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Acute portal hypertension using portal vein ligation abrogates TRAIL expression of liver-resident NK cells

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Abstract

The effects of acute portal hypertension (PHT), which is reported as poor prognostic factors in patients with hepatocellular carcinoma, are not well known on the liver immune system, including natural killer (NK) cells. The aim of this study, therefore, was to investigate how acute PHT influences the functions and characteristics of liver-resident NK (lr-NK) cells using an acute PHT mouse model. Acute PHT decreased the number of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL+) lr-NK cells by about 20% and attenuated cytotoxic activity against the Hepa1-6 cell line by about 40%. Among various cytokine, only interleukin-33 (IL-33), which inhibits NK activity, significantly increased after portal vein ligation (PVL). Because lr-NK cells highly expressed ST2/IL-33R, IL-33 co-culture significantly suppressed TRAIL expression on lr-NK cells by about 50%, and IL-33 administration markedly decreased TRAIL expression and cytotoxic activity of lr-NK cells. Furthermore, the TRAIL+ NK cells population was maintained by anti-IL33 antibody or following portosystemic shunt procedure even after PVL. Finally, we demonstrated that IL-33 decreased TRAIL expression in lr-NK cells via AKT–forkhead box O (FoxO) and mitogen-activated protein kinase (MAPK) signaling. *Conclusion*: This work demonstrates that PHT suppresses the TRAIL+ lr-NK cell population and antitumor activities in the liver. Additionally, Akt-FoxO and MAPK signaling pathways attenuate the TRAIL expression in lt-NK cells via IL-33 receptor in mice.

INTRODUCTION

Portal hypertension (PHT) adversely affects the prognosis of various liver disease. PHT negatively impacts the prognosis for patients with hepatocellular carcinoma (HCC) undergoing partial hepatectomy,[1–3] transarterial chemoembolization (TACE),[4] and awaiting liver transplantation for HCC.[5] Pre-operative portal vein embolization sometimes increased the size of tumors in the remnant liver lobes as a result of the potential tumor

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Yuki Imaoka and Koki Sato contributed equally to this work.

growth–stimulating environment provided by acute PHT.[6] The relative increase in hepatic arterial blood flow and increased secretion of inflammatory cytokines into the liver are associated with tumor growth.[7,8] At present, the relationship between acute PHT and intrahepatic immunity, and its role in tumor progression, have not been elucidated. Natural killer (NK) cells are a particularly important cell population that play a key role in the innate immune response, possessing both cytotoxic and cytokine-producing effector functions that act as the first line of defense against diseases.[9] In terms of intrahepatic immunity, immature NK cells are found at a higher proportion in the liver, producing higher amounts of cytokines and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL).[10–12] Particularly, DX5⁻ liver-resident (lr)-NK cells, which comprise a large portion of immature NK cells, express TRAIL and exert strong cytotoxicity against hepatoma cells via the TRAIL–TRAIL receptor pathway.[13–17] We have reported that partial hepatectomy and hepatic irradiation reduced TRAIL expression in lr-NK cells and decreased their antitumor activity.[17,18] However, the mechanism by which the TRAIL expression in lr-NK cells is down-regulated has not been fully elucidated.

IL-33 is a tissue-derived nuclear cytokine from the IL-1 family abundantly expressed in endothelial cells, epithelial cells, and fibroblast-like cells, both during homeostasis and inflammation.[19] The IL-33/ST2 complex mediates signals via the cytoplasmic domain toll receptor interleukin 1 to IL1RAcP, leading to activation of transcription factors such as nuclear factor kappa B via tumor necrosis factor receptor–associated factor 6, interleukin 1 receptor associated kinase-1/4 and mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase 1/2, Jun amino terminal kinase, p38, and phosphoinositide 3-kinase (PI3K)/AKT signaling.[20,21] In addition, Akt–forkhead box O1 (FoxO1) signaling, observed further downstream, is associated with immature lr-NK cells expressing high amounts of TRAIL.[12] However, despite recent progress in this field, we still know very little about the interleukin-33 (IL-33)–induced mechanism to mediate a reduction in the TRAIL expression on lr-NK cells. Therefore, we hypothesize that the damage inflicted on hepatocyte or liver endothelial cells by PHT increases the amount of released IL-33, which decreases TRAIL expression in lr-NK cells.

To test this hypothesis, we examined the impact of acute PHT on the activities of lr-NK cells using an acute PTH mouse model with portal vein ligation (PVL) and portosystemic shunt (PSS) mouse model that could attenuate acute PHT. Furthermore, our data revealed that IL-33/IL-33R signaling pathways are associated with the TRAIL expression in lr-NK cells.

METHODS

Animal experiments

C57BL/6J (B6) female mice that were 8–12 weeks old were purchased from CLEA Japan, Inc. Mice were housed in the animal facility of Hiroshima University, Japan, in a pathogen-free microenvironment. The study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals and the local committee for animal experiments. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University (Permit No. A20-97).

Development of the animal model

Mice were divided into three groups: the sham group as the control group, the ligation group subjected to right PVL as the PHT group, and the PSS + ligation group subjected to PSS procedure 4 weeks before right PVL as a decompression group. This PVL model imitated acute PHT, such as under physiological conditions, after balloon-occluded retrograde transvenous obliteration or massive liver resection, and small for size syndrome of liver transplantation. Under anesthesia, the ligation mice were laparotomized at the midline of the abdomen, and the right portal vein was separated from the common bile duct and the right hepatic artery and ligated securely, using 7–0 silk. Portions of the hepatic parenchyma, ranging from 25% to 35% of the total liver mass, were ligated in this manner (Figure S1A). On the contrary, portions of the hepatic parenchyma, ranging from 65% to 75% of the total liver mass, demonstrated increased portal flow and were correlated with high PHT. The PSS procedure was performed as described by several studies.[22,23] In the PSS model, a 0.5-cm incision in the left subcostal arch was created. A subcostal pouch was made in the subcutaneous tissue. The mouse spleen was pulled out and buried in this subcostal pouch. Then, the peritoneum was closed. Four weeks before PVL, the mouse spleen was placed into the subcutaneous pouch to induce PSS. PSS can prevent PHT even after PVL (Figure S1B).

Detailed methods for estimation of the portal vein pressure, isolation of lymphocytes, flow cytometric analysis, quantitative real-time polymerase chain reaction (PCR), NK cell isolation, cytotoxicity assay, histological analysis of liver tissue sections, and ingenuity pathway analysis (IPA) can be found in the Supporting Materials.

Hepatoma cell line

The mouse hepatoma cell line Hepa1-6 (derived from H-2b mice) was purchased from RIKENCell Bank. Hepa 1–6 cells express surface death receptor 5 but lack Decoy receptors (DcR1, DcR2).[12]

Histological evaluation of metastatic growth in the liver

To induce tumors *in vivo*, Hepa1-6 tumor cells in 0.2 ml of medium 199 (Sigma-Aldrich), at a concentration of 10^7 cells/ml, were injected into the spleen.[18] After the spleen was identified, Hepa1-6 tumor cells were slowly injected. The mice were sacrificed 7 days after tumor cell injection, and the liver was removed and fixed overnight in 10% formalin. For studies using a BZ-8000 microscope (Keyence), 4-μm tissue sections of the liver were stained with hematoxylin and eosin (HE). The relative areas occupied by the tumors were calculated as the percentage of the total scanned liver area.

