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The blockade of interleukin-33 released by hepatectomy would be a promising treatment option for cholangiocarcinoma

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Abstract

Interleukin-33 (IL-33), an alarmin released during tissue injury, facilitates the development of cholangiocarcinoma (CCA) in a murine model. However, it is unclear whether IL-33 is associated with human CCA. The aim of this study was to support the following hypothesis: IL-33 is released during hepatectomy for CCA, subsequently facilitating the development of subclinical CCA and eventually leading to recurrent disease. IL-33 expression was assessed in various samples from both humans and mice including resected liver and paired plasma samples collected at hepatectomy and after surgery, and its influences on recurrent disease and patient prognosis were determined. Homogenized human liver samples with high or low IL-33 expression were added to the culture medium of human CCA cells, and the changes in proliferation and migration were evaluated. To examine the effects of inhibiting the IL-33 release induced by hepatectomy, syngraft transplantation of murine CCA cells was performed in C57BL/6J mice with or without IL-33 blockade. The amount of IL-33 released into the plasma during hepatectomy correlated with the background liver expression. High expression of IL-33 in the liver was an independent risk factor for recurrence. Homogenized liver tissue strongly expressing IL-33 increased both the proliferation and migration of tumor cells. Mice who underwent hepatectomy exhibited CCA progression in the remnant liver, whereas blockade of IL-33 during hepatectomy inhibited tumor progression. Thus, we concluded that surgery for CCA with curative intent paradoxically induced IL-33 release, which facilitated CCA recurrence, and anti–IL-33 therapy during hepatectomy might reduce the risk of CCA recurrence.

KEYWORDS

cholangiocarcinoma, hepatectomy, interleukin-33, ST-2, syngraft murine model

1 | INTRODUCTION

Cholangiocarcinoma (CCA) is a lethal neoplasm originating from the biliary epithelium. Despite its increasing incidence worldwide,[1-4] therapeutic options are still limited, and overall survival rates are <10%.[3,5,6] Although surgical resection is a potentially curative treatment, more than half of CCA patients cannot undergo surgery because they have advanced disease.[3,7-11] Furthermore, the majority of patients develop recurrent disease even after surgical resection with curative intent.[8-10]

Interleukin-33 (IL-33), a member of the interleukin-1 (IL-1) family, is a crucial costimulatory agent in adaptive immune responses[12] and promotes cancer progression and metastasis by suppressing innate antitumor immunity in breast cancer and neck squamous cell carcinoma.[13-16] Recent reports have shown that short-term administration of IL-33 facilitates the development of a murine genetic model of CCA,[17,18] suggesting that exposure to IL-33 facilitates the development of CCA cells. However, IL-33 performs other functions as an alarmin, being rapidly released from cells upon tissue damage.[12] As the clinical role of IL-33 in human CCA has not been investigated thoroughly, there is a possibility that IL-33 is released during hepatectomy for CCA with curative intent. Given the above, we postulated the following sequence of events: IL-33 is released during hepatectomy; subsequently, the released IL-33 facilitates the development of CCA cells and disease recurrence.

We herein reported that hepatic IL-33 was released during hepatectomy, as determined by measuring the degree of hepatic expression, and high levels of hepatic IL-33 present in the liver were thus found to be a risk factor for CCA recurrence following surgery. Furthermore, to endorse the results of our retrospective study of human samples, we investigated the influence of hepatectomy on murine CCA cells in the remnant liver and examined whether blockade of IL-33 during hepatectomy inhibits CCA progression via a murine orthotopic transplant experiment using syngrafted CCA cells.

2 | MATERIALS AND METHODS

2.1 | Clinical samples

We examined resected specimens from patients who underwent surgery (hepatectomy or liver transplantation) for one of three different kinds of disease, CCA, hepatocellular carcinoma (HCC), or liver failure. Fifty resected CCA specimens were obtained from patients who underwent liver resection between 2000 and 2014 at Osaka University Hospital (Table 1). A set of primary and secondary liver resection samples were obtained from each HCC patient, and we investigated 50 sets of resected HCC specimens obtained between 2000 and 2014 at Osaka University Hospital. Six livers removed from patients who underwent liver transplantation were obtained between 2000 and 2014 at Osaka University Hospital. The liver specimens were preserved in paraffin blocks and cut into 3.5-μm-thick slices for immunohistochemistry, and a part of each resected liver specimen was preserved as frozen tissue at −250°C. Twenty-four pairs of human blood samples were obtained just before and just after hepatectomy for CCA, and six pairs of human blood samples were obtained just before and just after liver transplantation in the six recipient patients. The blood samples were centrifuged, and the plasma was collected for enzyme-linked immunosorbent assays (ELISAs). The use of samples was approved by the Human Ethics Review Committee of the Graduate School of Medicine, Osaka University. Written informed consent was obtained from each patient included in the study.

TABLE 1 Clinicopathological features in 50 patients with cholangiocarcinoma (CCA)

	mean ± SD
Age (Y)	62.4 ± 12.5
Sex (males:females)	32:18
Hepatitis (negative:HBV:HCV:HBV + HCV)	35:9: 6:0
CEA (≤5:>5 ng/mL)	42:8
CA19-9 (≤37:>37 U/mL)	31:19
Operation time (min)	433.0 ± 207.4
Blood loss (mL)	1278.6 ± 1271.0
pT (1:2:3:4)	1:21: 21:7
Tumor size (mm)	49.6 ± 36.0
Tumor number (single:multiple)	41:9
Vascular invasion (no:yes)	32:18
pN (0:1)	36:14
UICC pStage (1:2:3:4a:4b)	23:5: 2:20: 0
Histological type (tub1:tub2:por:others)	4:29: 12:5
Recurrence-free survival time (mo)	30.5 ± 33.5
Overall survival time (mo)	40.8 ± 35.0
Median follow-up time (mo)	30.3

Abbreviations: CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; por, poorly differentiated adenocarcinoma; SD, standard deviation; tub1, well-differentiated adenocarcinoma; tub2, moderately differentiated adenocarcinoma; UICC, Union for International Cancer Control.

