Effect of bevacizumab plus XELOX (CapeOX) chemotherapy on the liver natural killer cell activity in colorectal cancer with resectable liver metastasis

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ABSTRACT

Aim: We aimed to investigate chemotherapy effect of resectable colorectal cancer with liver metastasis (CRLM) on the functions of intrahepatic immune cells.

Methods: We classified patients into adjuvant chemotherapy (bevacizumab+CapeOX) after hepatectomy group (group A) and neoadjuvant chemotherapy followed by hepatectomy group (group B), and collected peripheral blood mononuclear cells (PBMCs) and liver mononuclear cells (LMNCs) to ascertain phenotypic and functional differences.

Results: There were no significant differences in lymphocyte fractions of either PBMCs or LMNCs between groups, except for the significantly lower percentage of natural killer (NK) cells in LMNCs in group B than in group A. Significantly higher percentage of NKG2D-positive NK cells in PBMCs, and percentage of TRAIL-, NKp30-, and SIRPβ-positive NK cells in LMNCs were found in group B. Furthermore, significantly higher expression of NKG2D and SIRPβ in peripheral blood NK cells and of NKp46 and CD122 in liver NK cells were found in group B. When LMNCs were incubated with IL-2 in vitro, no difference was observed in the expression of these molecules in NK cells between groups. Consistently, there was no difference in the cytotoxic activity of those LMNCs against a colon adenocarcinoma cell line between groups.

Conclusion: CRLM patients treated with neoadjuvant chemotherapy exhibited enhanced
expression of activation markers on peripheral blood and liver NK cells in comparison with patients who did not receive therapy; however, the difference in those function remains unclear. These results suggest that neoadjuvant chemotherapy does not have a negative impact on intrahepatic immune cells in resectable CRLM patients.

**KEYWORDS:**

Chemotherapy, colorectal cancer with liver metastasis, liver immunity, natural killer cells
INTRODUCTION

The primary clinical complication of colorectal cancer (CRC) is the invasion of tumor cells into distant organs and outgrowth of metastases. CRC with liver metastasis (CRLM) is a major prognostic factor for CRC patients (1, 2). The disease-specific mortality of progressive CRC has decreased significantly in past decades with novel chemotherapeutic agents, such as oxaliplatin and irinotecan, and molecular-targeted drugs, such as bevacizumab, a recombinant humanized monoclonal antibody that targets vascular endothelial growth factor (VEGF) (3). Adding bevacizumab to chemotherapy significantly reduces residual viable tumor cell volume in resected tumors and increases the proportion of patients eligible for liver metastasis resection, when compared with chemotherapy alone (4, 5).

Despite recent advancements in chemotherapy strategies for the treatment of advanced CRC patients, surgical resection of CRLM has been established as the treatment of choice and is the most effective and potentially curative therapy (6-10). Furthermore, surgical resection for CRLM combined with systemic adjuvant chemotherapy has a potential benefit to be curative for CRLM patients (11, 12). Meanwhile, the advantage of neoadjuvant chemotherapy in initially resectable CRLM patients is the treatment of undetected distant micrometastasis, thereby reducing recurrence risk after surgery (13). Although various studies have reported on the treatment strategy in resectable CRLM patients, the optimal treatment sequence remains
unclear.

Considering the impact of both surgical resection and chemotherapy on the host immunity is imperative in the treatment of resectable CRLM. Immune systems surrounding cancer cells are known to play crucial roles in regulating cancer cell proliferation, invasion, and metastasis through immunosurveillance (14, 15). Our group and other researchers have demonstrated that the decreased activity of immune cells in the liver after hepatectomy leads to tumor growth in mice (16, 17). While the influence of surgery on immune-surveillance against cancer cells has been occasionally investigated, the influence of chemotherapy on that remains to be elucidated. Studies have reported that the presence of tumor-infiltrating immune cells, such as natural killer (NK) cells and T cells, in CRLM patients improved the overall survival (OS) (6, 18). NK cells are part of the innate immune system and may provide a first line of defense against neoplastic cells by exerting an effector function without the necessity for priming (19). In addition, NK cells are abundant in human liver, and liver NK cells have remarkably higher cytotoxic activity against neoplastic cells than peripheral blood NK cells (20, 21). Such unique anatomical distribution and functional property of NK cells in the liver prompt us to investigate the influence of chemotherapy, particularly neoadjuvant chemotherapy, on the immunity of NK cells in the liver of CRLM patients.

