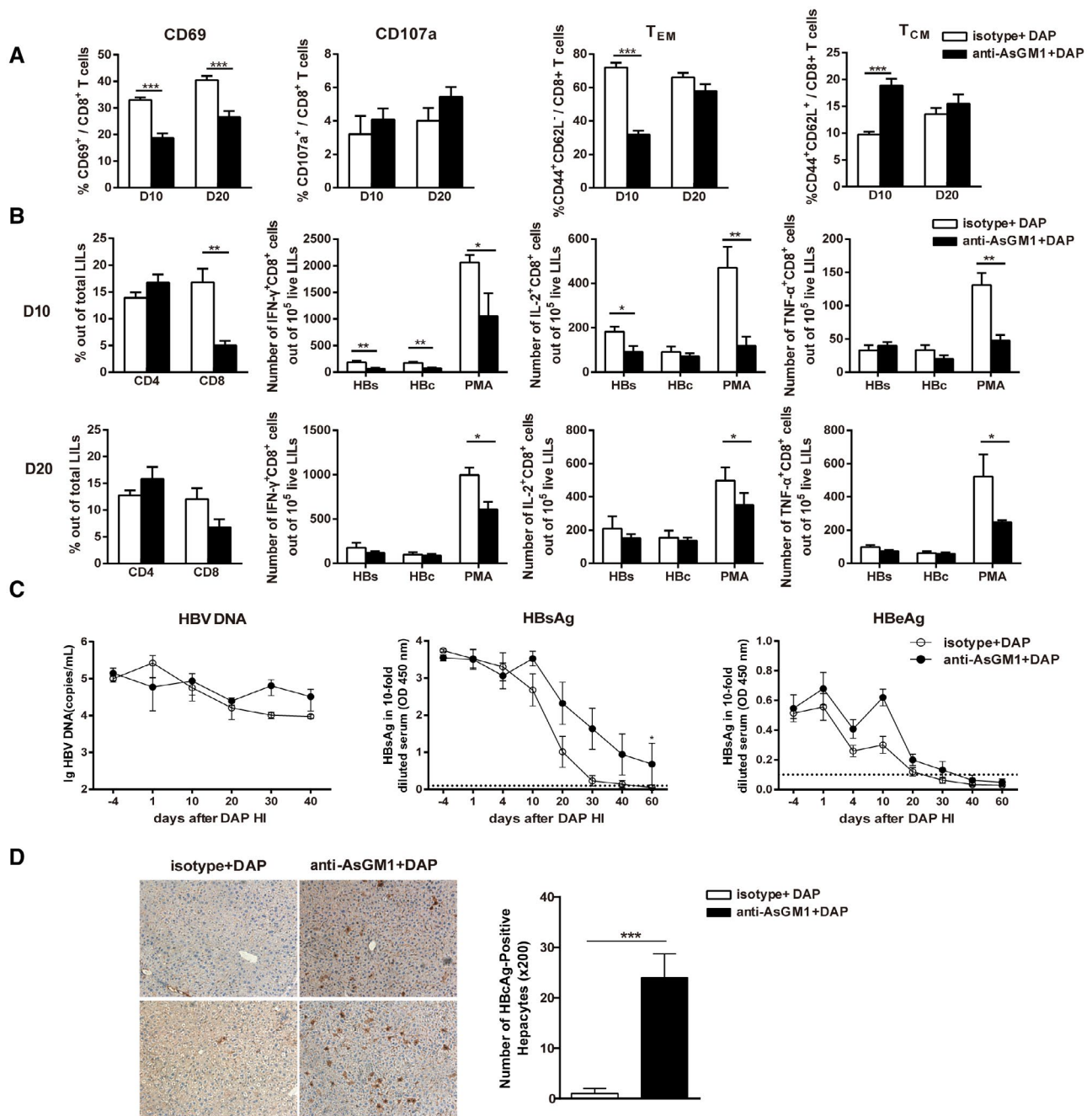


FIG. 4. NK cell depletion suppressed intrahepatic HBV-specific T-cell responses and inhibited HBV clearance. Study design is the same as shown in Fig. 3. LILs were isolated at days 10 and 20 after DAP HI. (A) Numbers of CD69⁺CD8⁺, CD107a⁺CD8⁺, CD62L⁻CD44⁺, and CD62L⁺CD44⁻CD8⁺ T cells are shown. (B) LILs were stimulated with 10 μg/mL of HBV peptide core93-100 or env208-216 in the presence of 1 μg/mL anti-CD28, Brefeldin A, and Monesin for 5 hours. The absolute number of CD8⁺ T cells producing IFN-γ, IL-2, or TNF-α is presented. (C) The kinetics of serum HBsAg, HBeAg, and HBV DNA levels are shown. Statistical differences of viral indicators were analyzed by repeated measures analysis. (D) Liver tissue sections at day 60 after DAP HI were stained with anti-HBc antibodies (magnification ×200). The number of HBeAg-positive hepatocytes was counted. Five to six mice were analyzed per group, and at least two independent experiments were performed. Data represent mean ± SEM, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, using unpaired Student *t* test. Abbreviations: D, days; Ig, log₁₀; OD, optical density; PMA, phorbol 12-myristate 13-acetate; T_{EM}, T cell-effector memory; T_{CM}, T cell-central memory.