2.2 | Immunohistochemistry

Immunohistochemical studies for IL-33 was performed with 50 resected liver specimens for CCA (Table 1) and 49 paired resected liver specimens for HCC, as described previously.[19] In brief, formalin-fixed, paraffin-embedded tissue samples were deparaffinized, boiled for antigen retrieval, incubated with each specific antibody (anti–IL-33 antibodies: rabbit polyclonal, 1:500 dilution, Medical & Biological Laboratories Co.) for 1 hour at room temperature, and detected with avidin-biotin complex reagents (Vector Laboratory Inc) and diaminobenzidine. All sections were counterstained with hematoxylin. The magnitude of IL-33 expression was evaluated as the number of IL-33–positive cells in the resected liver tissue in

the noncancerous region. The number of IL-33–positive cells was counted manually in five randomly selected areas at 40X magnification. Patients were divided into two groups by the median number of IL-33–positive cells in the immunohistochemistry assay: high expression, n = 25 and low expression, n = 25.

2.3 | Enzyme-linked immunosorbent assays

The protein levels of IL-33 and IL-6 were quantitated using ELISA kits (R&D Systems), as described previously.[20] Twenty-four pairs of human plasma samples obtained before and after hepatectomy for CCA, six pairs of human plasma samples obtained before and after liver transplantation, and five pairs of murine plasma samples obtained before and after hepatectomy were collected, and 100 μL of each sample was assayed for the IL-33 protein. Frozen liver tissue samples from the noncancerous regions of mouse/human samples were homogenized with 600 μL of PBS and adjusted to 50 μL (corresponding to 2.5 mg of murine liver tissue)/100 μL (corresponding to 5 mg of human liver tissue) aliquots of the supernatant to assay for the IL-33 and IL-6 proteins.

2.4 | Cytokine array analysis of human plasma collected before and after hepatectomy

Cytokine profiles were determined with the Quantibody Human Inflammatory Array 1 (RayBiotech), which permits the detection of cytokines, including IL-1α, IL-1β, IL-4, IL-6, IL-13, MCP-1, IFN-γ, and TNFα, in a single procedure, and 24 pairs of plasma samples obtained before and after hepatectomy for CCA were evaluated according to the manufacturer's protocol. The relative fluorescence strength was detected with a LuxScan 10 K-A microarray scanner (CapitalBio Corporation). Actual protein concentrations were calculated with the corresponding standard curve plotted from data for standard controls incorporated into the array.

2.5 | Murine hepatectomy and a syngraft transplantation model with/without IL-33 neutralization

Male C57BL/6J mice at 6 weeks of age were included in the following experiments:

To assess the changes in IL-33 expression in murine liver/blood induced by hepatectomy, mouse hepatectomy and blood collection were conducted under deep anesthesia with intraperitoneal injections (i.p.) of pentobarbital (40-85 mg/kg, NACALAI TESQUE). The abdominal cavity was opened by a midline approach, and the single left lobe of the liver was resected. The abdominal wall and skin were closed in separate layers with absorbable chromic 3-0 gut sutures. For blood collection, tail vein sampling or a tail snip was conducted as appropriate. Murine blood samples were obtained before and after surgery under deep anesthesia, and they were centrifuged to collect the plasma. The mice were anesthetized with pentobarbital (i.p.) at 28 days after surgery, and terminal blood and the remnant liver were obtained. The blood obtained before surgery and the primary resected liver were assessed as control subjects.

To assess the change in IL-33 production in the murine liver induced by IL-33 exposure, one-time administration of recombinant mouse IL-33 (rmIL-33; 1 μg/mouse, i.p., R&D Systems) was performed. Mice received IL-33 (i.p.) under short-term isoflurane anesthesia. PBS injection was conducted as a control. The mice were anesthetized with pentobarbital (i.p.) at 72 hours after injection and euthanized before the whole liver and spleen were collected.

To evaluate the influence of hepatectomy on CCA growth, a murine orthotopic transplant experiment using syngrafted CCA cells was conducted. Murine CCA cells from C57BL/6J mice were kindly provided by Dr Gregory J. Gores of Mayo Clinic (Rochester, MN, USA).[17] For this experiment, mice underwent two laparotomy surgeries under deep anesthesia with pentobarbital. In the first surgery, hepatectomy was performed on the mice in the hepatectomy/hepatectomy + anti–IL-33 groups as described above. As a control, surgery without hepatectomy (only laparotomy as a sham surgery) was conducted in the mice in the laparotomy group. Seven days after the first surgery, a tumor suspension solution (1×10^6 cells in 30 μL of PBS) was gently injected into the remnant liver under laparotomy. After the injection into the liver, a sterile cotton-tipped applicator was held over the injection site for approximately one minute to prevent leakage, and the abdominal wall was closed. For the experiment assessing neutralization of IL-33 during hepatectomy, each animal was injected intraperitoneally with an anti–IL-33 antibody (soluble ST2 [IL-1 receptor like 1, IL-33 receptor] antibody, 3.6 μg/1 mL/mouse; R&D Systems, hepatectomy + anti–IL-33 group) or PBS (1 mL/mouse, control, hepatectomy group, laparotomy group) before the second surgery. Each drug, the anti–IL-33 antibody or PBS, was administered on the day before the first surgery, the day of the first surgery, and postoperative days 1, 3, and 5. The mice were anesthetized with pentobarbital (i.p.) at 28 days after the second surgery and euthanized to obtain the remnant/whole liver.

Detailed additional information is provided in the Appendix S1.

2.6 | Cell lines, culture, and materials

Human CCA cell lines (HuCCT-1 and CCLP-1) were kindly provided by Dr Gregory J. Gores of Mayo Clinic and were incubated as described previously.[21-23] CCA cells were seeded at 60% confluence and changed into serum-free medium 24 hours later for treatment experiments. Subsequently, the cell lines were treated with each solution as follows: recombinant human IL-33 (rhIL-33, 10 ng/mL; R&D Systems) and an anti–IL-6 antibody (1.5 μg/mL; R&D Systems). For the experiment using extracted protein from a resected human liver, frozen liver tissue from the noncancerous regions of samples collected from human CCA patients was

homogenized with 600 μL of PBS, and the final protein concentration was adjusted to 10 ng/ml. In all experiments, cells were harvested after 72 hours of exposure.

2.7 | Quantitative real-time polymerase chain reaction

Real-time quantitative polymerase chain reaction (qRT-PCR) was performed as described previously.[19] Briefly, total RNA was isolated from frozen liver/spleen tissue using the RNeasy Plus Mini Kit (Qiagen), and complementary DNA was synthesized from 2.0 μg of total RNA. Using a LightCycler-FastStart DNA Master SYBR Green I kit (Roche Applied Science) with gene-specific oligonucleotide primers (Table S1), amplifications were performed in triplicate. Relative expression was calculated as the ratio of the specific mRNA level to the endogenous β-actin mRNA level in each sample.