Isolation of lymphocytes

Full details are outlined in the Supporting Materials. Liver lymphocytes were prepared according to a previously described method.[16]

Quantitative real-time PCR

Full details are outlined in the Supporting Materials. The PCR primers used for the gene analysis are listed in Table S1. All liver samples were collected from the left lobe.

Cytotoxicity assay

Full details are outlined in the Supporting Materials. Cell cytotoxicity was assessed as previously described.[17,18]

Statistical analysis

Unpaired Student's *t* tests and the nonparametric Mann–Whitney *U* test were performed to compare differences between the two independent groups; $p < 0.05$ was considered statistically significant. Values are expressed as mean ± SD. JMP 14 software (SAS Institute Inc.) was used for all calculations.
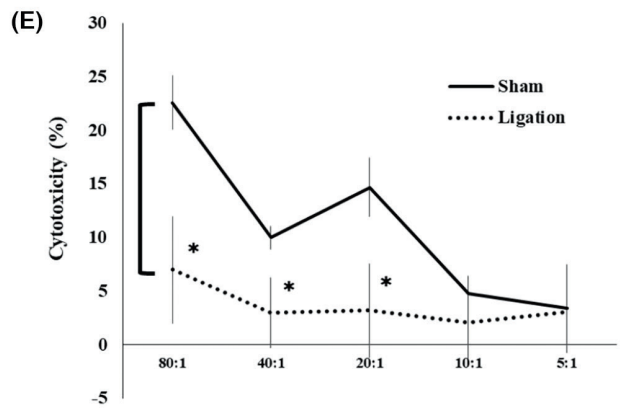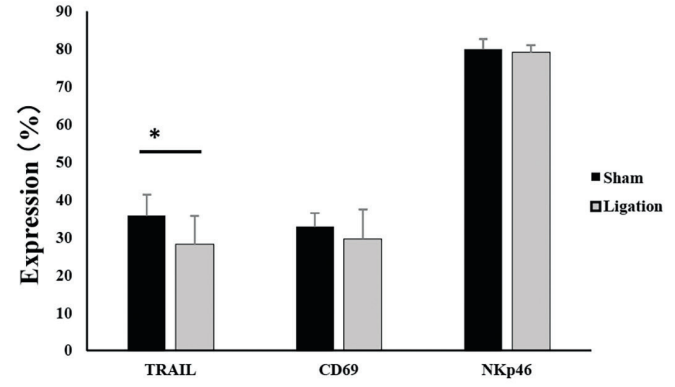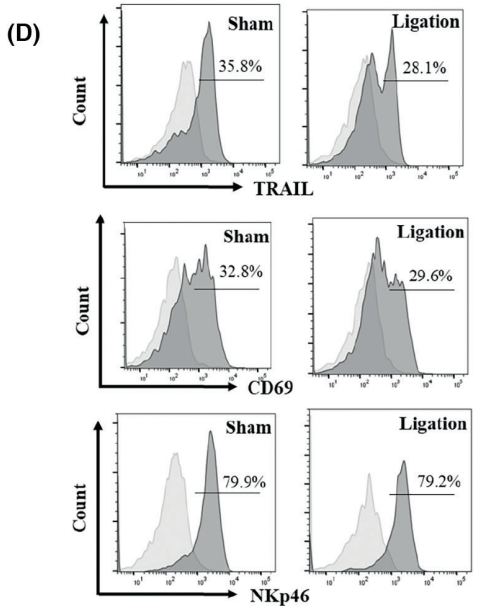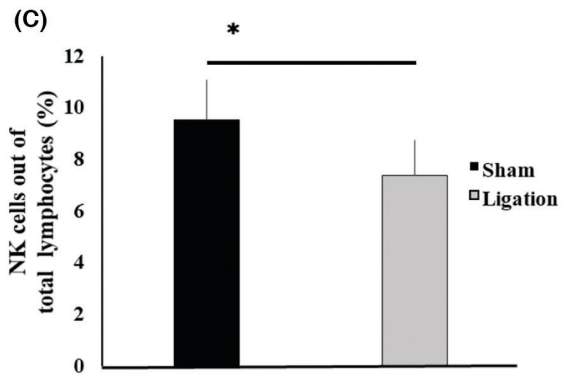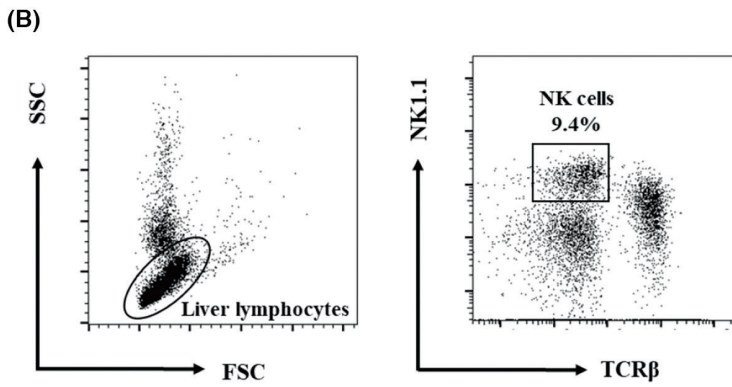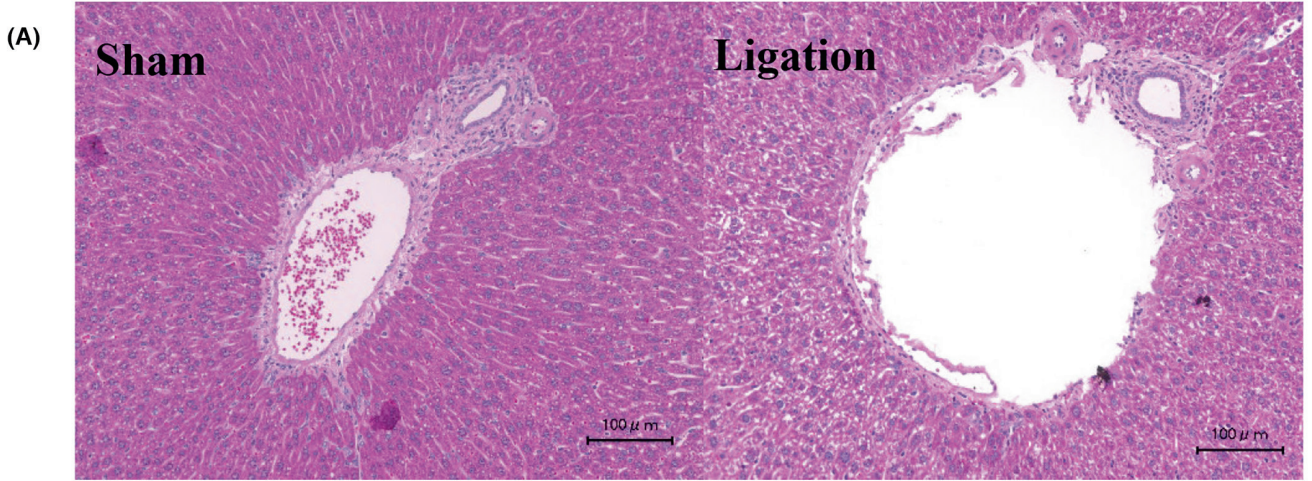
RESULTS

TRAIL^{+} NK cells decreased in the liver with acute PHT

First, we developed an acute PHT model by 30% PVL in mice to analyze the effect of increased portal pressure on intrahepatic immunity. The average portal vein pressure was 86.5 ± 8.9 mmH$_2$O before the right portal vein clump. After the clump, the average portal vein pressure was significantly increased to 114.0 ± 2.9 mmH$_2$O ($n = 4$, $p < 0.01$). Three days after PVL, the vascular endothelial cells of the interlobular veins of the portal branch were damaged within the Glisson's capsules (Figure 1A). Next, we assessed the influence of acute PHT on lr-NK cells using the PVL model. Both proportion of NK cells and activation markers including TRAIL on NK cells significantly decreased following PVL (Figure 1B–D), whereas CD69 and NKp46 expressions remained unchanged (Figure 1D). In addition, the cytotoxicity of lr-NK cells also significantly decreased in the acute PHT group compared with that of the sham group (Figure 1E).