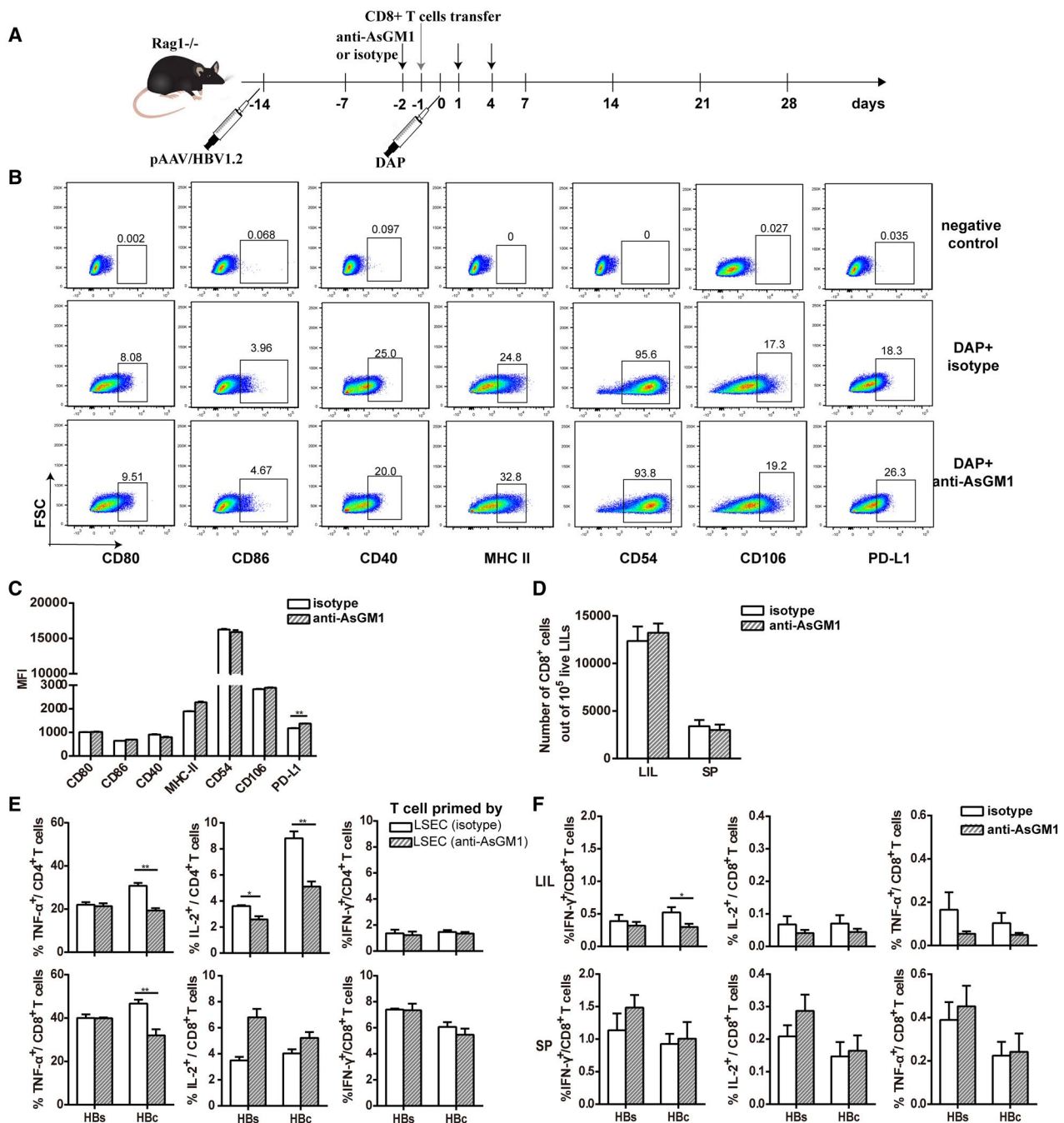


FIG. 5. NK cell depletion also impaired the antigen-presenting function of LSECs and HBV-specific CD8⁺ T-cell response in Rag1^{-/-} mice. (A) Study design: Rag1^{-/-} mice received anti-AsGM1 or isotype intraperitoneally at 12 days after HI of pAAV/HBV1.2. One day later, purified 5×10^6 CD8⁺ T cells from spleen of naive C57BL/6 mice were transferred to Rag1^{-/-} mice in a 1:1 ratio. The next day, mice were injected with DAP by HI. Anti-AsGM1 or isotype was injected every 3 days (3 times in total). (B–E) LILs and LSECs were isolated from the Rag1^{-/-} mice at day14 after DAP HI. (B) Representative staining plots and (C) MFI of surface markers on LSECs are shown. (D) The absolute number of CD8⁺ T cells in liver and spleen was determined. (E) The corresponding peptide-loaded LSECs were cocultured with splenocytes from pSM2-immunized mice for 3 days. IFN- γ , IL-2, or TNF- α production by T cells was measured by ICS. (F) LILs and splenocytes isolated at 28 dpi were stimulated with 10 μ g/mL of HBV peptide core93-100 or env208-216 for 5 hours. The percentage of CD8⁺ T cells producing IFN- γ , IL-2, or TNF- α is presented. Data represent mean \pm SEM, * P < 0.05, ** P < 0.01, using unpaired Student t test. Abbreviations: FSC, forward scatter; SP, spleen.

