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

Fumihiro Hirata[1], *Kohei Ishiyama[1,4], Yuka Tanaka[1], Tsuyoshi Kobayashi[1], Masakazu Hashimoto[1], Yoshihiro Saeki[1], Nobuki Ishida[1], Kazuhiro Taguchi[1], Junko Tanaka[3], Koji Arihiro[2], *Hideki Ohdan[1], and Hiroshima Surgical Study Group of Clinical Oncology (HiSCO)

[1]Department of Gastroenterological and Transplant Surgery, Graduate School of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan

[2]Department of Anatomical Pathology, Hiroshima University Hospital, Hiroshima, Japan

[3]Department of Epidemiology, Infectious Disease Control and Prevention, Graduate School of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan

[4]Department of Surgery, National Hospital Organization Kure Medical Center and Chugoku Cancer Center, Hiroshima, Japan

CORRESPONDING AUTHOR:

*Kohei Ishiyama

Department of Surgery, National Hospital Organization Kure Medical Center and Chugoku

Cancer Center

3-1, Aoyama, Kure, 737-0023 Hiroshima, Japan

Email: ishiyamak@kure-nh.go.jp, Phone: +81-823-22-3111, FAX: +81-823-21-0478

*Hideki Ohdan

Department of Gastroenterological and Transplant Surgery, Applied Life Sciences, Institute of Biomedical & Health Science, Hiroshima University

Email: hohdan@hiroshima-u.ac.jp, Phone: +81-82-257-5222, FAX: +81-82-257-5224

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/m^2 plus oxaliplatin 130 mg/m^2) 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 *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.

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 (1×10^5) 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:

% cytotoxicity = [(% experimental PKH^+ $DAPI^+$ targets) - (% spontaneous PKH^+ $DAPI^+$ targets)] / [100 - (% spontaneous PKH^+ $DAPI^+$ targets)] × 100.

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′ -CCTTGGCCATGAACGTCAGG-3′ (forward) and 5′ -CCTCTGAGGCCTCGCTGCG-3′ (reverse); *MICB*, 5′ -ACCTTGGCTATGAACGTCACA-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′ -CAACGGATTTGGTCGTATTGG-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/effector

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.

REFERENCES

1. Steele G, Jr., Ravikumar TS. Resection of hepatic metastases from colorectal cancer. Biologic perspective. Annals of surgery. 1989;210(2):127-38.

2. Lordan JT, Karanjia ND, Quiney N, Fawcett WJ, Worthington TR. A 10-year study of outcome following hepatic resection for colorectal liver metastases - The effect of evaluation in a multidisciplinary team setting. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology. 2009;35(3):302-6.

3. Ribero D, Wang H, Donadon M, Zorzi D, Thomas MB, Eng C, et al. Bevacizumab improves pathologic response and protects against hepatic injury in patients treated with oxaliplatin-based chemotherapy for colorectal liver metastases. Cancer. 2007;110(12):2761-7.

4. Okines A, Puerto OD, Cunningham D, Chau I, Van Cutsem E, Saltz L, et al. Surgery with curative-intent in patients treated with first-line chemotherapy plus bevacizumab for metastatic colorectal cancer First BEAT and the randomised phase-III NO16966 trial. British journal of cancer. 2009;101(7):1033-8.

5. Garcia-Alfonso P, Ferrer A, Gil S, Duenas R, Perez MT, Molina R, et al. Neoadjuvant and conversion treatment of patients with colorectal liver metastasis: the potential role of bevacizumab and other antiangiogenic agents. Targeted oncology.

2015;10(4):453-65.

6. Kanas GP, Taylor A, Primrose JN, Langeberg WJ, Kelsh MA, Mowat FS, et al. Survival after liver resection in metastatic colorectal cancer: review and meta-analysis of prognostic factors. Clinical epidemiology. 2012;4:283-301.

7. Kato T, Yasui K, Hirai T, Kanemitsu Y, Mori T, Sugihara K, et al. Therapeutic results for hepatic metastasis of colorectal cancer with special reference to effectiveness of hepatectomy: analysis of prognostic factors for 763 cases recorded at 18 institutions. Diseases of the colon and rectum. 2003;46(10 Suppl):S22-31.