To elucidate the relationship between the volume of the remnant liver and NK cell function, we also analyzed the NK function after the 70% PVL. Significant suppression of TRAIL-expressing NK cells was observed after 70% PVL (Figure S2A,B), whereas CD69 expression of NK cells remained unchanged (Figure S2B). These results indicated that a smaller volume of the remnant liver decreased the antitumor activity in conjunction with the increased portal pressure. The 70% PVL models had a very poor prognosis; therefore, we used a 30% PVL model for subsequent experiments.

IL-33 was detected as a key cytokine related to the decreased activity of lr-NK cells in the acute PHT model

We next analyzed the microarray of liver tissue to determine the inflammatory state in the liver caused by PVL. According to fold changes (>2) in microarray data sets for liver tissues obtained from the sham and the PHT model, the messenger RNA (mRNA) expressions of Spp1 (fold change = 3.15) related to the chemotaxis of NK cells[24] and IL-33 (fold change = 2.66) associated with the differentiation and maintenance of function in NK cells[19] were dynamically up-regulated (Figure 2A). Next, we comprehensively analyzed the expression of several inflammatory cytokines in the remnant lobes of the acute PHT model. IL-1α, IL-33, and interferon β (IFN-β) levels were significantly increased in the remnant lobes of the acute PHT model (Figure 2B). To confirm the functional role of IL-1α, IL-33, and IFN-β in lr-NK

(A)

(B)

(C)

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(E)

cells, liver mononuclear cells (LMNCs) were co-cultured with IL-1α, IL-33, or/and IFN-β *in vitro*. TRAIL expression on lr-NK cells cultured in the presence of IL-33 decreased over time compared to those cultured without IL-33 (as a control) (Figure 2C,D). To assess the effect of these cytokines on lr-NK cell activation, we incubated the LMNCs with IL-1α, IL-33 or IFN-β, separately and all together, for 4 h, and analyzed the expression of TRAIL and CD69 in the lr-NK cells. IL-33 was the only cytokine that down-regulated the TRAIL expression.

IL-33 decreased the antitumor activity of liver-resident NK cells

IL-33 is abundantly expressed in the endothelial cells during inflammation.[19] Therefore, we hypothesized that the damage of hepatocytes or liver endothelial cells attributed to acute PHT increases the amount of IL-33, which decreases the TRAIL expression in lr-NK cells. We analyzed changes in the intrahepatic IL-33 levels before and after PVL. First, the mRNA expression of IL-33 significantly increased in the liver tissue 3 days after PVL ($p < 0.05$; Figure 3A). Furthermore, immunostaining revealed that IL-33 was highly expressed on damaged endothelial cells and hepatocytes in the PHT group (Figure 3B). Next, we investigated the IL-33 receptor (ST2) on lr-NK cells. Lr-NK cells, especially immature Dx5$^-$ NK cells, strongly expressed the IL-33 receptor (Figure 3C). To confirm the functional role of IL-33 in lr-NK cells *in vitro*, isolated lr-NK cells were cultured in the presence of IL-33 *in vitro*. TRAIL expression on lr-NK cells significantly decreased after 4 h of culture with IL-33 (100 ng/ml). Especially, the ratio of mature Dx5$^+$ NK cells that demonstrated less TRAIL expression was significantly increased after culturing in the presence of IL-33 (Figure 3D). In addition, IL-33 decreased the cytotoxicity of LMNCs (Figure 3E).

To confirm the functional role of IL-33 in lr-NK cells *in vivo*, recombinant mouse IL-33 (rIL-33) was administered to mice. TRAIL, CD69, and Dx5 expressions of lr-NK cells were evaluated 4 h after administration through flow cytometric analysis. The expressions of TRAIL and CD69 significantly decreased in the rIL-33-treated mice compared with control mice. Furthermore, the population of mature Dx5$^+$ NK cells significantly increased in the rIL-33-treated mice (Figure 3F). In addition, the cytotoxicity of isolated lr-NK cells also decreased in the rIL-33-treated

mice (Figure 3G). As an alternative, oncostatin M (OSM) was used as an inducer of IL-33 expression in the mouse liver endothelial cells.[25] IL-33 mRNA expression increased in the OSM-treated mice (Figure S3A). TRAIL$^+$ and CD69$^+$ expression of lr-NK cells decreased in mice 4 h after OSM administration (Figure S3B). To analyze the influence of acute PHT on the antitumor activity of lr-NK cells, we used the PSS mouse to prevent acute PHT.[22,23] Although the mRNA expression of IL-33 significantly increased after ligation alone, PSS prevented the increase of IL-33 mRNA expression even after ligation (Figure 3H). The expressions of TRAIL and CD69 in lr-NK cells were maintained in PSS + ligation mice compared with that in the ligation mice (Figure 3I).

Inhibition of IL-33 maintained the antitumor activity of liver-resident NK cells

To clarify the influence of IL-33 on the antitumor activity of lr-NK cells, anti-mouse IL-33 antibody was administered to mice 1 day before PVL. The concentration of IL-33 in the remnant lobes 3 days after PVL significantly decreased in the anti-IL33-treated ligation model compared with the control ligation model (Figure 4A). The expressions of TRAIL and CD69 in lr-NK cells were improved in the anti-IL-33-treated ligation model. The population of DX5$^+$ NK cells did not significantly increase in the anti-IL-33-treated ligation model (Figure 4B). In addition, the cytotoxicity of NK cells did not decrease against hepatic tumors in the anti-IL-33-treated mice even after PVL (Figure 4C). Seven days after tumor injection, the relative areas occupied by the tumors decreased in the anti-IL-33-treated ligation model significantly (Figure 4D).