T-cell responses. As a result, serum HBeAg was much higher in anti-AsGM1-treated mice than in the isotype group (Supporting Fig. S5), although serum HBsAg and HBV DNA were almost unchanged with time between the two groups.

DAP STIMULATION COMBINED WITH NK CELLS PROMOTED T-CELL ACTIVATION PRIMED BY LSECs *IN VITRO*

Pretreatment of LSECs with DAP *in vitro* was not found to induce cytokine production by allogeneic T cells after 3 days coculture.⁽²⁰⁾ To investigate whether additional NK cells could promote T-cell immunity primed by LSECs, LSECs were incubated with 10 $\mu\text{g}/\text{mL}$ DAP with or without adding intrahepatic NK cells. We found that the production of IFN- γ and TNF- α by CD8⁺ T cells was only increased in the

presence of DAP accompanying NK cells (Fig. 6A), suggesting that NK cells could further promote functional maturation of LSECs. Moreover, DAP stimulation *in vitro* promoted the expression of CD69 and IFN- γ but not CD107a in NK cells with or without LSEC incubation (Fig. 6B).

TNF- α OR IFN- γ PROMOTED FUNCTIONAL MATURATION OF LSECs *IN VITRO*

As we had identified that the maturation of LSECs was dependent on NK cells both *in vivo* and *in vitro*, we next aimed to explore the mechanism by which NK cells regulate the maturation of LSECs. Activated NK cells secreted a profound amount of TNF- α and IFN- γ after HI of DAP in our study. Therefore, the LSEC phenotype and the ability of LSECs to activate T cells were investigated after stimulating