2.8 | Evaluation of malignant potency: proliferation, invasion, migration, and growth inhibition assays

A proliferation assay was performed with Cell Counting Kit-8 (Dojindo Molecular Technologies), as described previously.[24] In brief, the viable cell number was determined from the absorbance value. An invasion assay was performed with Transwell cell culture chambers (BD Biosciences), as described previously.[25] In brief, 1×10^5 cells were seeded in triplicate on a Matrigel-coated membrane. After 48 hours, the cells that had invaded the undersurface of the membrane were fixed with 100% methanol and stained with 1% toluidine blue. Four microscopic fields were randomly selected for cell counting. A migration assay was performed with cells that had been seeded at a density of 5×10^5 cells per well in 6-well plates. A scratch was made in the cell monolayer with a 200-μL pipette tip, and the cells were then cultured under standard conditions. Cell migration was evaluated by measuring the open area between the wound edges. Growth-inhibiting effects were tested using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as described previously.[26] Cells were incubated in medium supplemented with gemcitabine (GEM, 0.5 to 320 ng/mL) for 72 hours. The proportion of MTT-positive cells in cultures incubated without drugs was defined as 100% viability. These assays were repeated at least three times, and similar results were obtained each time.

2.9 | Statistical analyses

Kaplan-Meier analysis and the log-rank test were used to construct survival curves and evaluate differences in a univariate analysis. Logistic regression was performed for both a multivariate analysis and a partition analysis of the detected factors. The data are expressed as the mean ± standard deviation (SD) of at least three independent experiments. The chi-squared test and Student's *t*-test were used for comparing categorical variables, as appropriate. An unpaired Student's *t*-test was used to examine differences in growth-inhibiting effects in vitro. *P*-values < 0.05 were considered statistically significant. All statistical analyses were completed using the JMP 13.0 software program (SAS Institute).

3 | RESULTS

3.1 | IL-33 was released into the plasma from the liver during hepatectomy, and high expression of IL-33 in the liver was a significant risk factor for CCA recurrence

The IL-33 expression in the background liver (the noncancerous region) of resected CCA specimens varied widely (Figure 1A, B). Most IL-33–positive cells in resected liver tissue were localized in the liver stroma. The number of IL-33–positive cells was not associated with any tumor factors or patient background characteristics (Table 2). The amount of IL-33 released into the plasma following hepatectomy directly correlated with the background liver expression, as assessed by immunohistochemistry (Figure 1C). To assess the correlations between IL-33 and other inflammatory cytokines, a cytokine array was performed. No cytokines which were related to the number of IL-33–positive cells in the resected liver were found. (Figure S1). Furthermore, increases in the IL-33 level in the plasma were not observed with liver transplantation surgery, which did not involve liver injury during the surgical procedure (Figure 1C).

To assess IL-33 expression after hepatectomy, we compared secondary resected liver specimens with primary resected liver specimens. As re-resection was not performed for CCA recurrence, we investigated HCC livers and compared sets of primary and secondary resected liver samples in noncancerous regions. The median interval between primary and secondary surgery for HCC was 3.03 years. IL-33 expression was significantly increased in the secondary resected liver specimens compared with the primary resected liver sections (Figure 1D, E).

These findings suggested that hepatectomy for CCA induced the release of IL-33 into the plasma, with the amount released correlating with the number of IL-33–positive cells, and hepatectomy increased the population of IL-33–positive cells in the liver for several years. Thus, we hypothesized that the number of IL-33–positive cells is a risk factor for CCA recurrence.

When patients were divided into two groups according to their level of IL-33 expression by the median number of 35 cells/high-power field, the recurrence-free survival (RFS) of the group with high IL-33 expression was significantly shorter than that of the low IL-33 expression group (Figure 1F; median survival time for the high expression group vs. the low expression group, 13.2 vs. 40.3 months, $P = 0.014$). Furthermore, IL-33 expression was identified as a