Candidate genes approach identified the TRAIL pathway in liver-resident NK cells

To provide additional insight into the relevant genes affecting TRAIL expression on NK cells, the activation *z* score depicts the amplitude of the difference between TRAIL$^+$ and TRAIL$^-$ NK cell subsets. The upstream regulator analysis (URA) predicted that various pathways were differentially activated between TRAIL$^+$ versus TRAIL$^-$ NK cell subsets (Figure 5A). We detected
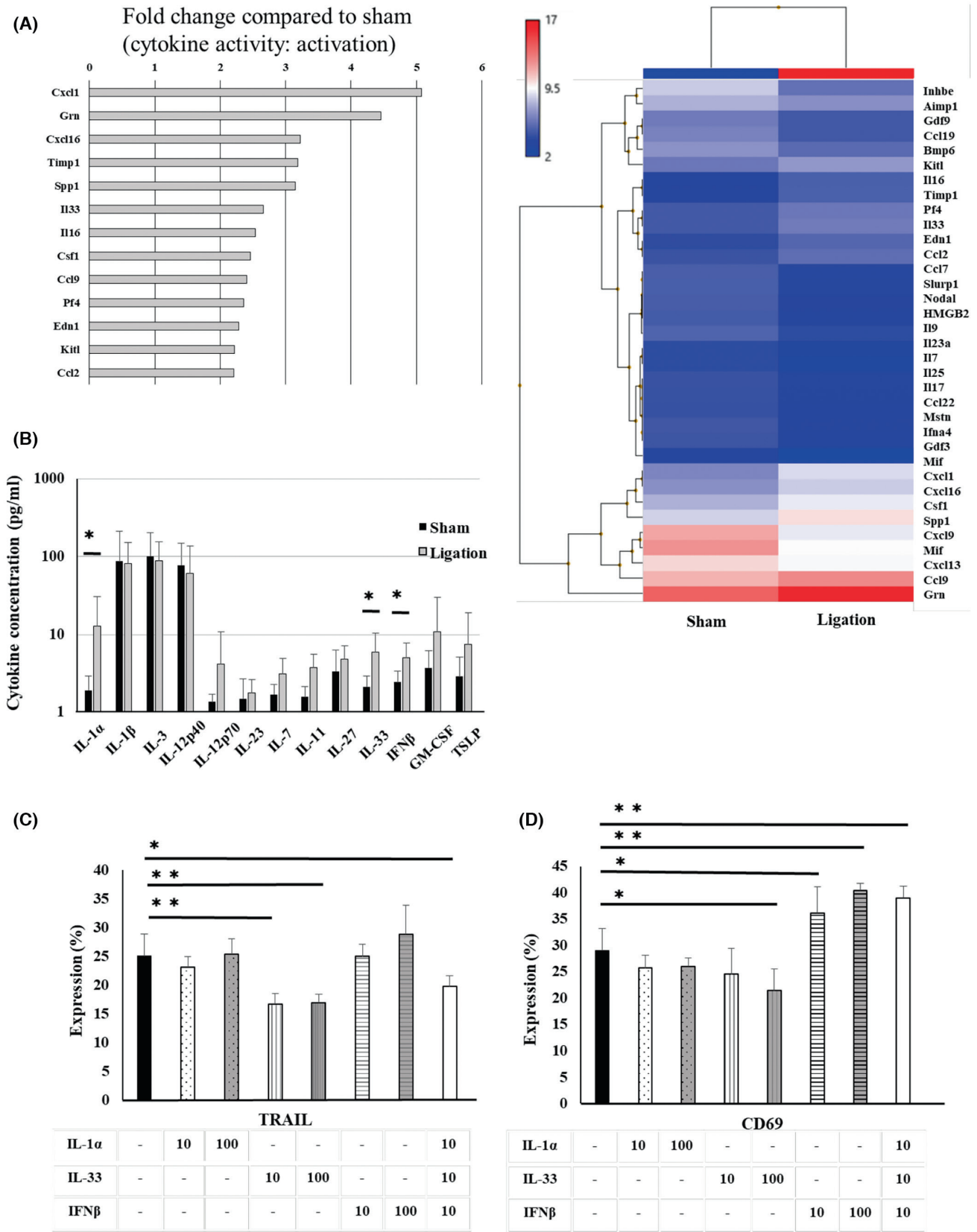
FIGURE 2 Interleukin-33 (IL-33) was detected as a factor contributing to decreased TRAIL expression on lr-NK cells after PVL. (A) Predicted functional cytokine genes were associated with PHT. Top 40 genes with the highest positive and negative correlation genes of cytokine were selected; the heat map is shown. (B) Various cytokines levels were evaluated between the sham model and the PHT model (sham, $n = 5$; ligation, $n = 10$). (C,D) Liver mononuclear cells (LMNCs) were co-cultured with recombinant mouse IL-1α, IL-33, and interferon β (IFN-β; 0, 10, and 100 ng/ml) (4 mice per group). TRAIL and CD69 expressions in lr-NK cells were evaluated with time (4 h) via flow cytometric analysis. Statistical differences were detected by paired t-test or Wilcoxon test when appropriate. *$p < 0.05$; **$p < 0.01$.

(A)

(B)