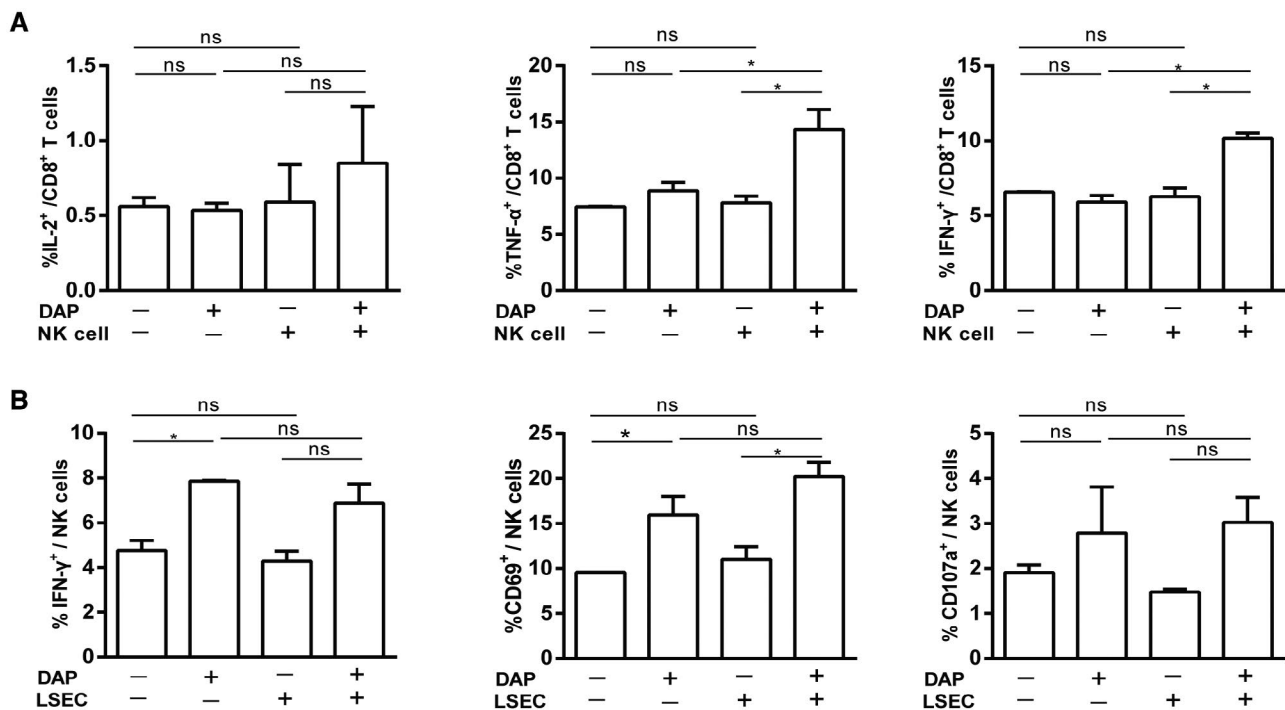


FIG. 6. NK cells combined with DAP promoted T-cell activation primed by LSECs *in vitro*. Primary isolated LSECs were cocultured with NK cells from the liver of naive mice with or without DAP for 16 hours. The LSECs/NK cell ratio is 5:1. NK cells were then collected, and LSECs were washed by phosphate-buffered saline. (A) T-cell activation primed by LSECs was determined by coculturing with splenocytes under the stimulation of CD3 and CD28 for 3 days. IFN- γ , IL-2, or TNF- α production by T cells was measured by ICS. (B) NK cell activation markers were measured. Data shown represent the mean \pm SEM of one of three representative experiments. Differences among multiple groups were analyzed by one-way ANOVA with least significant difference; * $P < 0.05$. Abbreviation: ns, not significant.

LSECs overnight by 100 ng/mL TNF- α or IFN- γ . We found that TNF- α stimulation up-regulated the expression of CD54 and CD106 in LSECs, while IFN- γ up-regulated CD54 and PD-L1 in LSECs (Fig. 7A,B). Moreover, the joint stimulation of both cytokines further promoted the expression of CD106