8. Kohne CH, van Cutsem E, Wils J, Bokemeyer C, El-Serafi M, Lutz MP, et al. Phase III study of weekly high-dose infusional fluorouracil plus folinic acid with or without irinotecan in patients with metastatic colorectal cancer: European Organisation for Research and Treatment of Cancer Gastrointestinal Group Study 40986. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2005;23(22):4856-65.

9. Garden OJ, Rees M, Poston GJ, Mirza D, Saunders M, Ledermann J, et al. Guidelines for resection of colorectal cancer liver metastases. Gut. 2006;55 Suppl 3:iii1-8.

10. Watanabe T, Itabashi M, Shimada Y, Tanaka S, Ito Y, Ajioka Y, et al. Japanese Society for Cancer of the Colon and Rectum (JSCCR) Guidelines 2014 for treatment of colorectal cancer. International journal of clinical oncology. 2015;20(2):207-39.

11. Cucchetti A, Ferrero A, Cescon M, Donadon M, Russolillo N, Ercolani G, et al. Cure

model survival analysis after hepatic resection for colorectal liver metastases. Annals of surgical oncology. 2015;22(6):1908-14.

12. Figueras J, Valls C, Rafecas A, Fabregat J, Ramos E, Jaurrieta E. Resection rate and effect of postoperative chemotherapy on survival after surgery for colorectal liver metastases. The British journal of surgery. 2001;88(7):980-5.

13. Ellis LM, Curley SA, Grothey A. Surgical resection after downsizing of colorectal liver metastasis in the era of bevacizumab. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2005;23(22):4853-5.

14. Schwitalle Y, Kloor M, Eiermann S, Linnebacher M, Kienle P, Knaebel HP, et al. Immune response against frameshift-induced neopeptides in HNPCC patients and healthy HNPCC mutation carriers. Gastroenterology. 2008;134(4):988-97.

15. Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, et al. Adaptive immunity maintains occult cancer in an equilibrium state. Nature. 2007;450(7171):903-7.

16. Morimoto H, Nio Y, Imai S, Shiraishi T, Tsubono M, Tseng CC, et al. Hepatectomy accelerates the growth of transplanted liver tumor in mice. Cancer detection and prevention. 1992;16(2):137-47.

17. Ohira M, Ohdan H, Mitsuta H, Ishiyama K, Tanaka Y, Igarashi Y, et al. Adoptive transfer of TRAIL-expressing natural killer cells prevents recurrence of hepatocellular carcinoma after partial hepatectomy. Transplantation. 2006;82(12):1712-9.

18. Donadon M, Hudspeth K, Cimino M, Di Tommaso L, Preti M, Tentorio P, et al. Increased Infiltration of Natural Killer and T Cells in Colorectal Liver Metastases Improves Patient Overall Survival. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2017;21(8):1226-36.

19. Trinchieri G. Biology of natural killer cells. Advances in immunology. 1989;47:187-376.

20. Ishiyama K, Ohdan H, Ohira M, Mitsuta H, Arihiro K, Asahara T. Difference in cytotoxicity against hepatocellular carcinoma between liver and periphery natural killer cells in humans. Hepatology. 2006;43(2):362-72.

21. Ohira M, Nishida S, Tryphonopoulos P, Tekin A, Selvaggi G, Moon J, et al. Clinical-scale isolation of interleukin-2-stimulated liver natural killer cells for treatment of liver transplantation with hepatocellular carcinoma. Cell transplantation. 2012;21(7):1397-406.

22. Tanimine N, Tanaka Y, Abe T, Piao J, Chayama K, Ohdan H. Functional Behavior of NKp46-Positive Intrahepatic Natural Killer Cells Against Hepatitis C Virus Reinfection After Liver Transplantation. Transplantation. 2016;100(2):355-64.

23. Tanimine N, Tanaka Y, Abe T, Piao J, Ishiyama K, Kobayashi T, et al. MELD and Child-Pugh Scores Are Related to Immune Status of Intrahepatic Natural Killer Cells in Liver Transplant Candidates. Transplantation proceedings. 2017;49(1):98-101.

24. Kim GG, Donnenberg VS, Donnenberg AD, Gooding W, Whiteside TL. A novel multiparametric flow cytometry-based cytotoxicity assay simultaneously immunophenotypes effector cells: comparisons to a 4 h 51Cr-release assay. Journal of immunological methods. 2007;325(1-2):51-66.

25. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer. 2009;45(2):228-47.