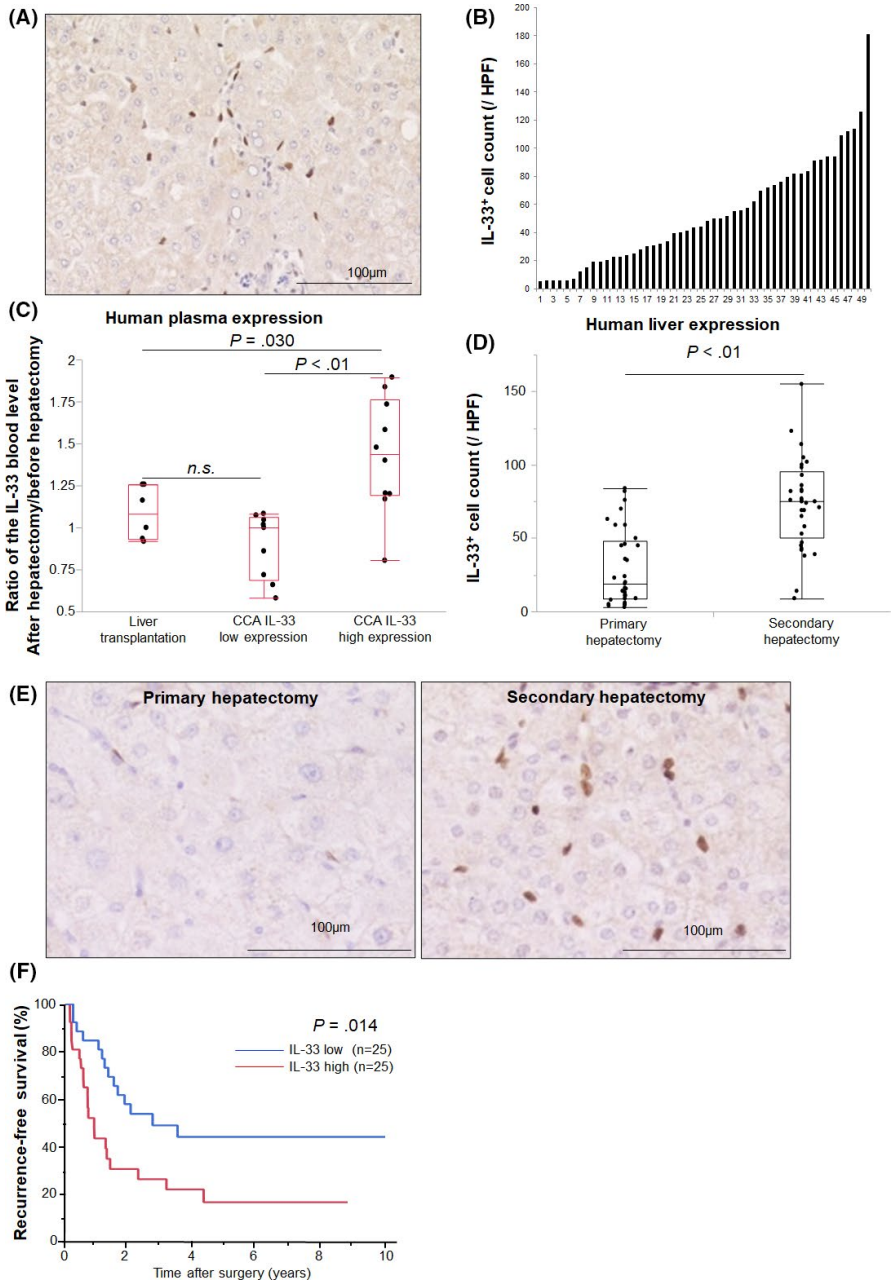


FIGURE 1 Hepatic interleukin-33 (IL-33) was released into the circulation during hepatectomy, increased IL-33 expression in the remnant liver for several years, and facilitated cholangiocarcinoma (CCA) recurrence. A, B The numbers of IL-33–positive cells in noncancerous regions of the liver of patients with CCA were evaluated. Representative immunohistochemical staining images (A) and the average numbers within five random high-power fields (HPFs) of each sample (B) are depicted. Scale bar, 100 μm. C, The ratio of cytokines in the blood was calculated by dividing the plasma value obtained just after surgery by that obtained just before surgery for each patient. The change in IL-33 expression in each patient is depicted. Patients undergoing liver transplantation in the same era are shown as controls because they suffered surgical trauma but did not undergo hepatectomy (liver injury). Patients undergoing hepatectomy for CCA were divided into two groups according to the median number of IL-33–positive cells determined in an immunohistochemistry assay: high expression, n = 25 and low expression, n = 25. D, E, Representative immunohistochemical staining images of IL-33 expression in noncancerous regions of the liver of patients with hepatocellular carcinoma (HCC) collected during primary or secondary resection. Scale bar, 100 μm. Boxplots of the average number of IL-33–positive cells within five random HPFs of each sample. F, Kaplan-Meier curves for recurrence-free survival. Patients were divided into two groups according to the median number of IL-33–positive cells determined in an immunohistochemistry assay: high expression, n = 25 and low expression, n = 25

TABLE 2 Clinicopathological features based on interleukin-33 (IL-33) expression

	IL-33 expression		
	High (n = 25)	Low (n = 25)	*P* value
Age (≤65:>65 y)	12:13	13:12	.7773
Sex (males:females)	12:13	20:5	.0169
Hepatitis (no:yes)	17:8	18:7	.7576
CEA (≤5:>5 ng/mL)	19:6	23:2	.1157
CA19-9 (≤37:>37 U/mL)	15:10	16:9	.7707
Operation time (≤500:>500 min)	17:8	12:13	.1504
Blood loss (≤1500:>1500 mL)	19:6	15:10	.2234
pT (1:2:3:4)	0:10: 11:4	1:11: 10:3	.6538
Tumor size (≤50:>50 mm)	17:8	19:6	.5282
Tumor number (single:multiple)	19:6	22:3	.2085
Vascular invasion (no:yes)	16:9	16:9	1.0000
pN (0:1)	18:7	18:7	1.0000
UICC pStage (1 + 2:3 + 4)	14:11	14:11	1.0000
Histological type (tub1 + tub2:por + others)	18:7	15:10	.3695

Abbreviations: CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; por, poorly differentiated adenocarcinoma; tub1, well-differentiated adenocarcinoma; tub2, moderately differentiated adenocarcinoma; UICC, Union for International Cancer Control.

significant risk factor for RFS in a multivariate analysis following a univariate analysis of 50 CCA patients (Table 3).

3.2 | Remnant liver after hepatectomy facilitated CCA growth in mice, and perioperative IL-33 blockade significantly ameliorated the effect of hepatectomy

To validate the clinical findings that hepatectomy induces the release of IL-33 from the liver and facilitates CCA progression, we performed a mouse experiment. Hepatectomy in C57BL/6J mice significantly increased the IL-33 level in the blood (Figure 2A), and IL-33 expression was significantly increased in the remnant liver tissue at 1 month after hepatectomy compared with the primary resected liver tissue (Figure 2B). To assess the change in IL-33 production in the murine liver induced by transient IL-33 exposure, one administration of rmIL-33 was performed. The rmIL-33 injection, mimicking the situation after hepatectomy, increased IL-33 mRNA expression in the murine liver after 72 hours (Figure S2A). To validate the influence of hepatectomy on CCA proliferation, orthotopic transplantation using a syngraft of CCA cells in C57BL/6 mice was conducted. In the experiment, tumor progression was facilitated in the remnant liver after hepatectomy (Figure 2C-E). To evaluate the effect of anti–IL-33 treatment on this outcome, anti–IL-33 antibody administration was performed in the murine model. Perioperative administration of the anti–IL-33 antibody significantly inhibited the elevation in the IL-33 level in the remnant liver (Figure 2F); furthermore, perioperative IL-33 blockade significantly ameliorated CCA development in the remnant liver after hepatectomy (Figure 2E).

3.3 | Homogenized human liver tissue containing many IL-33–expressing cells increased proliferation and facilitated migration in human CCA cells

To examine what type of cells the IL-33–positive cells were, we evaluated CD8 expression on IL-33–positive cells by using the available frozen sections because previous report demonstrated that IL-33–positive cells in liver tissue were a kind of CD8 + T cells, and their immunohistochemical findings for IL-33 were similar to our own.[16] Both human and murine liver tissue showed positivity to CD8 (Figure S3).