In clinical setting, we are conducting a phase II/III randomized clinical trial in Hiroshima Surgical Study Group of Clinical Oncology (HiSCO) to determine whether neoadjuvant
chemotherapy followed by hepatectomy is superior to adjuvant chemotherapy after hepatectomy in resectable CRLM patients regarding progression-free survival (PFS), OS, and time to treatment failure. Since this clinical trial provided a good opportunity to obtain samples from resectable CRLM patients, who were either previously exposed or not exposed to chemotherapy, we additionally but separately designed an independent study to investigate the effect of neoadjuvant chemotherapy in resectable CRLM patients on liver NK cell activity as subanalysis of a clinical trial.
MATERIALS AND METHODS

Study Design and Procedures

We have been conducting a phase II/III randomized clinical trial in HiSCO (Hiroshima, Japan) registered with national review board (HiSCO-01, University Hospital Medical Information [UMIN] 00000378) to elucidate whether the neoadjuvant chemotherapy, bevacizumab combined with XELOX (CapeOX: capecitabine plus oxaliplatin), followed by hepatectomy is superior to adjuvant chemotherapy after hepatectomy in resectable CRLM patients. From June 2010, a planned cohort of 260 macroscopically-resectable CRLM patients who fulfilled the inclusion criteria (Table 1) were randomly assigned to either an adjuvant chemotherapy (eight courses of bevacizumab 7.5 mg/kg with capecitabine 2000 mg/m² plus oxaliplatin 130 mg/m²) after hepatectomy group (group A) or neoadjuvant chemotherapy followed by hepatectomy group (group B) based on the discretion of the reference physician in the HiSCO group. In group A, adjuvant chemotherapy was administered within 8 weeks after hepatectomy. CapeOX therapy was initiated, of which eight courses (each lasting 3 weeks) were administered. Bevacizumab was administered in the second and subsequent courses. In group B, neoadjuvant chemotherapy comprised eight courses of bevacizumab plus CapeOX therapy and each course lasted for 3 weeks. Bevacizumab was withdrawn in the final course. Surgical resection was performed within 2
and 8 weeks after the completion of neoadjuvant chemotherapy.

This study was additionally designed as subanalysis of a randomized clinical trial in HiSCO-01 to investigate the effect of chemotherapy on the functions of intrahepatic immune cells in resectable CRLM patients. From February 2011, we conducted this additional study in 30 consecutive patients. The number of samples was determined on the basis of our previous study, which demonstrated the relationship between clinical pathology and activation status of liver and peripheral blood NK cells (22, 23). We calculated sample size to detect the difference among percentage positive NK cells under the assumption that expected difference 20%, SD 15%, alpha level 5% and 80% power, and it was 10 for each group. Assuming a dropout rate of 33%, target sample size was set as 15 for each group. We obtained clinical data, including patients’ characteristics, and data obtained by analysis for comparative assessment. The research was conducted in compliance with the Declaration of Helsinki published by the World Medical Association and the Ethical Guidelines for Clinical Research published by Ministry of Health, Labor, and Welfare, Japan. In addition, this study was approved by the Institutional Review Board (IRB) of all institutions participating in this study. We obtained written informed consent from all patients before enrollment.

Collection of Mononuclear Cells

We analyzed the contents of immune cell and phenotype using peripheral blood
mononuclear cells (PBMCs) and liver mononuclear cells (LMNCs) obtained from eligible patients in both groups. We collected blood samples at the time of hepatectomy, and PBMCs were isolated by gradient centrifugation with Separate-L (Muto Pure Chemicals Co., Ltd, Tokyo, Japan) from 40 mL heparinized peripheral blood. In addition, LMNCs were obtained by \textit{ex vivo} perfusion through the portal vein of resected livers from CRLM patients as previously described (20). Effluents were condensed by centrifugation, and LMNCs were isolated by gradient centrifugation with Separate-L.

\textit{Flow Cytometric Analyses}

We performed flow cytometric (FCM) analyses using FACSCalibur cytometer (BD Biosciences, San Jose, CA) and FlowJo 7.6.5 software (TreeStar Inc., Ashland, OR). Based on a previous study (23), the monoclonal antibodies (mAbs) used for surface staining of lymphocytes to assess the phenotypic properties of NK cells were as follows: fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (HIT3a), anti-CD56 (B159), anti-CD19 (HIB19); phycoerythrin (PE)-conjugated anti-NKp30 (p30-15), anti-NKp46 (9E2), anti-CD122 (Mik-β3), and anti-CD56 (B159), anti-CD11b (ICRF44); and allophycocyanin (APC)-conjugated anti-CD3 (HIT3a), anti-natural-killer group 2, member D (NKG2D; 1D11), purchased from Becton Dickinson (San Jose, CA); PE-conjugated antitumor necrosis factor-related apoptosis-inducing ligand (TRAIL; RIK-2; eBioscience, Santa Clara, CA); and
PE-conjugated anti-signal regulatory protein β (SIRPβ; B4B; BioLegend, San Diego, CA).