(C) **(D)** **(E)**
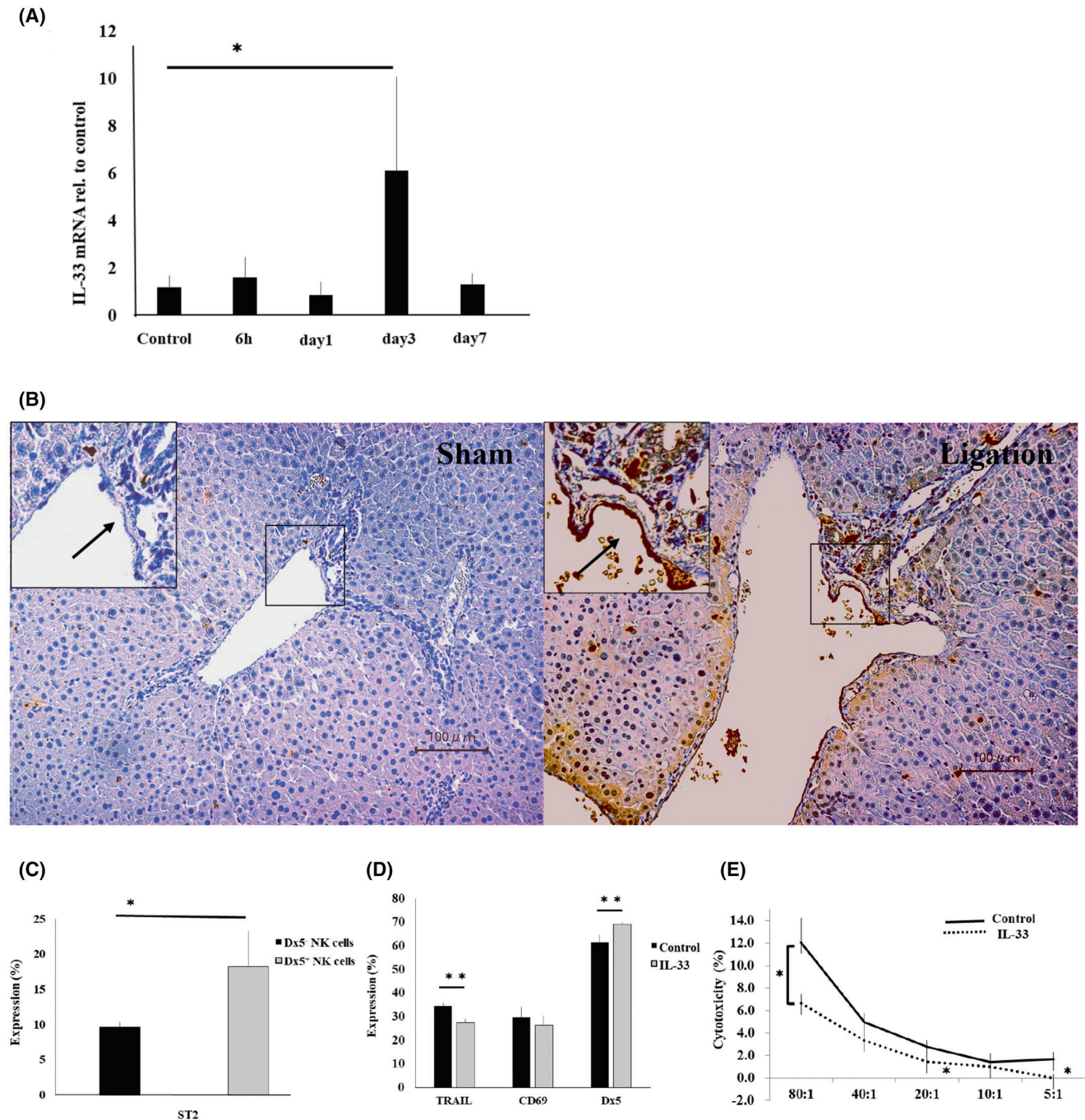
FIGURE 3 IL-33 decreased TRAIL expression of lr-NK cells *in vitro* and *in vivo*. (A) Relative fold change in messenger RNA (mRNA) expression of IL-33 after 6 h, day 1, day 3, and day 7 compared with that observed at 0 h as a control in the ligation group (4−5 mice per group); data are expressed as means ± SD. (B) Expression of IL-33 in the liver tissues of the sham and the PHT groups. Immunolocalization of IL-33 was performed using primary antibody rat immunoglobulin G (IgG) anti-mouse IL-33 in sections of the sham models and the ligation models (day 3); scalebar in small micrographs indicates 100 μm. (C) IL-33 receptor (ST2) expression of Dx5$^-$ lr-NK cells and Dx5$^+$ lr-NK cells in mice are shown in flow cytometric analysis. (D) Isolated lr-NK cells were co-cultured with rIL-33 (100 ng/ml) for 4 h. (isolated from 10 mice). TRAIL, CD69, and Dx5 expressions of hepatic NK cells were decreased following co-culturing with IL-33 after 4 h, as shown in flow cytometric analysis. (E) The cytotoxicity of LMNCs is shown. lr-NK cells co-cultured with rIL-33 (100 ng/ml) had lower cytotoxicity compared with that observed with phosphate buffered saline (PBS) (IL-33 0 ng/ml as a control). Data are expressed as means ± SD (3 mice per group). (F) Recombinant mouse IL-33 (rIL-33) (400 ng/200 μl) or PBS (200 μl as a control) was administered to B6 mice. TRAIL, CD69, and Dx5 expressions of hepatic NK cells were evaluated 4 h after injection via flow cytometric analysis. (G) The cytotoxicity of isolated lr-NK cells is shown. IL-33 injection decreased the cytotoxicity of lr-NK cells. Data are expressed as means ± SD (4 mice per group). (H) Relative fold change in the mRNA expression of IL-33 observed at day 3 in ligation model, portosystemic shunt (PSS)+sham model, and PSS+ligation model compared with B6 mice (control) (4−5 mice per group). (I) Representative histograms of TRAIL expression in NK1.1 + TCRβ− lr-NK cells in PSS+sham model and PSS+ligation model. The proportions of TRAIL$^+$ and CD69$^+$ cells among NK1.1$^+$TCRβ$^-$ lr-NK cells are shown in the sham model, the ligation model, PSS+ sham model, and PSS+ ligation model (5−6 mice per group). Statistical differences were detected by paired t-test or Wilcoxon test when appropriate. *$p < 0.05$; **$p < 0.01$.
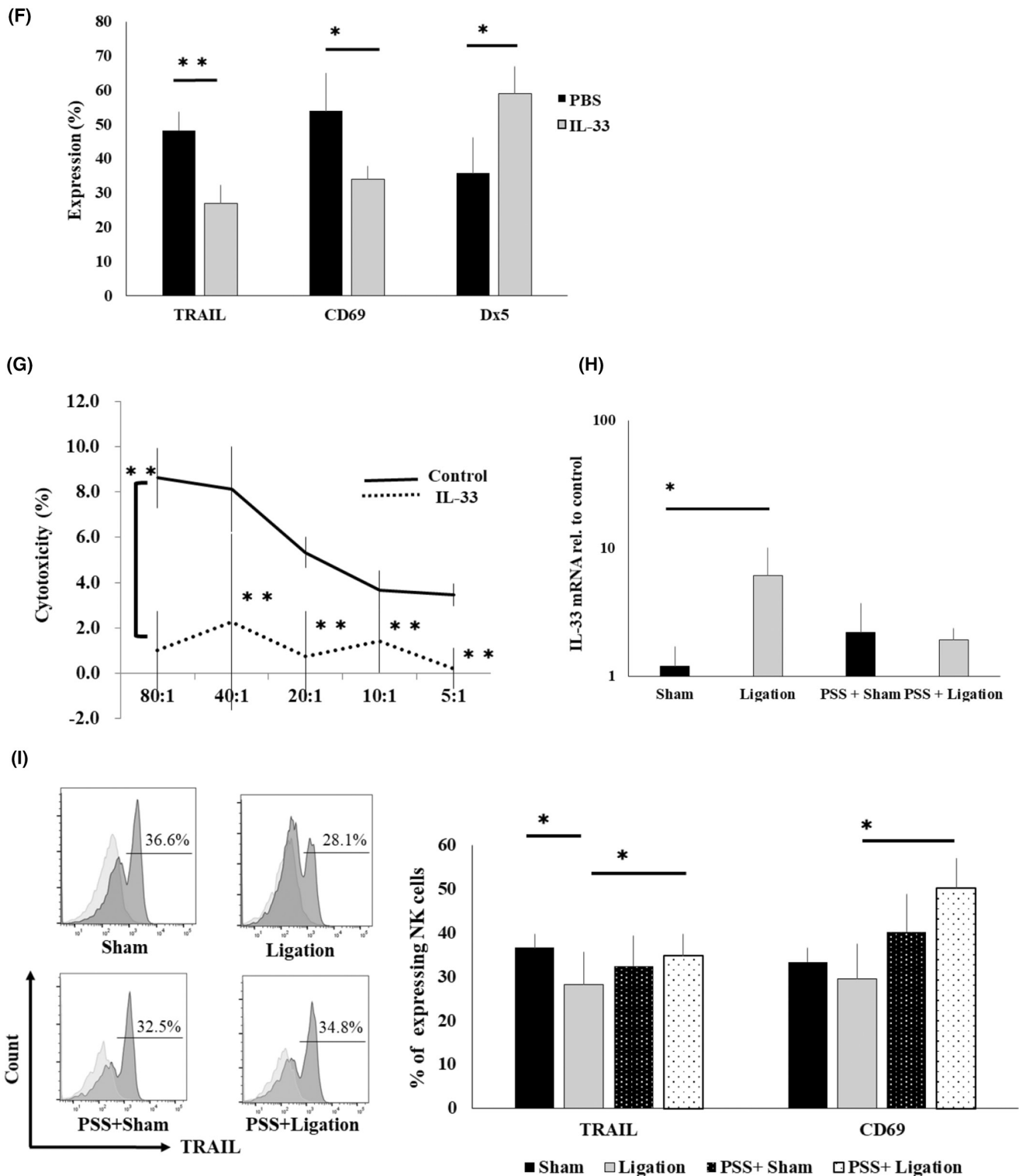
(F)

(G)


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FIGURE 3 (Continued)