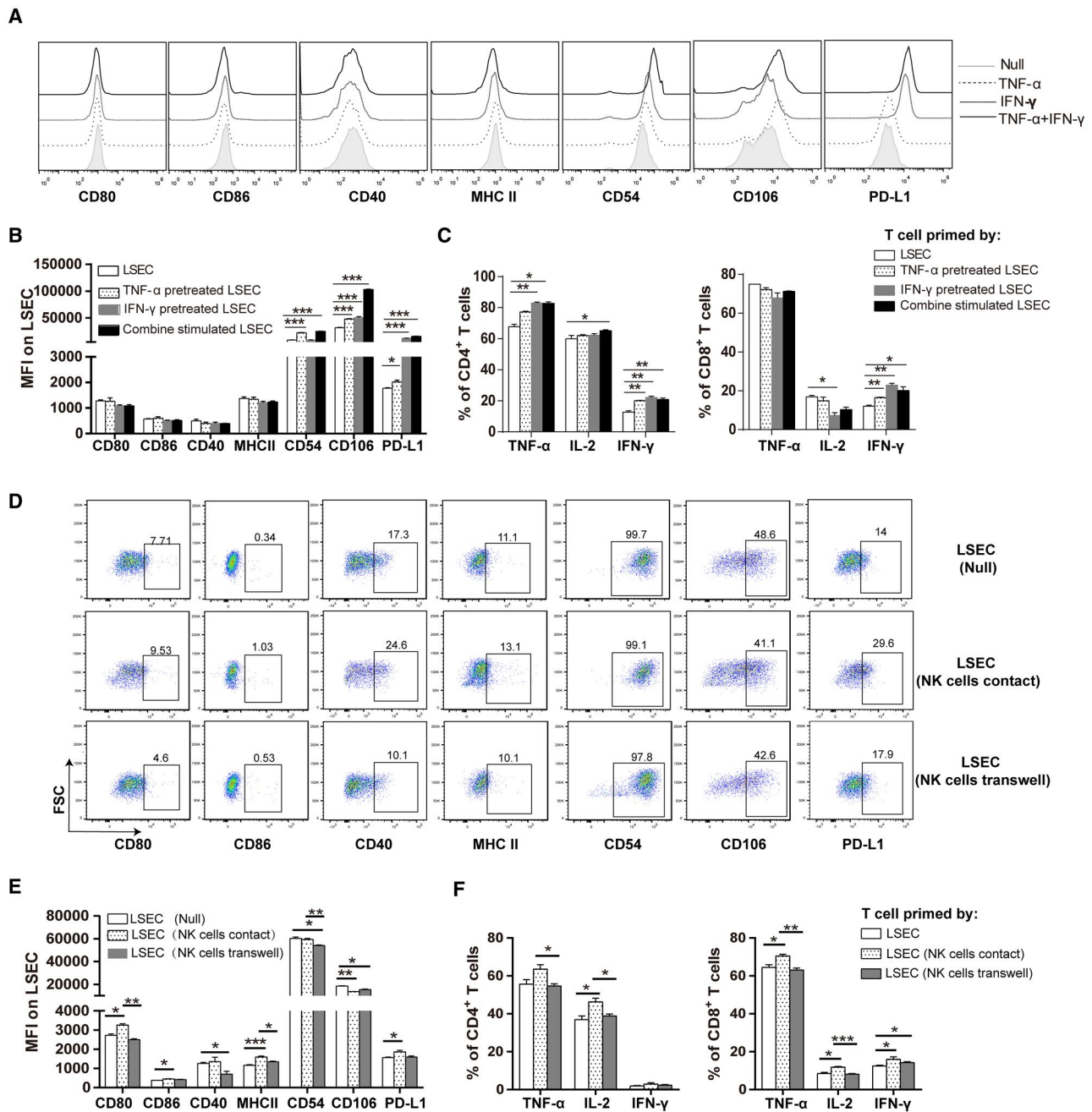


FIG. 7. Soluble TNF- α , IFN- γ , and direct contact with NK cells are involved in T-cell activation primed by LSECs. (A-C) LSECs isolated from naive mice were stimulated with 100 ng/mL murine recombinant TNF- α , IFN- γ , or both for 16 hours. (D-F) Purified intrahepatic NK cells from naive mice were cocultured with LSECs at ratios of 1:5 (LSECs, 5×10^5 /well; NK cells, 1×10^5 /well) either by direct contact or by transwell for 16 hours. (A) representative histogram or (D) pseudocolor plots and (B,E) MFI of surface markers on LSECs were shown. (C,F) T-cell activation primed by LSECs was determined by coculturing with splenocytes under the stimulation of CD3 and CD28 for 3 days, and the percentage of producing IFN- γ , IL-2, or TNF- α T cells from cocultured splenocytes was detected by flow cytometry. Data shown represent mean \pm SEM of one of three representative experiments. Differences among multiple groups were analyzed by one-way ANOVA with least significant difference. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Abbreviation: FSC, forward scatter.

in LSECs (Fig. 7A,B). More importantly, TNF- α - or IFN- γ -pretreated LSECs induced a substantial increase of IFN- γ and TNF- α in CD4⁺ T cells and IFN- γ production in CD8⁺ T cells (Fig. 7C). This suggested that IFN- γ and TNF- α could promote T-cell activation primed by LSECs, which might be one of the ways for NK cells to affect LSEC maturation.

CELL-CELL CONTACT WAS REQUIRED FOR NK CELLS TO REGULATE THE FUNCTIONAL MATURATION OF LSECs *IN VITRO*

To investigate whether cell-cell contact is also required for NK cells to regulate LSECs, NK cells were cocultured with LSECs either by transwell or direct contact in the presence of a low concentration of IL-15. Compared to the null group, the expression of CD80, CD86, and MHC-II on LSECs in contact with intrahepatic NK cells was up-regulated while the expression of CD40, CD106, and CD54 was even further suppressed on LSECs cocultured with NK cells by transwell (Fig. 7E). In addition, LSECs in contact with intrahepatic NK cells enhanced their ability to stimulate IL-2 and IFN- γ production by CD4⁺ and CD8⁺ T cells. In contrast, a similar change was not found for LSECs cocultured with NK cells by transwell (Fig. 7F). Taken together, this finding illuminated that cell-cell contact is also critical for NK cells to regulate the phenotypic and functional maturation of LSECs.