Mouse IgG1κ was used as an isotype-matched control. Dead cells were excluded from the analysis by light scatter analysis and propidium iodide staining.

**Cytotoxicity Assays**

LMNCs were suspended in DMEM medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Sanko Chemical Co., Tokyo, Japan), 25 mM HEPES buffer (Gibco), 50 µM mercaptoethanol (Katayama Chemical Co., Osaka, Japan), 50 U/mL penicillin, and 50 µg/mL streptomycin (Gibco). In addition, LMNCs were used for phenotypic analyses and cultured with or without human recombinant interleukin (IL)-2 (100 Japanese reference U/mL; Takeda, Tokyo, Japan) for 3 days at 37°C in 5% CO2. Cultured cells were harvested and used for phenotypic analyses and cytotoxicity assays, which were performed using FACSARiaII (BD Biosciences) and FlowJo 7.6.5 software. Furthermore, we used DLD-1 cells (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan), established from a colon adenocarcinoma cell line (Dukes type C), as target cells. The FCM assay was performed to evaluate cell-mediated cytotoxicity, as described previously (24) with minor modifications. DLD-1 cells were labeled with PKH using the PKH26 Fluorescent Cell Linker Kits (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instructions. PKH-labeled target cells (1 × 10⁵) and prepared effector cells were added to a total of 200 µL
in a round-bottom, 96-well microtiter plates (BD Falcon, San Diego, CA) in duplicate wells. After 4-h incubation, cells were harvested and stained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). As a control, target cells were incubated in culture medium alone to determine spontaneous cell death. The number of PKH-labeled target cells that were killed was determined by analyzing PKH and DAPI double-positive cells using FCM. The cytotoxicity percentage was calculated using the following equation:

\[
\text{% cytotoxicity} = \left[ \left( \% \text{ experimental PKH}^+ \text{ DAPI}^+ \text{ targets} \right) - \left( \% \text{ spontaneous PKH}^+ \text{ DAPI}^+ \text{ targets} \right) \right] / \left[ 100 - \left( \% \text{ spontaneous PKH}^+ \text{ DAPI}^+ \text{ targets} \right) \right] \times 100.
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**Real-time Polymerase Chain Reaction**

Total RNA was isolated from resected tumor tissues of CRLM using RNeasy Mini kit (Qiagen, Limburg, the Netherlands) and reverse-transcribed using ReverTra Ace qPCR RT Kit (Toyobo Life Science, Tokyo, Japan) according to the manufacturer’s instructions. We determined the relative copy numbers of NKG2D ligands, such as MHC class I polypeptide-related sequence A (MICA), MHC class I polypeptide-related sequence B (MICB), and UL16-binding protein 2 (ULBP2), by real-time polymerase chain reaction (PCR) using NKG2D ligand-specific primer pairs and normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). We amplified the resulting cDNA with Rotor-Gene 3000 and Rotor-Gene SYBR Green PCR Kits (Qiagen) and analyzed data...
using ΔCt method for relative quantification. We performed all RT-PCR experiments using 2 µL cDNA with the following cycling parameters: 40 cycles at 95°C for 5 s and 60°C for 10 s. The following primers were used: MICA, 5′ -CCTTGCCATGAACGTCAGG-3′ (forward) and 5′ -CCTCTGAGCCTCGCTGCG-3′ (reverse); MICB, 5′ -ACCTTGCTATGAACGTCACA-3′ (forward) and 5′ -CCCTCTGAGACCTCGCTGCA-3′ (reverse); ULBP2, 5′ -CAGAGCAACTGCGTGACATT-3′ (forward) and 5′ -CATGCCCATCAAGAAGTCCT-3′ (reverse); and GAPDH (used as an internal control), 5′ -CAACGGATTTGGTCTGTAATGG-3′ (forward) and 5′ -CCATGGGTGGAATCATATTGG-3′ (reverse).

Image-Confirmed and Pathological Chemotherapy Response Rates

We evaluated the image-confirmed chemotherapy response rate according to the response evaluation criteria in solid tumors (RECIST v1.1) (25) as follows: complete response (CR), partial response (PR), progressive disease (PD), and stable disease (SD). Furthermore, the pathological chemotherapy response rate was defined according to the criteria of the Japanese Society for Cancer of the Colon and Rectum (JSCCR) (10) as follows: grade 0, grade 1a, grade 1b, grade 2, and grade 3. In this study, the pathologist randomly selected CRLM slides stained with hematoxylin-eosin, and evaluated chemotherapy effectiveness without
knowledge about clinical history of the patients.