FIGURE 4 Anti-IL-33 antibody maintained TRAIL expression in hepatic NK cells in the ligation model. (A) The concentration of IL-33 in the remnant lobes of the anti-IL-33-treated ligation model compared with that observed in PBS-treated ligation models (5 mice per group). (B) Representative histograms of TRAIL expression in NK1.1$^+$TCRβ$^-$ lr-NK cells in PBS-treated sham mice, PBS-treated ligation mice, anti-IL-33-treated sham mice, and anti-IL-33-treated ligation mice. The proportions of TRAIL$^+$, CD69$^+$, and Dx5$^+$ cells among NK1.1$^+$TCRβ$^-$ lr-NK cells are shown in PBS-treated sham mice, PBS-treated ligation mice, anti-IL-33-treated sham mice, and anti-IL-33-treated ligation mice (5 mice per group). (C) The cytotoxicity of LMNCs is shown. The cytotoxicity of LMNCs in anti-IL-33-treated ligation mice did not decrease. Data are expressed as means ± SD (4 mice per group). (D) Representative histopathological findings of liver specimens after tumor injection (stained with HE). Scalebar in small micrographs indicate 100 μm. Specimens are shown from PBS-treated ligation mice (left) and anti-IL-33-treated ligation mice (right) (4 mice per group). Statistical differences were detected by Wilcoxon test when appropriate. *$p < 0.05$; **$p < 0.01$.
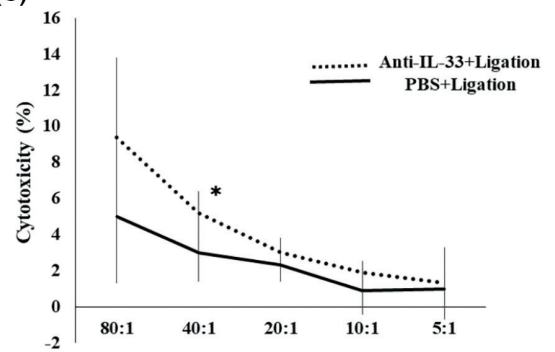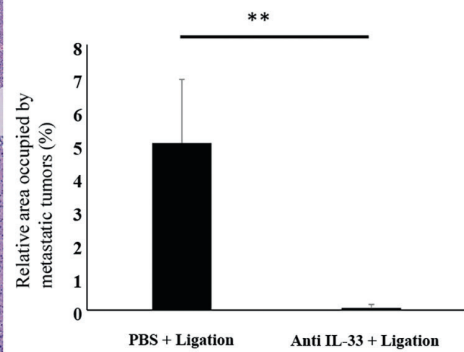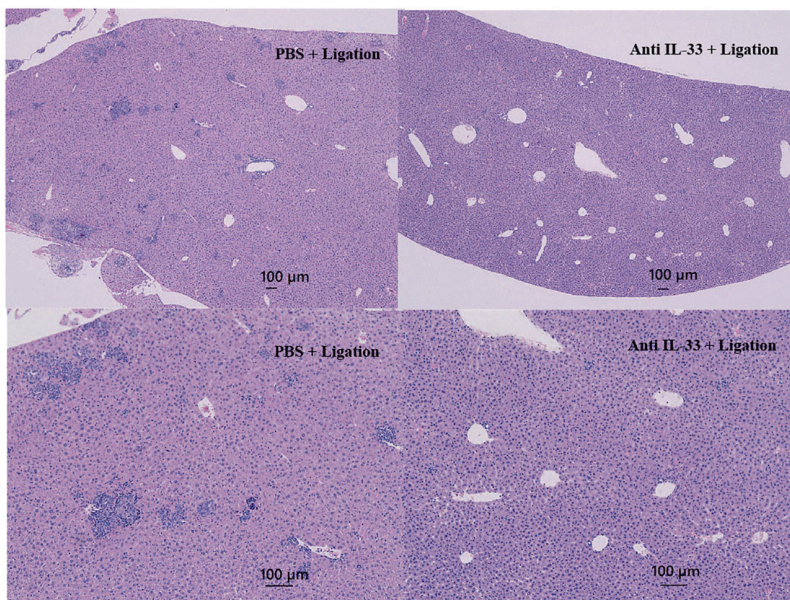
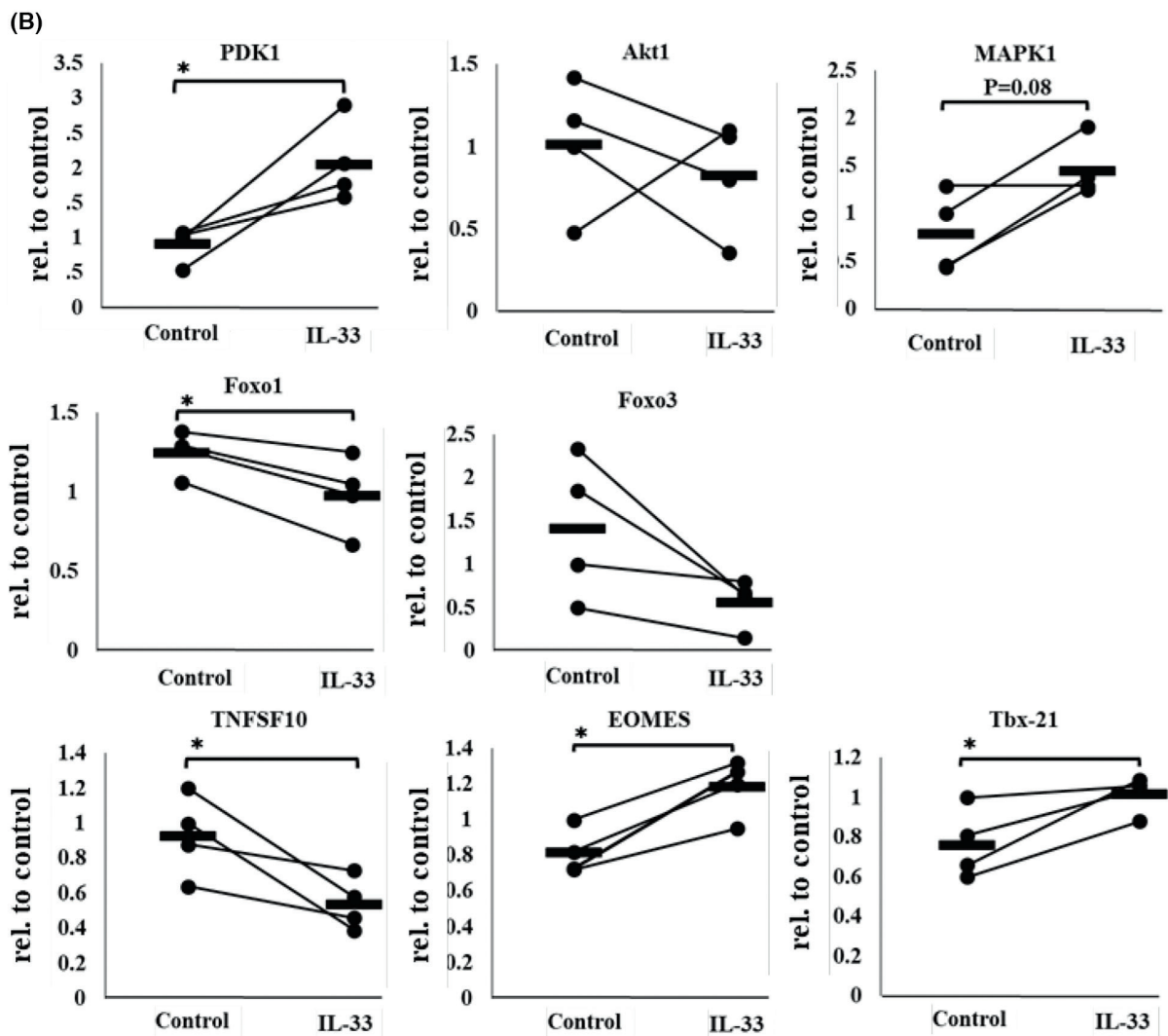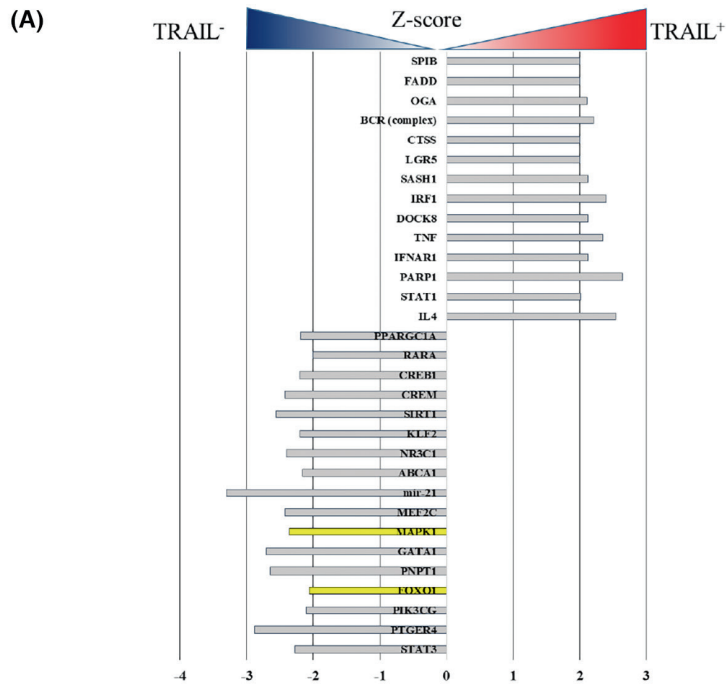**(A)**