26. Dietrich J, Cella M, Seiffert M, Buhring HJ, Colonna M. Cutting edge: signal-regulatory protein beta 1 is a DAP12-associated activating receptor expressed in myeloid cells. J Immunol. 2000;164(1):9-12.

27. Piccio L, Vermi W, Boles KS, Fuchs A, Strader CA, Facchetti F, et al. Adhesion of human T cells to antigen-presenting cells through SIRPbeta2-CD47 interaction costimulates T-cell proliferation. Blood. 2005;105(6):2421-7.

28. Zamai L, Ahmad M, Bennett IM, Azzoni L, Alnemri ES, Perussia B. Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells. The Journal of experimental medicine. 1998;188(12):2375-80.

29. Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, et al. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and

stimulate NK cytotoxicity through the NKG2D receptor. Immunity. 2001;14(2):123-33.

30. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science. 1999;285(5428):727-9.

31. Komada Y, Zhang SL, Zhou YW, Hanada M, Shibata T, Azuma E, et al. Cellular immunosuppression in children with acute lymphoblastic leukemia: effect of consolidation chemotherapy. Cancer immunology, immunotherapy : CII. 1992;35(4):271-6.

32. Fujiwara Y, Shiba H, Furukawa K, Iida T, Sakamoto T, Gocho T, et al. Perioperative change in white blood cell count predicts outcome of hepatic resection for hepatocellular carcinoma. Journal of hepato-biliary-pancreatic sciences. 2010;17(6):892-7.

33. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nature medicine. 2004;10(9):942-9.

34. Haruki K, Shiba H, Fujiwara Y, Furukawa K, Wakiyama S, Ogawa M, et al. Perioperative change in peripheral blood monocyte count may predict prognosis in patients with colorectal liver metastasis after hepatic resection. Journal of surgical oncology. 2012;106(1):31-5.

35. Coca S, Perez-Piqueras J, Martinez D, Colmenarejo A, Saez MA, Vallejo C, et al. The prognostic significance of intratumoral natural killer cells in patients with colorectal

carcinoma. Cancer. 1997;79(12):2320-8.

36. Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Che X, Iwashige H, et al. Prognostic value of intratumoral natural killer cells in gastric carcinoma. Cancer. 2000;88(3):577-83.

37. Lanier LL. NK cell receptors. Annual review of immunology. 1998;16:359-93.

38. Menon AG, Morreau H, Tollenaar RA, Alphenaar E, Van Puijenbroek M, Putter H, et al. Down-regulation of HLA-A expression correlates with a better prognosis in colorectal cancer patients. Laboratory investigation; a journal of technical methods and pathology. 2002;82(12):1725-33.

39. Cordon-Cardo C, Fuks Z, Drobnjak M, Moreno C, Eisenbach L, Feldman M. Expression of HLA-A,B,C antigens on primary and metastatic tumor cell populations of human carcinomas. Cancer research. 1991;51(23 Pt 1):6372-80.

40. Vitale M, Cantoni C, Pietra G, Mingari MC, Moretta L. Effect of tumor cells and tumor microenvironment on NK-cell function. European journal of immunology. 2014;44(6):1582-92.

41. Champsaur M, Lanier LL. Effect of NKG2D ligand expression on host immune responses. Immunological reviews. 2010;235(1):267-85.

42. Groh V, Rhinehart R, Randolph-Habecker J, Topp MS, Riddell SR, Spies T. Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells. Nature immunology. 2001;2(3):255-60.

43. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. The Journal of biological chemistry. 1996;271(22):12687-90.

44. Galligan L, Longley DB, McEwan M, Wilson TR, McLaughlin K, Johnston PG. Chemotherapy and TRAIL-mediated colon cancer cell death: the roles of p53, TRAIL receptors, and c-FLIP. Molecular cancer therapeutics. 2005;4(12):2026-36.

45. Showalter A, Limaye A, Oyer JL, Igarashi R, Kittipatarin C, Copik AJ, et al. Cytokines in immunogenic cell death: Applications for cancer immunotherapy. Cytokine. 2017;97:123-32.

46. McGilvray RW, Eagle RA, Watson NF, Al-Attar A, Ball G, Jafferji I, et al. NKG2D ligand expression in human colorectal cancer reveals associations with prognosis and evidence for immunoediting. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15(22):6993-7002.

47. Klinger M, Tamandl D, Eipeldauer S, Hacker S, Herberger B, Kaczirek K, et al. Bevacizumab improves pathological response of colorectal cancer liver metastases treated with XELOX/FOLFOX. Annals of surgical oncology. 2010;17(8):2059-65.