Statistical Analysis

Data are presented as the mean ± standard deviation (SD). We performed statistical analyses using JMP 11 for Windows (SAS Institute, Inc., Cary, NC). Statistical significance of the differences observed between groups was evaluated by Mann-Whitney U-test and ANOVA analysis with Scheffe F test. $P < 0.05$ was considered statistically significant.
RESULTS

Patient Demographics and Clinicopathological Characteristics

In this prospective open-label study, we enrolled CRLM patients previously untreated with chemotherapy. In group B, 11 of 15 patients were evaluable for preoperatively administered bevacizumab combined with CapeOX at the time of sampling. In group A, 15 patients were used as controls without the influence of chemotherapy. The differences in the number of cases between these both groups were the exclusion of some cases because of complications of chemotherapy, treatment refusal, and another carcinoma occurrence. Table 2 summarizes patients’ characteristics in this study. No significant differences existed in patients’ characteristics, including sex, age, synchronous/metachronous, location of primary tumor, clinical stage, tumor differentiation, surgical procedure for hepatectomy, mean resected liver weight, and count of intrahepatic immune cells collected by perfusion between these groups. Synchronous liver resection with primary resection was performed in one patient in Group A (1/10) and another patient in Group B (1/5) with synchronous metastasis. These patients showed no characteristic findings.

Neoadjuvant Chemotherapy did not Affect Cell Numbers or the Proportion of Lymphocytes in PBMCs and LMNCs, Except for the Proportion of Liver NK Cells
A comparison of the numbers of PBMCs, lymphocytes, and monocytes revealed no significant differences between groups A and B (Fig. 1a, c, e), and no significant differences were observed between the two groups in terms of the numbers of LMNCs, lymphocytes, and monocytes collected during liver perfusion from resected livers (Fig. 1b, d, f). We compared proportion of NK cells, NKT cells, T cells, and B cells in both PBMCs and LMNCs between both groups to analyze the effect of neoadjuvant chemotherapy on lymphocyte populations. No significant differences were found in the proportion of NK cells in PBMCs (Fig. 1g). However, the proportion of NK cells in LMNCs was significantly lower in group B than in group A (17.1 ± 10.0 versus 28.2% ± 12.2%; \( P = 0.03 \); Fig. 1h). The proportion of NKT cells, T cells, and B cells in both PBMCs and LMNCs did not differ significantly between two groups (Fig. 1i-n). Further, we evaluated the correlation of NK and T cells to examine the relationship between innate and adaptive immunities. In both groups, a positive correlation was noted between the number of peripheral blood NK cells and that of T cells. Moreover, although there was a positive correlation between the number of liver NK cells and that of T cells in group B, this was not observed in group A (Supplemental Figure S1).

**Potential Augmentation of the NK Cell Activity in the Liver of Patients Receiving Neoadjuvant Chemotherapy Followed by Hepatectomy**

We assessed the phenotypic differences in NK cells, which play a pivotal role in tumor
surveillance, to investigate the effect of neoadjuvant chemotherapy on the innate-immune system in CRLM patients. The mean fluorescent intensity (MFI) of NKG2D and SIRPβ, which are associated with the small transmembrane adapter protein DAP12; transduce stimulatory signals (26); and are expressed on activated NK cells (27) on peripheral blood NK cells, was significantly higher in group B than in group A ($P = 0.03$ and $P = 0.04$, respectively; Fig. 2a). The proportion of NKG2D-positive NK cells was also significantly elevated in PBMCs from group B compared with group A ($P = 0.04$; Fig. 2b). The proportion of TRAIL-positive NK cells significantly increased in LMNCs from group B compared with that from group A ($P = 0.03$; Fig. 2d). Reportedly, TRAIL can induce either apoptosis by Fas-associated death domain-dependent mechanism or necrosis through receptor-interactive peptide-dependent cascade through the ligation of its death domain-containing receptors under physiological conditions (28). MFI of NKp46 and CD122, known as IL-2Rβ, on liver NK cells was significantly higher in Group B than in group A ($P = 0.04$ and $P = 0.03$, respectively; Fig. 2c). Proportion of NKp30 and SIRPβ-positive NK cells significantly increased in LMNCs from group B compared with LMNCs from group A ($P = 0.04$ and $P = 0.02$, respectively; Fig. 2d). Overall, the expression of cytotoxic and activation molecules in peripheral blood and liver NK cells tended to be enhanced in group B compared with group A. Further, we confirmed whether the adverse events of neoadjuvant chemotherapy is involved in the functions of NK cells. Notably, in group B, there was no difference in terms of the
expression of surface molecules on NK cells between cases with myelosuppression (n = 4) and those without myelosuppression (n = 7) (Supplemental Figure S2).