We examined a liver lysate solution to evaluate the influence of IL-33–positive cells on CCA cells. In response to culture with the homogenized liver solution containing many IL-33–positive cells, CCA cell lines exhibited significant increases in cell proliferation, migration, and invasion. However, no significant changes in GEM sensitivity were noted (Figure 3A-D).

3.4 | IL-6 is a key cytokine involved in CCA progression in liver tissue with a high number of IL-33–positive cells

We assessed the candidate cytokine IL-6 to elucidate the potential mechanism underlying IL-33–mediated tumor development because IL-33 is assumed to increase IL-6 production[27] and IL-6 is a well-known facilitator of CCA development.[17,21]

After hepatectomy, the liver in both humans and mice showed increased IL-6 production (Figure 4A, B), and the protein expression of IL-6 in the liver was significantly correlated with the number of IL-33–positive cells (Figure 4C). Furthermore, short-term exposure of mice that did not undergo hepatectomy to IL-33 induced IL-6 production in the liver (Figure S2A).

To assess the role of IL-6 in IL-33–mediated processes, CCA cells cultured with homogenized liver tissues containing many or few IL-33–positive cells were treated with an anti–IL-6 antibody. The anti–IL-6 treatment markedly reduced the effects on malignancy (migration, invasion, and proliferation) induced by the homogenized liver tissue containing many IL-33–positive cells, whereas no marked changes were noted in the experiment using samples extracted from a liver with fewer IL-33–positive cells (Figure 4D, E).

To assess the effects of IL-6 on IL-33–mediated tumor growth in the mouse models, we examined anti–IL-6 antibody treatment (Appendix S1). The administration of anti–IL-6 antibody inhibited

TABLE 3 Univariate and multivariate analyses of recurrence-free survival

	Univariate	Multivariate		
	P value	HR	95% CI	*P* value
Age (≤65:>65 y)	0.51			
Sex (males:females)	0.58			
Hepatitis (no:yes)	0.12			
CEA (≤5:>5 ng/mL)	<0.001	3.47	0.95-14.8	.060
CA19-9 (≤37:>37 U/mL)	0.56			
pT	<0.001			.0074
pT1/2	-			
pT3	0.31	1.21	0.51-2.87	.66
pT4	0.0010	6.18	1.96-18.8	.0025
Tumor size (≤50:>50 mm)	<0.001			NA
Tumor number (single:multiple)	<0.001			NA
Vascular invasion (no:yes)	<0.001			NA
pN (0:1)	0.010	1.34	0.38-3.89	.40
UICC pStage (1 + 2:3 + 4)	0.0022			NA
Histological type (tub1 + tub2:por + others)	0.30			
IL-33 (low:high)	0.014	2.19	1.01-4.85	.046

Abbreviations: CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CI, confidence interval; HR, hazard ratio; IL-33, interleukin-33; NA, not applicable; por, poorly differentiated adenocarcinoma; tub1, well-differentiated adenocarcinoma; tub2, moderately differentiated adenocarcinoma; UICC, Union for International Cancer Control.

tumor growth despite the lack of influence on the increase of IL-33–positive cells in the remnant liver (Figure S4).

4 | DISCUSSION

Hepatectomy for CCA is a potentially curative treatment;[3,7-11] however, the majority of patients develop recurrent disease.[8-10] Although liver transplantation as a curative option for CCA is controversial, some patients undergoing liver transplantation for CCA show a favorable prognosis.[3,28,29] We suspected that some cytokines are released during hepatectomy and exacerbate tumor development in CCA patients, and thus we focused on the alarmin IL-33. In our study, IL-33 was released by hepatectomy, with the amount released correlating with the number of IL-33–positive cells and significantly related to CCA recurrence. Additionally, there were no other inflammatory cytokines that were released by hepatectomy with the amount released correlating with the number of IL-33–positive cells and significantly related to CCA recurrence (data not shown). From our findings, human liver tissue with CCA/HCC basically maintained some IL-33–positive cells, and thus hepatectomy for CCA/HCC usually induced IL-33 release. However, the IL-33 level in the plasma of recipient patients did not increase during liver transplantation surgery despite the patients undergoing whole-liver resection. Although the resected whole liver from the recipient patients contained a certain number of IL-33–positive cells, the removal of the liver without hepatectomy (without liver injury) did not increase the IL-33 level in the plasma. These findings may suggest that blockade of IL-33 on hepatectomy perioperative days will improve the prognosis of CCA patients by decreasing CCA recurrence.

The mechanisms underlying CCA recurrence associated with IL-33 release induced by hepatectomy are of interest. Although the amount of released IL-33 in the plasma is reduced by degradation and does not remain high for long after surgery,[12,27,30] it has been reported that released IL-33 increases the number of cells expressing IL-33 to prepare for future events. McHedlidze et al reported that rmIL-33 was trapped in murine liver tissues by IL-33 exposure,[31] and Pichery et al reported that IL-33 induced an increase in IL-33 production in liver tissues via inflammation related to IL-33.[31,32] In our study, both human and murine livers exhibited an increased number of IL-33–positive cells in the remnant liver after hepatectomy, and transient IL-33 administration increased IL-33 production in the murine liver. Thus, we considered that the cells constitutively expressing IL-33 in the remnant liver maintained conditions that facilitated the development of CCA recurrence, and we assessed liver lysate solutions to evaluate the influence of IL-33–positive cells on CCA cells.

A liver lysate solution including a high number of IL-33–positive cells increased the proliferation and migration of CCA cells; however, the role of the cells that constitutively produce IL-33 in the liver has been unclear. Current thinking holds that IL-33–positive cells may induce an increase in the levels of inflammatory cytokines, including