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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

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

CRC: colorectal cancer CRLM: colorectal cancer with liver metastasis PS:performance status

Table 2. Patients' characteristics

Patients' characteristics

		Group A	Group B	*P* value
Eligible patients / enrolled patients		15 /15	11 / 15	
Sex	Male	11	9	
	Female	4	2	1.00
Age	Median (range)	66 (27 - 77)	66 (45 - 78)	0.78
Relations of CRC and CRLM	Synchronous	10	5	
	Metachronous	5	6	0.43
Location of Primary tumor	Colon	9	6	
	Rectum	5	4	
	Colon and Rectum	1	1	0.82
Clinical Stage	I	0	1	
	II	3	3	
	III	2	2	
	IV	10	5	0.55
Tumor Differentiation	Well	2	2	
	Moderately	12	9	
	Poorly	0	0	
	Mucinous	1	0	0.66
Hepatectomy	Partial resection	5	7	
	Segmentectomy	8	2	
	Lobectomy	2	2	0.18
Resection liver weight(g)	Median (range)	183 (26 - 845)	200 (10 - 638)	0.80
The number of LMNCs ($\times 10^6$)	Median (range)	127 (5 - 945)	42 (3 - 634)	0.34

CRC：colorectal cancer, CRLM：colorectal cancer with liver metastasis, LMNCs: liver mononuclear cells

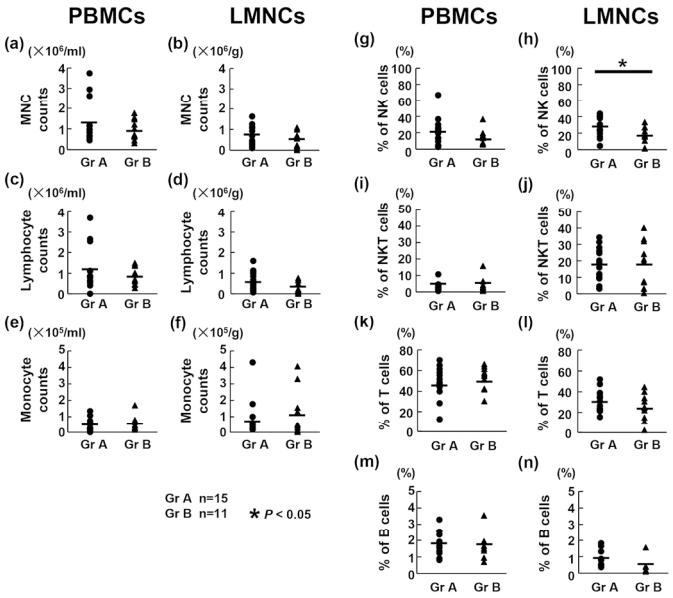

Figure 1

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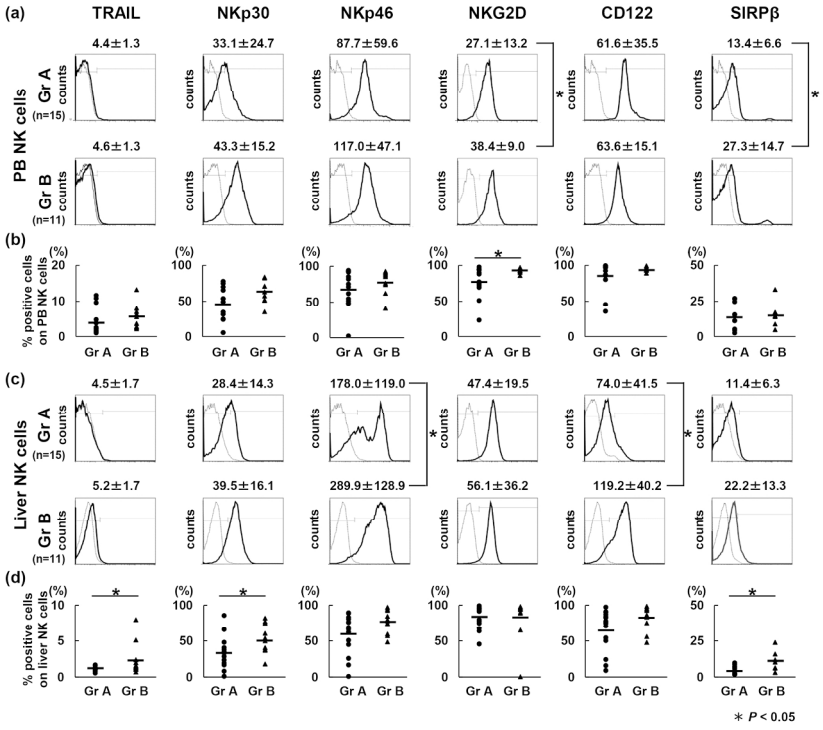