Then, we investigated the influence of the cytotoxic activity of LMNCs against DLD-1 CRC cells. We stimulated LMNCs obtained from each group with IL-2 and evaluated phenotypic alterations in these cells using the FCM assay. Of note, IL-2-stimulated LMNCs were used as effector cells rather than freshly isolated LMNCs because of limited level of cytotoxic activity of freshly isolated LMNCs (data not shown). The IL-2 stimulation increased the expression of activated molecules such as TRAIL, NKp30, NKp46, and NKG2D, on liver NK cells compared to the naive condition in both groups, and the remarkable difference in the expression levels of surface molecule in IL-2-stimulated LMNCs from both groups was no longer observed (Fig. 3a, b). Consequently, the cytotoxic activity of IL-2-stimulated LMNCs obtained from both groups showed no significant differences (Fig. 3c).

*Neoadjuvant Chemotherapy did not Affect the Expression Levels of NKG2D Ligands in Liver Tumors.*

We then evaluated the influence of chemotherapy on the expression of tumor-specific antigens that were theoretically recognized by NK cells on tumors from CRLM patients. The ligands for NKG2D comprised human class I-like molecules MICA, MICB, and ULBPs, which are stress-induced molecules expressed by tumors of epithelial origin and activate the
NK cell cytotoxicity through their NKG2D receptor (29, 30). Although we performed immunohistochemistry using frozen sections as a preliminary experiment, the evaluation of the expression of MICA, MICB, and ULBP2 in tumor tissue was challenging. Hence, we examined the expression levels of these molecules in liver specimens from patients in each group by RT-PCR. Ligands for NKG2D were evaluable in 13 of 15 samples in group A and 5 of 11 samples in group B. However, we could not perform complete sequencing on other samples because of a lack of DNA or degradation of DNA. The difficulty in extracting DNA from liver tissue because of tumor necrosis accounted for small number of samples in group B. Furthermore, although we could not exclude the possibility of inconsistencies arising from this limitation, no significant differences were observed in expression levels of these three targets between two groups (Fig. 4).

*No Association between NK Cell Activation Marker Expression and Clinically Evaluated Chemotherapy Response Rate*

To investigate the effect of neoadjuvant chemotherapy on tumor shrinkage, we assessed the correlation between NK cell surface activation markers, such as TRAIL, NKp30, NKp46, NKG2D, CD122, and SIRPβ, in PBMCs and LMNCs and the chemotherapy response clinically evaluated using RECIST or the histological treatment response (grade classification) were studied in patients receiving neoadjuvant chemotherapy followed by
hepatectomy. The evaluation of RECIST in 11 patients revealed no CR cases, whereas there were 6 PR cases (54.6%), 4 SD cases (36.4%), and 1 PD case (9.0%). Four cases were classified as grade 1a (36.4%), 2 cases as grade 1b (18.2%), 0 case as grade 2a (0%), 4 cases as grade 2b (36.4%) and 1 case as grade 3 (9.0%). Despite confirming the treatment effect in various cases, we observed no significant correlation between the expression levels of each activated molecule in NK cells and image-confirmed or pathological chemotherapy response rates (Fig. 5).
DISCUSSION

The effect of chemotherapeutic agents on the immune cell function is considered to be involved in the immunosuppression associated with myelosuppression or drug cytotoxicity (31). However, the immune cell function, particularly regarding liver immunity at the local anatomical site with distant CRC metastasis, during chemotherapy, remains unclear. To resolve this clinical question, we separately designed a subanalysis study of a phase II/III randomized clinical trial, which was conducted by the HiSCO group, to investigate whether chemotherapeutic or surgical precedence as a primary treatment has a clinical advantage for treatment of resectable CRLM patients.