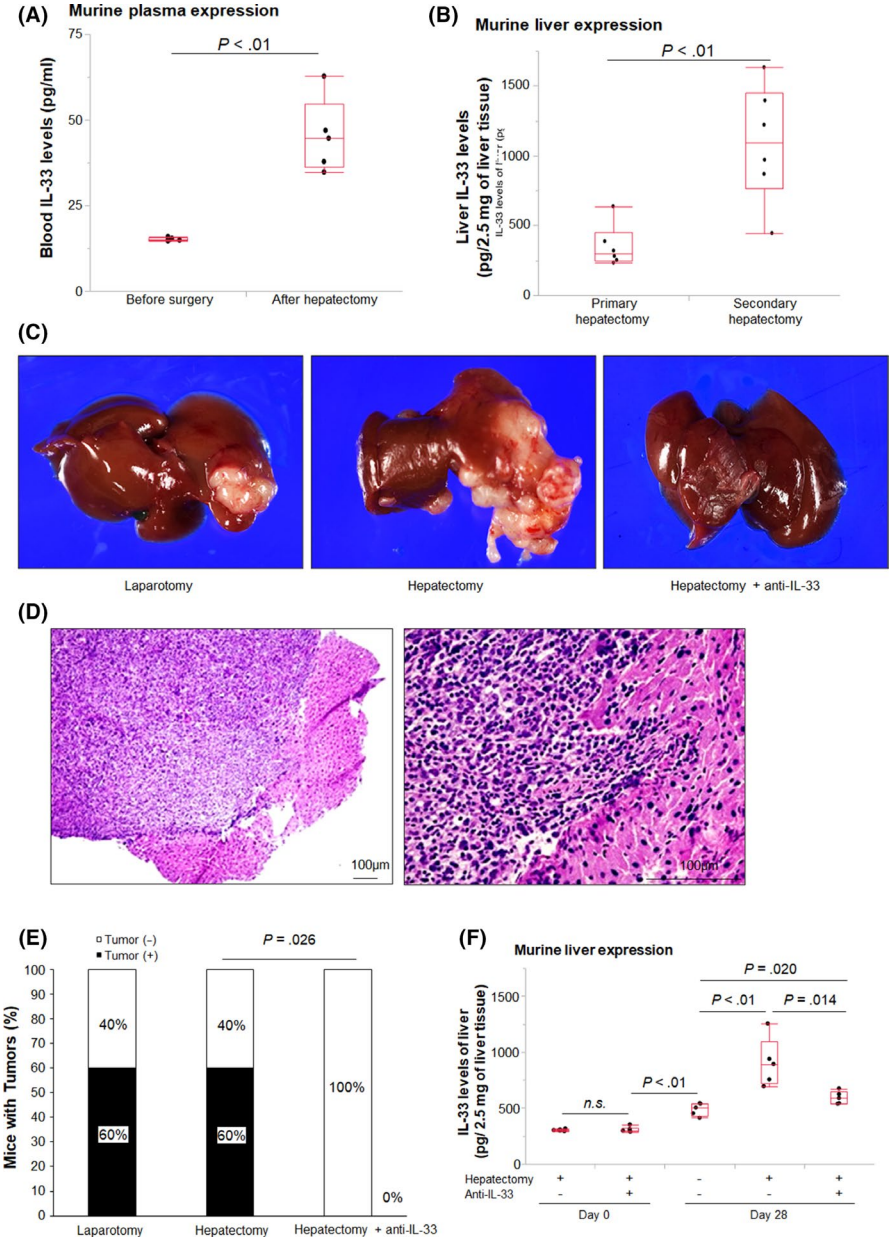


FIGURE 2 The effect of interleukin-33 (IL-33) neutralization on a murine hepatectomy and syngraft transplantation model. A, B, Hepatic IL-33 was released into the circulation during hepatectomy and increased IL-33 expression in the remnant liver in mice. A, IL-33 protein expression in the murine plasma. The plasma was collected before and after hepatectomy (n = 6). B, IL-33 protein expression in 2.5 mg of murine liver lysate. The lysate was extracted from the liver at the first laparotomy and from the remnant liver collected 28 d after hepatectomy (n = 6). C-F, IL-33 blockade dramatically inhibited cholangiocarcinoma (CCA) development in the remnant murine liver after hepatectomy. In a syngraft transplantation experiment, 16 mice were divided into three groups: laparotomy (n = 5, a tumor solution was injected into the liver after laparotomy), hepatectomy (n = 5, the tumor solution was injected into the liver after laparotomy, followed by administration of PBS), and hepatectomy + anti-IL-33 treatment (n = 6, the tumor solution was injected into the liver after laparotomy, followed by administration of an anti–IL-33 antibody). C, Liver appearances of mice at 8 w after syngraft transplantation of murine CCA cells. D, A representative photomicrograph of hematoxylin and eosin-stained sections of murine CCA (peripheral region) tissue at 8 w after transplantation. The right panel shows a high-power field view of the tumor that is shown in the left panel. The tumor findings are similar to those for human CCA. E, Percentage of animals with tumors. F, A comparison of IL-33 expression in 2.5 mg of murine liver lysate. The lysate was extracted from the liver tissue collected at the first laparotomy and the remnant liver collected at 7 d after hepatectomy

IL-6.[2,12,17,33-43] We therefore focused on the IL-33–relevant cytokine IL-6 because IL-6 is also a well-known facilitator of CCA.[44,45] Indeed, anti–IL-6 treatment ameliorated the effect of the liver lysate solution containing a large population of IL-33–positive cells, and the administration of anti–IL-6 antibody inhibited tumor growth in murine hepatectomy and syngraft transplantation models. Thus, we suspect that IL-6 is a dominant cytokine in this mechanism, and the cells constitutively expressing IL-33 in the remnant liver establish conditions that facilitate the development of CCA recurrence with increasing IL-6 expression.