Discussion

In the present study, we demonstrated that DAP administration into the liver promoted the recruitment and activation of cNK cells in a CXCR3-dependent manner, which in turn maintained the maturation of LSECs and enabled control of HBV infection (Fig. 8). Depleting cNK cells by anti-AsGM1 impaired the maturation and antigen-presenting function of LSECs, which resulted in suppressed intrahepatic T-cell response and finally inhibited HBV clearance. Moreover, adding NK cells enhanced T-cell responses induced by LSECs pretreated with DAP *in vitro*. The potential mechanisms by which NK cells regulate the maturation of LSECs may involve soluble cytokines as well as cell-cell contact.

Previous studies have demonstrated that NK cells express low levels of NOD1 and that DAP stimulation

in vitro promotes the gene expression of IFN- γ but does not yield significant up-regulation of IFN- γ release on NK cells.⁽²¹⁾ This coincides with our study that DAP slightly promotes the secretion of IFN- γ on NK cells *in vitro*. However, DAP administration into liver by HI has no impact on NK cell number or IFN- γ production at day 1 and day 4 after HI (data not shown), which may exclude the direct effect of DAP on NK cell activation *in vivo*. On the other hand, intrahepatic mature MHC-II⁺CD11c⁺ DCs were significantly increased at 10 dpi after DAP HI, which is earlier than NK cell accumulation at 20 dpi. Indeed, the crosstalk between NK cells and DCs has recently been elucidated. Mature DCs could promote NK cell proliferation and cytokine production by releasing IL-12, IL-15, IL-18, type I IFN (IFN-I)⁽¹¹⁾ or prolonged cell-cell contact.⁽²²⁾ The increased mature DCs may partly promote NK cell activation at later time points in our study. However, the specific mechanism is yet to be elucidated.

We found that NK cell migration induced by DAP depends on the CXCR3 axis, which coincides with a previous study that CXCR3-deficient NK cells failed to migrate to cancer cells.⁽²³⁾ In fact, CXCR3 is also preferentially expressed on other immune cells, including monocytes, DCs, and T cells.⁽²⁴⁾ However, we did not observe a significant change in these immune cells between CXCR3^{-/-} mice and wild-type mice (Supporting Fig. S2A). The ligands of CXCR3 seem to preferentially recruit and influence certain lymphocyte populations under different conditions. For example, CXCL9 and CXCL10 cooperatively induced the recruitment of NK cells and controls to the spinal cord during herpes simplex virus 2 infection,⁽²⁵⁾ while CXCL11 selectively induced regulatory T cells.⁽²⁶⁾ HI of DAP mainly up-regulated CXCL9 and CXCL10 instead of CXCL11 in liver,⁽⁷⁾ which may partly explain why the CXCR3 axis mainly influences NK cell migration after DAP HI. In addition, it has been reported that CD8⁺ T-cell recruitment into infected brain in a murine cerebral malaria model⁽²⁷⁾ or inflamed joints in arthritic animals⁽²⁸⁾ depends on CXCR3. In our study, the number of CD8⁺ T cells was significantly increased in the spleen of CXCR3^{-/-} mice, although the number of intrahepatic CD8⁺ T cells was not significantly decreased (Supporting Fig. S2A). This might also be due to the reduced migration of CD8⁺ T cells into the liver in CXCR3^{-/-} mice.