To date, several studies have reported correlations between immunosuppressed status and tumor recurrence or patient prognosis in various malignant tumors. Reportedly, preoperative variables, including decreased lymphocyte count, increased monocyte count, and elevated neutrophil-to-lymphocyte ratio, are associated with poor prognosis in cancer patients (32, 33). In addition, perioperative changes in peripheral blood monocyte counts are independent risk factors for OS after hepatectomy and may reflect an immunosuppressive state (34). In this study, PBMC and LMNC counts, including those of lymphocytes and monocytes, of the group receiving neoadjuvant chemotherapy were not less than those of the group primarily receiving hepatectomy. Hence, preoperative introduction of bevacizumab combined with
CapeOX chemotherapy for the treatment of CRLM may not weaken the immune system function in these patients. Generally, the innate and adaptive immune systems can protect the host against tumor development via immunosurveillance. In this study, we showed a positive correlation between the number of liver NK cells and that of T cells in the group receiving neoadjuvant chemotherapy followed by hepatectomy, possibly indicating the effect of the innate immune system against the adaptive immune system. However, further investigation is warranted to describe the precise role of neoadjuvant chemotherapy in adaptive immunity following NK cell activation in patients with resectable CRLM.

Several investigators have reported that patients with a relatively dense infiltration of malignant tumors by NK cells exhibited better clinical outcomes (18, 35, 36). The outcomes of NK cell-target cell interactions are regulated by fine integrative balance between inhibitory and activating receptors expressed on NK cells and their ligands on target cells (37). In addition, several studies have demonstrated that a majority of CRCs exhibit diminished MHC class I expression, making them particularly vulnerable to NK cell-mediated killing and these patients show survival benefit (38, 39). However, the precise mechanism of NK cell function in CRLM tissues remains unclear. In this study, proportion of peripheral blood NK cells did not significantly differ between two groups, although the proportion of liver NK cells was significantly lower in neoadjuvant chemotherapy group. We collected NK cells in the liver by nondestructive perfusion method to avoid possibility of enzyme-induced alteration or
disruption of specific epitopes and evaluate the effect of chemotherapy on NK cells in the liver. In this case, the tumor infiltrated mononuclear cells, among which NK cell fraction might be enriched through potential chemotaxis from the tumor endothelium, are likely to be present in the perfusate, hence the collected LMNCs contain tumor infiltrated immunocytes. Because >50% of the patients displayed PR in the neoadjuvant chemotherapy group, the number of liver NK cells that infiltrated into the tumor decreased due to tumor shrinkage. Consequently, the proportion of NK cells among LMNCs possibly reduced in these patients. Another possibility might be that the chemotherapy comprising bevacizumab plus CapeOX directly influences the existence of NK cells in the liver. To address this issue, further investigation may be required.

The efficacy of NK cell-mediated tumor clearance depends on the type of NK cells that are present in the tissue, or that have migrated to the tumor site from peripheral blood (40). The non-classical MHC class I antigens, such as MICA, MICB, and ULBPs, can engage a stimulatory receptor on NK cells comprising a heterodimeric complex of NKG2D/DAP10, a cell-surface adaptor molecule involved in signal transduction (29, 41). The activation signal resulting from the engagement of NKG2D-DAP10 overrides the inhibitory signal from MHC class I molecules leading to target cell lysis (30, 42). Reportedly, high density of intratumoral NKp46-expressing NK cells was associated with OS in CRLM patients who received neoadjuvant chemotherapy (18). In this study, the expression of activation markers/effecto
molecules on both peripheral and liver NK cells of patients treated with neoadjuvant chemotherapy was enhanced than that in patients who did not receive chemotherapy; however, the proportion of NK cells among LMNCs was reduced. Our results indicated the increased NKG2D-expressing peripheral blood NK cells and NKp46-expressing liver NK cells in the neoadjuvant chemotherapy group. Furthermore, the proportion of TRAIL-positive NK cells in LMNCs significantly increased in the neoadjuvant chemotherapy group. Studies have shown TRAIL to be critical among the TNF family members in the NK cell-mediated antitumor function (20, 43). Besides, CRC cells are susceptible to TRAIL-induced apoptosis, both as a single agent of recombinant TRAIL and combined with chemotherapy and targeted therapies (44). Thus, administration of preoperative chemotherapeutic agents phenotypically activated liver NK cells; however, underlying mechanism remains unclear. This seems consistent with a previous finding that dying tumor cells treated with chemotherapeutic agents release proinflammatory cytokines that are crucial in the stimulation of protective anti-cancer immune responses (45). Thus, it is possible that neoadjuvant chemotherapy enhances NK cell-mediated cytotoxicity against tumor cells; however, we could not demonstrate the enhancement of cytotoxic activity in our experimental system. Its clinical significance remains completely unknown. Furthermore, we confirmed the effect of neoadjuvant chemotherapy on NK cells and showed that myelosuppression did not affect at least the expression of the surface molecules on NK cells in cases wherein chemotherapy
could be continued in the group receiving neoadjuvant chemotherapy followed by hepatectomy. However, the effects of the adverse events of neoadjuvant chemotherapy on intrahepatic immune cells are still unknown.