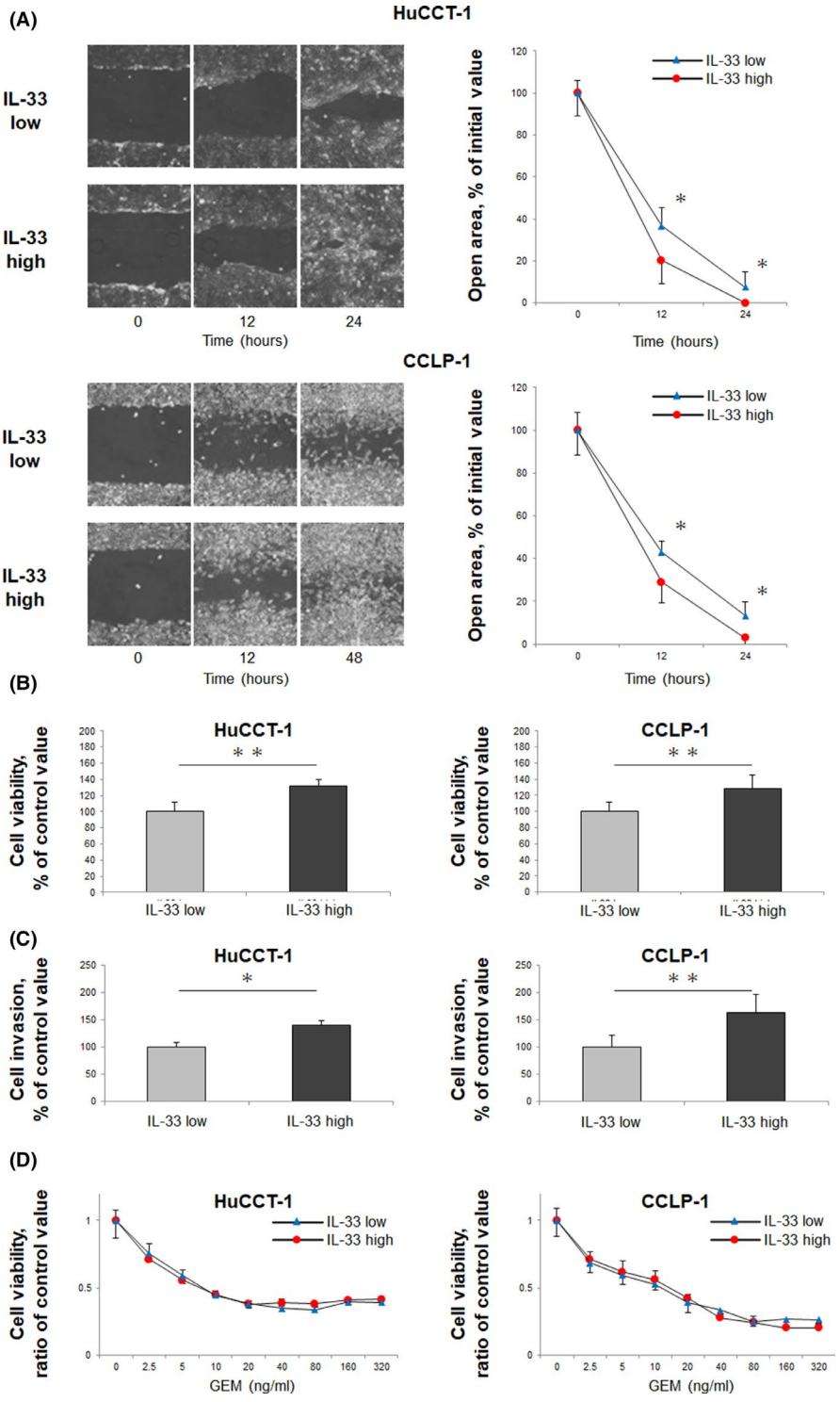


FIGURE 3 Factors from liver lysates expressing substantial amounts of IL-33 increased cell migration, proliferation, and invasion. HuCCT-1 and CCLP-1 cells were cultured with lysates from a pair of liver samples resected from the same person. The liver sample collected during the first surgery was used for the extraction of an "IL-33 low" lysate, whereas the sample collected during the second surgery from the same patient was used for extraction of an "IL-33 high" lysate. Experiments were performed in triplicate and repeated three times with similar results. Values are presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. A, Representative time-lapse images of wound-healing assay treatment groups and the calculated percentage of the open area. B, Cell viability is reported as the percentage of the control value, and cells were treated for 72 h. The viability of cells cultured with the "IL-33 low" lysate was set as the control value. C, Changes in the number of invaded cells, with the control group used as the comparator. The number of cells cultured with the "IL-33 low" lysate was set as the control value. D, Ratios of cell viability after treatment with each gemcitabine (GEM) concentration for 72 h

Supporting the above findings, short-term blockade of IL-33 in mice dramatically reduced the risk of CCA development for 1 month by inhibiting the transient IL-33 elevation that followed hepatectomy. Although the efficacy of soluble ST2 administration (which works as an antagonist for IL-33 blockade) in humans has not yet been established, our findings thus far suggest that perioperative ST2 administration to CCA patients may provide a promising clinical effect on patient outcomes.

It was regarded that IL-33 promotes cancer-associated inflammation, tumor progression, and metastasis in many cancer types. In breast cancer, lung cancer, pancreatic cancer, gastric cancer, and colorectal cancer, IL-33 was reported as a possible mediator of carcinogenesis or of tumor progression.[46-50] The influence of IL-33 on biliary tract cancer has been consistently reported. Jorge Bezerra et al had demonstrated that IL-33 is a biliary mitogen, and it was reported that systemic IL-33 administration facilitated CCA

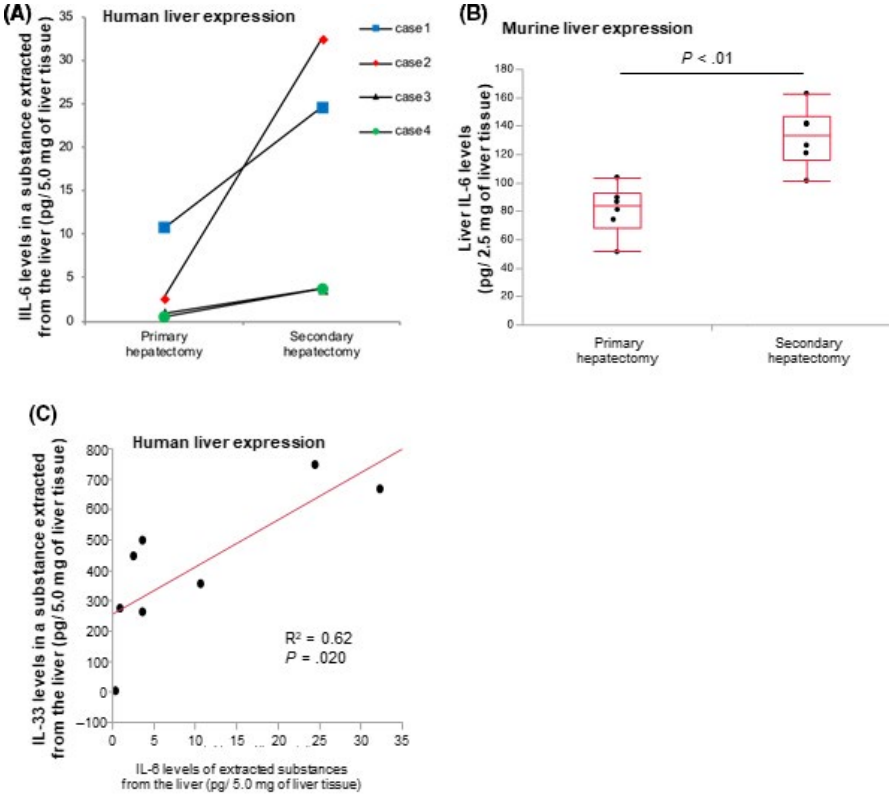
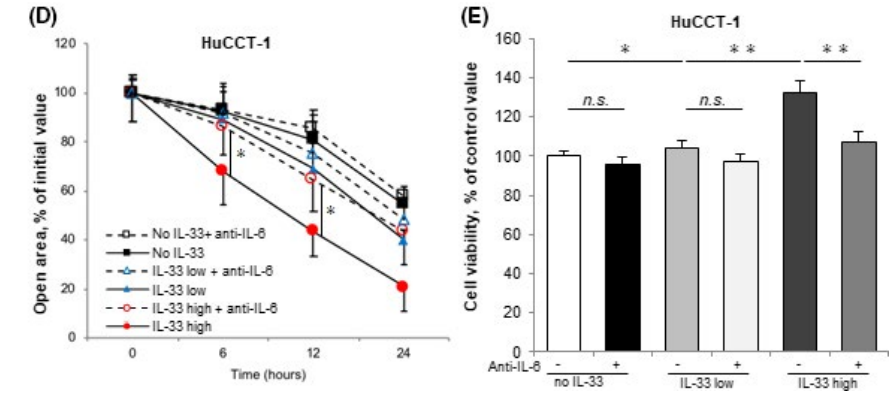