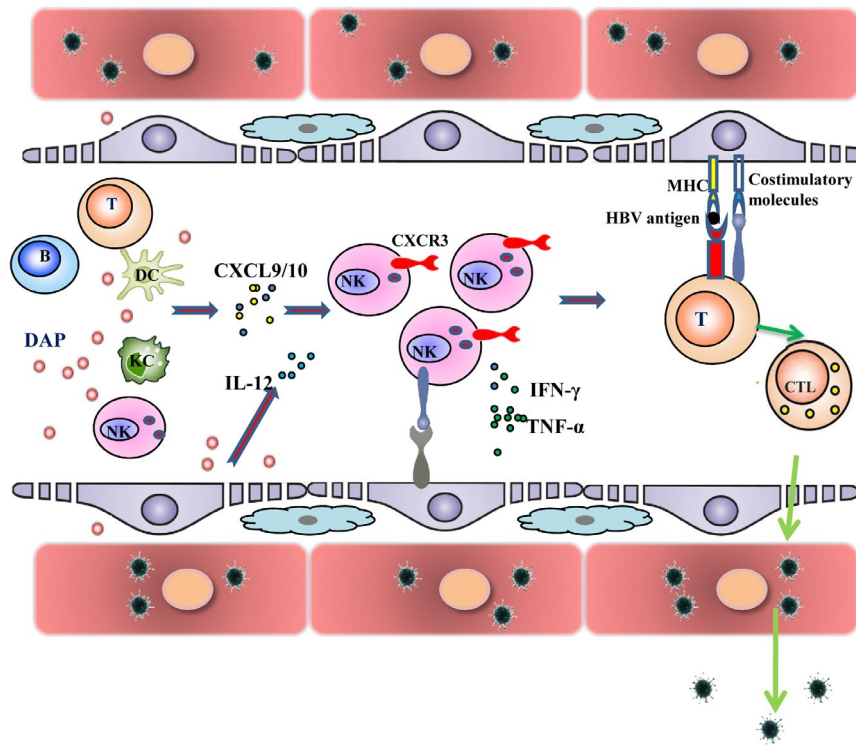


FIG. 8. NK cell activation and accumulation induced by DAP promoted the maturation of LSECs and HBV-specific T-cell responses in a mouse model. DAP administration into liver by HI not only promoted the release of IL-12 by LSECs but also enhanced the secretion of chemokines CXCL9 and CXCL10 by other immune cells. cNK cells migrated into liver through the CXCR3 axis. These NK cells were further activated by the released IL-12. Activated NK cells then secreted a large amount of IFN- γ and TNF- α . These two cytokines together with the immune synapse between NK cells and LSECs promoted the phenotypic and functional maturation of LSECs, which consequently enhanced HBV-specific T-cell responses and promoted HBV clearance. Abbreviations: B, B cell; CTL, cytotoxic T cell; KC, Kupffer cell; T, T cell.

HI of DAP has been shown to promote the maturation of LSECs, characterized by up-regulation of MHC-II, CD40, CD54, CD106, CD80, and CD86 and down-regulation of PD-L1.^(7,20) In our study, activated NK cells secreted profound amounts of IFN- γ and TNF- α after DAP HI (Fig. 1D). It has been reported that TNF- α enhances the expression of costimulatory molecules on DCs and contributes to production of IL-12 by DCs when synergizing with IFN- γ .^(29,30) TNF- α combined with protease 3 promotes the expression of adhesion molecules CD54 and CD106 on renal endothelial cells.⁽³¹⁾ Consistent with these studies, TNF- α and IFN- γ stimulation *in vitro* promoted the expression of CD54 and CD106 on LSECs (Fig. 7A,B) while NK cell depletion reduced the expression of CD40, CD54, and CD106 and up-regulated PD-L1 (Fig. 3A,B). This validates that NK cells may promote the phenotypic maturation

of LSECs by IFN- γ and TNF- α . Moreover, NK cells that secreted significantly lower TNF- α and IFN- γ in CXCR3^{-/-} mice induced down-regulation of CD40 and CD54 and up-regulation of PD-L1 on LSECs (Supporting Fig. S2C), which supports our assumption again. In addition, hepatic NK cells promoted the up-regulation of CD80, CD86, and MHC-II in LSECs and T-cell immunity induced LSECs in a cell-cell contact manner (Fig. 7E,F). This is consistent with the results found in DCs.⁽²²⁾ Taken together, both soluble cytokines and cell-cell contact are involved for NK cells to regulate LSEC maturation. However, the underlying mechanisms of cell-cell contact between LSECs and NK cells remain to be elucidated.

Of note, LSEC phenotypic and functional changes in Rag1^{-/-} mice after CD8⁺ T-cell transfer and NK cell depletion were not completely consistent with

those changes in wild-type mice. This might have been due to the lack of assistance from B cells and T cells. Apart from producing antigen-specific antibodies, B cells also influence multiple aspects of immunity through cytokine production or cell–cell contact.⁽³²⁾ For example, B cells could guide the development of lymphoid tissues by producing lymphotoxin $\alpha 1\beta 2$ ⁽³³⁾ and activate DCs by secreting granulocyte-macrophage colony-stimulating factor.⁽³⁴⁾ It has also been reported that preactivated B cells induce NK cells to produce greater amounts of IFN- γ through direct contact.⁽³⁵⁾ Furthermore, although we transferred CD8⁺ T cells into mice, these Rag1^{-/-} mice still lack CD4⁺ T cells. Previous information suggests that CD4⁺ T cells could also promote NK cell activation through secreting IL-2 or the crosstalk with APCs.⁽³⁶⁾ Therefore, lacking B and T cells in Rag1^{-/-} mice might weaken NK cell/LSEC axis through direct or indirect ways.