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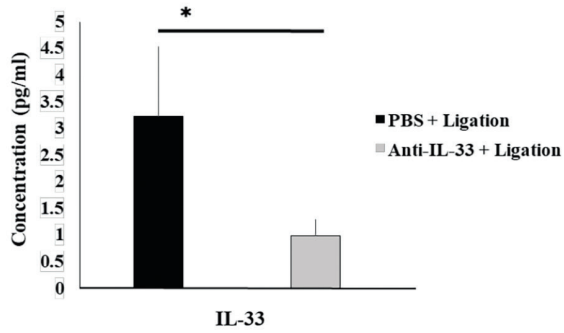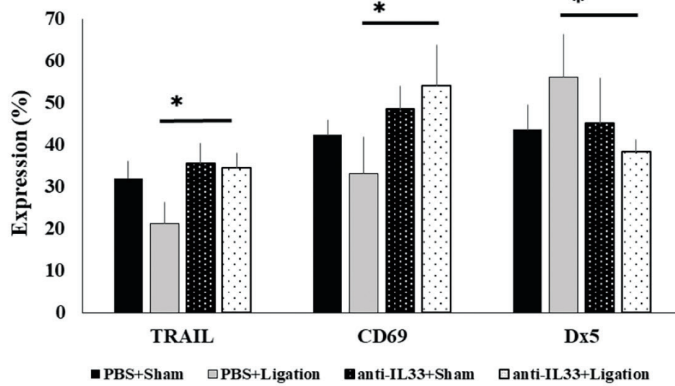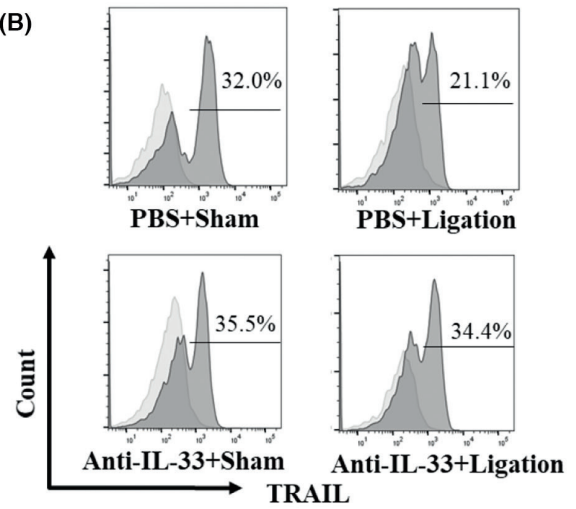
F I G U R E 5   Upstream regulator analysis of differential expression genes between TRAIL positive and negative NK cells. (A) Fourteen predictive activated genes and 17 predictive inhibited genes associated with the expression of TRAIL in the positive cell population. (B) Akt–forkhead box O (FoxO) and mitogen-activated protein kinase (MAPK) signaling in lr-NK cells were analyzed using quantitative real-time polymerase chain reaction (PCR) 1 h after co-culturing with IL-33 (n = 4 mice). Statistical differences were detected by Wilcoxon test when appropriate. *$p < 0.05$.

14 predictive activated genes and 17 predictive inhibited genes associated with the expression of TRAIL in the positive cell population. As common genes observed in these microarray results and IL-33/ST2 downstream signals reported in the previous study, we focused on two genes, Foxo1[26,27] and MAPK1,[20,28] whose expression was induced by IL-33. Akt-FoxO and MAPK signaling pathways in lr-NK cells were analyzed through quantitative real-time PCR 1 h after culturing in the presence of IL-33 (Figure 5B). Consistent with the stimulation of IL-33 in lr-NK cells, mRNA expression of phosphoinositide-dependent protein kinase 1, Tbx-21 (T-bet), and EOMES in lr-NK cells increased significantly 1 h after culturing with IL-33. In contrast, mRNA expression of FoxO1 in lr-NK cells decreased significantly 1 h after culturing with IL-33. The mRNA expression of TNFSF10, the primary gene associated with TRAIL expression, demonstrated significant decrease (p-value < 0.05). In addition, mRNA expression of MAPK1 increased (p-value = 0.08). Akt-FoxO and MAPK signaling in lr-NK cells are summarized in Figure 6.

DISCUSSION

In this study, we proved that acute PHT damaged liver tissues and increased the amount of released IL-33 in the liver. IL-33 decreased the proportion of TRAIL$^+$ NK cells in the liver and cytotoxicity of lr-NK cells. PSS, which reduced acute PHT and IL-33 down-regulation, canceled these attenuations. Akt-FoxO and MAPK signaling negatively impacted TRAIL expression in lr-NK cells through IL-33/ST2.

First, we observed that acute PHT decreased TRAIL expression in lr-NK cells, depending on the amount of the remnant liver. We observed that both the proportion of NK cells and TRAIL expression rate were reduced after PVL. The absolute number of NK cells in all liver lymphocytes in the remnant liver was also reduced. As both the number and TRAIL expression of NK cells in the liver were reduced by acute PHT, there were no compensatory changes due to migration from outside the liver associated with inflammation.