In addition, the NKG2D ligand expression is reportedly correlated with better clinical prognosis in CRC (46). In this study, neoadjuvant chemotherapy did not affect the expression of non-classical MHC class I antigens on CRLM tumor tissues, suggesting that chemotherapeutic agents might not diminish susceptibility to NK cell-mediated anti-tumor cytotoxicity. Cytotoxic activity of IL-2-stimulated LMNCs from neoadjuvant chemotherapy group was not significantly different from that of the group that received surgery first. These results suggest that chemotherapy does not have a negative impact on intrahepatic immune cells in resectable CRLM patients.

Recently, a complete pathological response was shown to be correlated with high rates of the OS and PFS in advanced CRC patients who had undergone neoadjuvant and conversion chemotherapy before resection of CRLM (47). In the present study focusing on the early limited phase until hepatectomy, we found no significant correlation between the expression strength of activating markers on NK cells and the image-confirmed and pathological chemotherapy response rates at the time of surgical treatment. The main HiSCO-01 trial is still under long-term follow-up observations, thereby not allowing us to evaluate the correlation between the expression strength of the activating markers on NK cells and
survival benefits.

In summary, this study suggests that neoadjuvant chemotherapy for the treatment of resectable CRLM induces the activation of peripheral blood and liver NK cells; however, its clinical significance remains unknown.
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DISCLOSURE STATEMENT

The protocol for this research project was approved by a suitably constituted Ethics Committee of the institution and it conforms to the provisions of the Declaration of Helsinki. Committee of Hiroshima University Clinical Research Ethics Review, Approval No. Clinical 186. Written informed consent was obtained from subjects and/or guardians.
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FINANCIAL SUPPORT INFORMATION

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FIGURE LEGENDS

Figure 1. Effects of neoadjuvant chemotherapy on lymphocyte subsets in both peripheral blood mononuclear cells (PBMCs) and liver mononuclear cells (LMNCs).

The numbers and proportions of PBMCs and LMNCs were analyzed by the FCM assay in groups A and B (n = 15 and 11, respectively). a, A comparison of the number of PBMCs per mL of blood between the two groups; b, a comparison of the number of LMNCs collected by liver perfusion per g of resected liver between the two groups; c-f, a comparison of the number of lymphocytes and monocytes in PBMCs and LMNCs between the two groups.; g-n, a comparison of the percentages of NK cells, NKT cells, T cells, and B cells in PBMCs and LMNCs between the two groups. *P < 0.05.

Figure 2. Neoadjuvant chemotherapy increased the expression of activation markers in peripheral blood and liver NK cells.

The FCM analysis of PBMCs and freshly isolated LMNCs obtained from liver perfusate after staining with CD3 and CD56 mAbs together with additional mAbs was performed (n = 15 [group (Gr) A]; n = 11 [Gr B]). a, Histograms represent the log fluorescence intensities obtained by staining for TRAIL, NKp30, NKp46, NKG2D, CD122, and SIRPβ after gating of CD3^+CD56^+ peripheral blood NK cell subsets obtained from patients. Dotted lines,
negative control staining with isotype-matched mAbs; numbers above each histogram, mean± SD of the MFI of targeted molecule expression on peripheral blood NK cells. b, Each point indicates the percentage of peripheral blood NK cells in each group that was positive for the TRAIL, NKp30, NKP46, NKG2D, CD122, and SIRPβ expression. c, Histograms representing the log fluorescence intensities obtained by staining for TRAIL, NKp30, NKP46, NKG2D, CD122, and SIRPβ after gating of CD3−CD56+ liver NK cell subsets obtained from patients. Dotted lines, negative control staining with isotype-matched mAbs; numbers above each histogram, mean± SD of the MFI of targeted molecule expression on liver NK cells. d, Each point indicates the percentage of liver NK cells in each group that was positive for the TRAIL, NKp30, NKP46, NKG2D, CD122, and SIRPβ expression. *P < 0.05.

Figure 3. Neoadjuvant chemotherapy did not suppress the cytotoxic activity of cytokine-stimulated LMNCs.