In the current study, a time lag was observed between LSEC maturation and NK cell accumulation; LSECs were activated at 10 dpi, but NK cells mainly accumulated and were activated at 20 dpi. Indeed, the interaction between LSECs and NK cells seems complex. NK cells can be activated through various cytokines, including IFN-I, IL-2, IL-12, IL-15, and IL-18, or through sensing of sudden imbalances in ligands for activating and inhibitory receptors.⁽³⁷⁾ Data in our group have illustrated that DAP stimulation promotes the secretion of IFN-stimulated gene 15, IFN- β , and IL-12 by LSECs,^(7,20) and this may subsequently participate in the activation of NK cells. CD54 on LSECs forms an adhesion molecule pair with lymphocyte function-associated antigen 1 on NK cells and thus promotes the migration of NK cells.⁽³⁸⁾ Yang et al.⁽³⁹⁾ has suggested that LSECs could also combine with NK cells in a carbohydrate-dependent manner and promote the cytotoxicity of NK cells. Moreover, LSEC depletion leads to fewer NK cells accumulating in the liver.⁽⁷⁾ Taken together, LSECs also play a crucial role in NK cell accumulation and activation. In addition, intrahepatic CD8⁺ T cells were activated at 10 dpi after DAP HI,⁽⁷⁾ characterized by increased amounts of antiviral cytokines IL-2, TNF- α , and IFN- γ . These cytokines may also induce NK cell activation and accumulation at the later time point of day 20 after DAP HI. Therefore, T cells may also participate in the regulation of crosstalk between LSECs and NK cells.

Effective HBV-specific T-cell responses are known to be crucial for HBV clearance. However, NK cells

play a dual role in T-cell immunity. On the one hand, NK cells would promote T-cell immunity by secreting cytokines or by affecting the maturation of APCs. In our study, T-cell response primed by LSECs was significantly suppressed in anti-AsGM1-treated mice, which coincides with suppressed intrahepatic HBV-specific T-cell response (Fig. 5E,F). This indicates that NK cells could indirectly affect T-cell responses by regulating LSECs. In addition, we found IFN- γ production by cNK cells was significantly increased at 10 and 20 dpi after DAP HI. IFN- γ secreted by NK cells has been reported to promote CD4⁺ T-cell differentiation into T helper 1 cells,⁽⁴⁰⁾ induce maturation of DCs,⁽²⁹⁾ and control effector CD8⁺ T-cell mobilization to virus-infected tissue,^(41,42) which leads to enhanced CD8⁺ T-cell response and virus or tumor clearance.⁽⁴³⁾ These findings suggest that NK cells not only promote LSEC maturation but may also mediate T-cell differentiation, recruitment, as well as functional responses. In addition, HBV-specific T-cell responses were enhanced at 10 and 20 dpi after DAP HI while LSECs were mainly activated at 10 dpi.⁽⁷⁾ The enhanced T-cell responses might be partially due to the activation of NK cells at 20 dpi. However, the number of intrahepatic CD8⁺ T cells was not significantly increased after DAP HI (Supporting Fig. S1A), indicating that NK cells in our study might mainly affect functional responses of CD8⁺ T cells. On the other hand, activated NK cells also negatively regulate T-cell response by killing activated T cells through death receptor ligand pathways, such as TRAIL or Fas L.⁽⁴⁴⁾ However, IFN-I signaling protected T cells against NK cell-mediated elimination.⁽⁴⁵⁾ In our study, DAP had no impact on the expression of TRAIL on NK cells, and NK cell depletion inhibited T-cell activation, indicating that the positive regulation of NK cells toward T cells overcomes their negative effects.

In conclusion, our study demonstrated for the first time that functional maturation of LSECs following NOD1 activation depends on NK cells through the soluble cytokines TNF- α and IFN- γ and cell–cell contact, and thus leads to T-cell responses and HBV clearance. Although our study focused on chronic HBV, the interaction between NK cell activation and LSEC maturation may also play an important role in other hepatotropic virus or liver tumors. A limitation regarding this study is whether the interaction between LSECs and NK cells in a mouse model reflects the

real situation in humans with chronic HBV infection. Therefore, additional studies to investigate the crosstalk between LSECs and NK cells in human liver and other hepatotropic virus will be required. Our findings provide important clues for future studies of immunotherapy for hepatotropic virus based on LSEC maturation.

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