We also proved that the damage of hepatocytes or liver endothelial cells attributed to acute PHT increases the amount of IL-33 released, which decreased the TRAIL expression in lr-NK cells. Our results showed that lr-NK cells, especially immature Dx5$^-$ NK cells, strongly expressed ST2, which is the receptor of IL-33. IL-33 is normally released by damaged or necrotic barrier cells.[29–31] In contrast, after performing the PSS procedure to prevent acute PHT, the damage associated with acute PHT was reduced, and the expression of IL-33 decreased in the liver tissues. The down-regulation of IL-33 performed using PSS or anti-IL-33 antibody maintained the TRAIL expression in lr-NK cells successfully even after PVL. These results indicated that the effects of acute PHT on intrahepatic immunity mediated via IL-33/ST2 signaling, especially lr-NK cells. Hence, the substantial reduction in the TRAIL$^+$ NK cell population in the liver after acute PHT can be explained by TRAIL$^+$ NK cell instability via IL-33/ST2 signaling.

The proportion of TRAIL$^+$ NK cells in lr-NK cells decreased by stimulation with IL-33. A similar result was observed by stimulation with OSM, which is an inducer of IL-33.[25] The major targets of IL-33 are tissue-resident immune cells, including NK cells.[19] In mice, the expression of endothelial IL-33 appears to be restricted to the adipose tissue, liver, and reproductive tract of females.[32] The function of IL-33 can vary depending on the expressing organs. Therefore, the effect of IL-33 on NK cells is controversial. IL-33 is reported to activate both human[33] and mouse[34] NK cells. IL-33 also increases the cytotoxic activity of spleen NK cells.[35] However, several studies have reported that IL-33 plays different roles in NK cells within the tumor microenvironment, depending on the levels of IL-33 and expressing tissue.[36–39] IL-33 decreases the number of lr-NK cells and the cytotoxic activity of NK cells.[38–40] The IL-33 also decreases frequencies of tumoricidal NK cells.[41] We hypothesized that the role of IL-33 on NK cells was dependent on the different maturation pathways of NK cells in the tissues. In the liver, which contains immature Dx5$^-$ NK cells, IL-33 decreased the TRAIL expression of lr-NK cells and increased the population of mature Dx5$^+$ TRAIL$^-$ NK cells. Our results support the negative function of IL-33 in lr-NK cells.

In this study, IL-33 increased the expression of T-bet and EOMES in lr-NK cells. The transcription factor T-bet determines the developmental stability in immature NK cells with constitutive expression of TRAIL.[42] In addition, the process of maturation, in which the expression of TRAIL is reduced and that of the integrin DX5 is induced, requires the transcription factor EOMES.[42] Activation of PI3K-AKT inactivates Foxo transcription factors.[26,27] In addition, AKT signaling suppresses the expression of FoxO targets involved in the induction of the expression of TRAIL and FASL.[43–45] NK cells in mice model are known to express Foxo1, and to a lesser extent Foxo3.[46] Foxo1 inhibits Tbx21 expression via
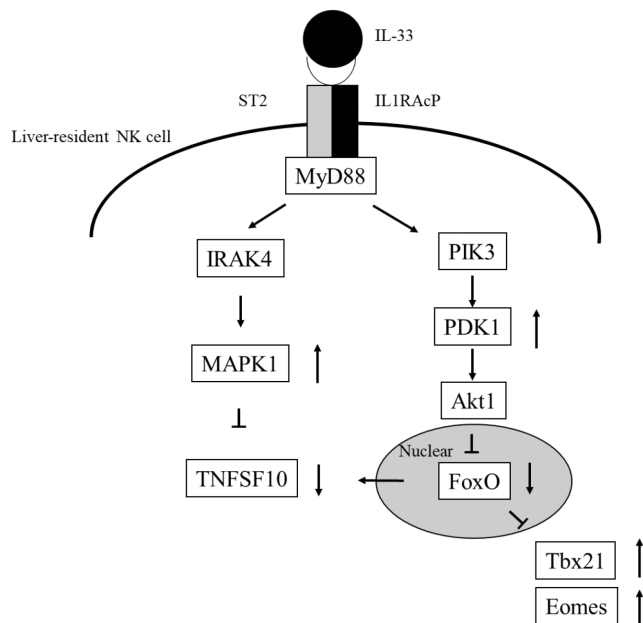
FIGURE 6 Summary of IL-33/IL-33R signaling in lr-NK cells. Akt-FoxO and MAPK signaling in lr-NK cells are summarized according to the results of quantitative real-time PCR. IRAK4, interleukin 1 receptor associated kinase; MyD88, myeloid differentiation factor 88; PDK1, phosphoinositide-dependent protein kinase 1; PI3K, phosphoinositide 3-kinase; TNFSF10, TNF superfamily menber 10.

direct association with the Tbx21 promoter and acts as a negative checkpoint in NK cell maturation.[46,47] IL-33 also activates p38 MAPK signaling in NK cells.[20,28] In the IPA-based URA, FoxO1 and MAPK1 were detected as predictive inhibited genes of TRAIL^{+} NK cells. However, the reduction of Tbx21 and EOMES levels increased the populations of immature lr-NK cells, demonstrating high TRAIL expression via increased FoxO1.[12] Our results also indicated that mRNA expression of FoxO1 in lr-NK cells decreased after culturing with IL-33. The inconsistent result may be attributed to the peculiarities of lr-NK cells, which were not reflected in the database. On the contrary, mRNA expression of MAPK-1, which was detected as an inhibitory gene of TRAIL^{+} NK cells, increased after stimulation of IL-33. The mRNA expression of MAPK1was induced by IL-33 in support of the IPA results. These results supported strongly that Akt-FoxO and MAPK signaling pathways negatively impacted the TRAIL expression in lr-NK cells via IL-33/ST2.

There are, however, some limitations to the present study. First, the expressions of EOMES and T-bet, depending on the maturity of lr-NK cells, are known to be likely different between mice and humans. These issues should be assessed in future human studies. Second, the role of other immune cells expressing the IL-33 receptor, such as regulatory T cells and innate lymphoid cell type 2 cells, was not evaluated in detail. Third, intestinal congestion caused by acute PVL and

the production of intestinal-derived inflammatory cells and inflammatory cytokines can also affect the intrahepatic immunity. Although these indirect mechanisms should be responsible for suppressing the activity of lr-NK cells, our results indicated a direct mechanism in which IL-33 suppresses the activity of lr-NK cells. These indirect mechanisms should be assessed in future studies.

Finally, these results will have important implications for the future treatment of HCC and underscore the damage that acute PHT can exert on the immune system. The mechanism by which IL-33 suppressed the antitumor activity of lr-NK cells can explain the negative effects of acute PHT in various liver diseases. The blockade of IL-33 during hepatectomy for cholangiocarcinoma in mice model inhibited tumor progression.[48] Anti-IL-33 treatments in humans are focused on allergy and/or asthma. Etokimab, an anti-IL-33 biologics, has previously demonstrated a good safety profile and favorable pharmacodynamic properties in many clinical studies.[49] Our results in the mice model indicate the need for further verifications in humans to prove the usefulness of the anti-IL-33 treatments among patients with HCC or acute PHT.

We found that acute PHT decreased the antitumor activity of lr-NK cells. Overall, these findings indicated that Akt-FoxO and MAPK signaling pathways negatively impact the TRAIL expression in lr-NK cells via the IL-33 receptor in mice.

AUTHOR CONTRIBUTIONS
Data analysis and manuscript draft: Yuki Imaoka, Koki Sato, and Masahiro Ohira. Investigation: Yuki Imaoka, Koki Sato, Masahiro Ohira, Kouki Imaoka, Ryosuke Nakano, and Takuya Yano. *Study design*: Masahiro Ohira, Yuka Tanaka, and Hideki Ohdan.

CONFLICT OF INTEREST
Nothing to report.

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