FIGURE 4 The relationships between interleukin-6 (IL-6) expression and hepatectomy/IL-33. A, B, The expression of the IL-6 protein in 5.0 mg of human (A) or 2.5 mg of murine (B) liver lysate. A, Lysates were extracted from the liver tissue collected during the first and second hepatectomies. B, In the murine model, the samples obtained during the first laparotomy and the remnant liver at 28 d after hepatectomy were evaluated (n = 6). C, The results of a correlation analysis of the protein expression of IL-6 and IL-33 in 5.0 mg of human liver lysate. D, Change in the percentage of the open area in a wound-healing assay for each treatment. E, Cell viability as a percentage of the control value. Cells were treated for 72 h. The viability of cells treated with PBS was set as the control value. Experiments were performed in triplicate and repeated three times with similar results. D, E, Each value is shown as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$



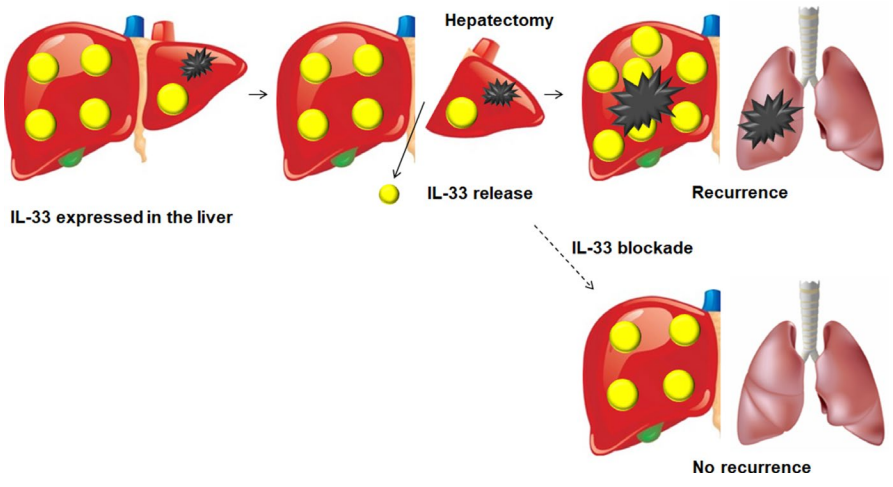


FIGURE 5 The role of interleukin-33 (IL-33) in cholangiocarcinoma (CCA) recurrence. Hepatic IL-33 is released during hepatectomy, increasing hepatic IL-33 expression in the remnant liver and facilitating CCA recurrence following surgery by changing the environment. Our results suggest that anti–IL-33 therapy during hepatectomy reduces the risk of CCA recurrence following surgery

carcinogenesis from 20% to 70% in a mouse model developing CCA by using oncogene transfection.[17,18] Furthermore, a recent study reported that IL-33 mediated extrahepatic CCA development.[51] By contrast, the reported influence of IL-33 on HCC is controversial.[52-55] Dominik Bergis et al demonstrated that the serum level of IL-33 did not differ among the patients with HCC or liver cirrhosis (LC), whereas sST2 (IL-33 receptor) was significantly higher in the patients with HCC than in those with LC,[54] indicating that the sensitivity of IL-33 was affected by the condition of the background liver. We suspected that the degree of infection in the background liver might affect the results concerning IL-33 effect, and thus patients with CCA in LC (eg primary sclerosing cholangitis) might show different sensitivity for released IL-33.

In the present study, we had several limitations. We did not evaluate the detail of what type of cells the IL-33–positive cells were. Brunner et al found that IL-33–positive cells in liver tissue were a kind of T cells, their immunohistochemical findings for IL-33 were similar to our own,[16] and IL-33 administration to mice increased both IL-33 and IL-6 production in the spleen as well (Figure S2B). We thus suspected that the cells producing IL-33 in the liver were lymphocytes, although further experiments are needed. To confirm whether IL-6 is the most dominant cytokine in the underlying mechanisms associated with IL-33–positive cells in the remnant liver, we intend to perform additional cytokine arrays. We did not assess whether the cells expressing IL-33 regulated the environment by modulating the immune response, but we are planning future experiments to address this point.

We concluded that surgery for CCA with curative intent paradoxically released IL-33, which facilitated CCA recurrence, and anti–IL-33 therapy during hepatectomy might reduce the risk of CCA recurrence (Figure 5).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest related to this article.

ETHICAL APPROVAL

The use of resected samples was approved by the Human Ethics Review Committee of the Graduate School of Medicine, Osaka University (17 494).

CONSENT TO PARTICIPATE

All animal experiments were performed in accordance with the Regulations on Animal Experimentation at Osaka University.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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