The FCM analyses of LMNCs cultivated with recombinant IL-2 for 3 days were performed after staining with mAbs against CD3 and CD56 (n = 11 [Gr A]; n = 5 [Gr B]). a, Histograms represent the log fluorescence intensity obtained by staining for TRAIL, NKp30, NKP46, NKG2D, CD122, and SIRPβ after gating of CD3−CD56+ IL-2-stimulated liver NK cell subsets obtained from patients. Dotted lines, negative control staining with isotype-matched mAbs; numbers above each histogram, mean± SD of the MFI of targeted molecule
expression on IL-2-stimulated liver NK cells. b, Each point indicates the percentage of IL-2-stimulated liver NK cells in each group that was positive for the TRAIL, NKP30, NKP46, NKG2D, CD122, and SIRPβ expression. c, the NK cytotoxic activity of IL-2-stimulated LMNCs against DLD-1 target cells was compared between group A (dotted line) and group B (solid line) and analyzed using an FCM-based cytotoxic assay. All data are expressed as mean ± SD (n = 4 [Gr A]; n = 4 [Gr B]).

**Figure 4.** Neoadjuvant chemotherapy did not affect the expression levels of MICA, MICB, and ULBP2 in liver tumors.

The expression levels of NKG2D ligands (MICA, MICB, and ULBP2) in resected liver specimens of CRLM from each group were investigated (n = 13 [Gr A]; n = 5 [Gr B]). RNA from liver tumors was extracted and reverse-transcribed. Relative copy numbers of NKG2D ligands were determined by real-time PCR using each NKG2D ligand-specific primer pair and normalized to the expression of GAPDH.

**Figure 5.** Neoadjuvant chemotherapy did not correlate with the clinical/pathological response rates and the expression levels of surface molecules on NK cells.

Clinical evaluation of response by the RECIST and histological evaluation of treatment response (grade classification) were performed in group B (n = 11). a, the relationships
between TRAIL, NKG2D, and SIRPβ on peripheral blood NK cells and clinical response
determined by the RECIST or histological treatment response (grade classification) were
studied. b. The relationships between TRAIL, NKp30, NKp46, CD122, and SIRPβ on liver
NK cells and clinical determined by the RECIST or histological treatment response (grade
classification) were studied.
Table 1. Eligibility Criteria

<table>
<thead>
<tr>
<th>Eligibility Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) a primary lesion histologically diagnosed as CRC</td>
</tr>
<tr>
<td>(2) the presence of CRLM of stage H1 or H2</td>
</tr>
<tr>
<td>(3) the metastatic lesion in the liver required less than 60 % of liver resection and allowed resection with a microscopically negative margin</td>
</tr>
<tr>
<td>(4) no distant/peritoneal metastasis other than CRLM</td>
</tr>
<tr>
<td>(5) the primary lesion had already been or could be resected with a microscopically negative margin</td>
</tr>
<tr>
<td>(6) no history of local therapy such as radiofrequency ablation or chemotherapy/radiotherapy for the CRLM</td>
</tr>
<tr>
<td>(7) no history of chemotherapy involving the use of oxaliplatin</td>
</tr>
<tr>
<td>(8) the liver disease could be classified as Child-Pugh class A</td>
</tr>
<tr>
<td>(9) no evident hemorrhage or obstruction arising from CRC</td>
</tr>
<tr>
<td>(10) the patients were aged between 20 and 80 years during enrollment</td>
</tr>
<tr>
<td>(11) the patient’s PS was either 0 or 1</td>
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</table>

CRC: colorectal cancer  | CRLM: colorectal cancer with liver metastasis  | PS: performance status
## Table 2. Patients’ characteristics

<table>
<thead>
<tr>
<th>Patients’ characteristics</th>
<th>Group A</th>
<th>Group B</th>
<th>P value</th>
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<tbody>
<tr>
<td>Eligible patients / enrolled patients</td>
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<td>11 / 15</td>
<td></td>
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<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>11</td>
<td>9</td>
<td>1.00</td>
</tr>
<tr>
<td>Female</td>
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<td></td>
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<tr>
<td>Age</td>
<td></td>
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<tr>
<td>Median (range)</td>
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<td>66 (45 - 78)</td>
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<td>Relations of CRC and CRLM</td>
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<td>Lobectomy</td>
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<td>Resection liver weight(g)</td>
<td>Median (range)</td>
<td>183 (26 - 845)</td>
<td>200 (10 - 638)</td>
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<tr>
<td>The number of LMNCs ($\times 10^6$)</td>
<td>Median (range)</td>
<td>127 (5 - 945)</td>
<td>42 (3 - 634)</td>
</tr>
</tbody>
</table>

CRC: colorectal cancer, CRLM: colorectal cancer with liver metastasis, LMNCs: liver mononuclear cells
Figure 1
169x127mm (300 x 300 DPI)
Figure 2

209x157mm (300 x 300 DPI)
Figure 3

209x157mm (300 x 300 DPI)
Figure 4

160x120mm (300 x 300 DPI)
Figure 5

169x127mm (300 x 300 DPI)