Figure 2

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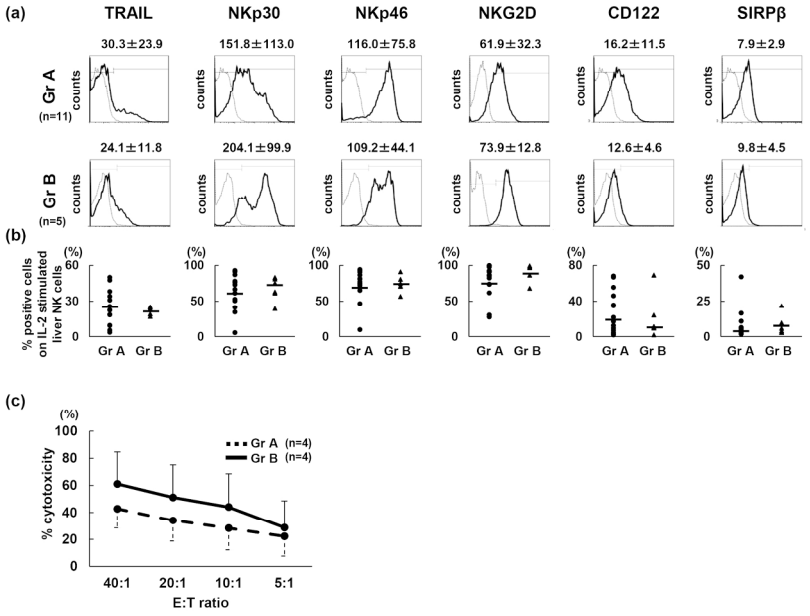

Figure 3

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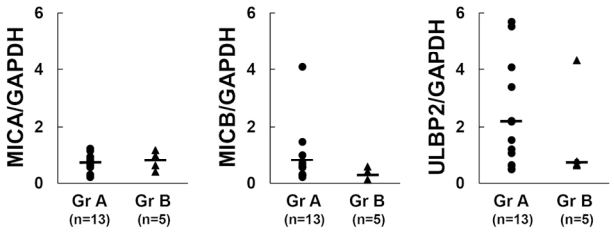

Figure 4

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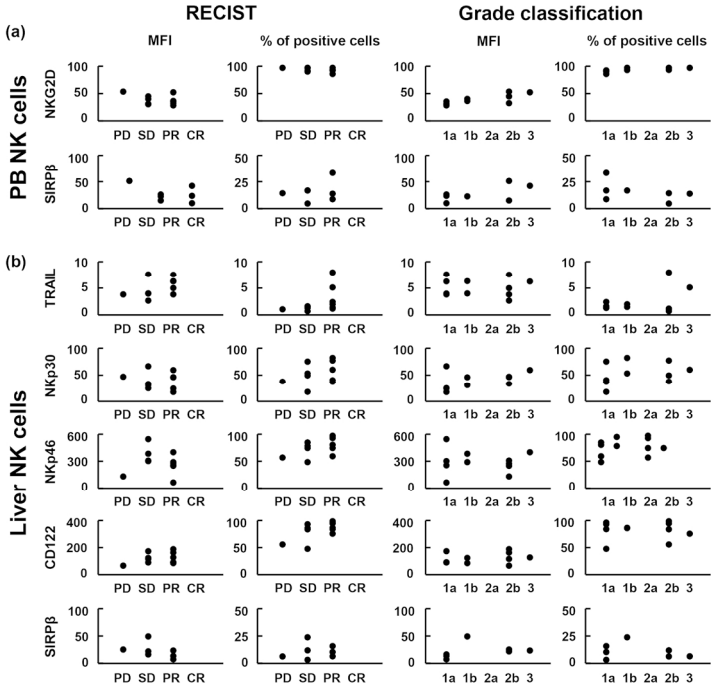

Figure 5

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