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HLA-C Matching Status Does Not Affect Rituximab-Mediated Antibody-Dependent Cellular Cytotoxicity by Allogeneic Natural Killer Cells

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Risk of leukemia relapse after T cell-depleted hematopoietic stem cell transplantation is lower in the "HLA-C mismatched" recipient-donor combinations. This might be attributable to increased natural killing by allogeneic NK cells carrying a KIR that does not bind to HLA-C on target cells (HLA-C-uncoupled KIR). Considering a new strategy of allogeneic NK cell transfer with rituximab to treat B-cell lymphomas, however, it is unknown whether the HLA-C matching status also affects rituximab-mediated antibody-dependent cellular cytotoxicity (ADCC). To address this issue, we investigated the levels of ADCC by purified NK cells carrying an HLA-C-uncoupled KIR, where the NK cell donors had either matched or mismatched HLA-C combination with target cells. Purified NK cells carrying an HLA-C-uncoupled KIR consistently showed enhanced ADCC against target cells when NK cell donors had an HLA-C-mismatch. When NK cell donors did not have an HLA-C mismatch, it was inconsistent whether HLA-C-uncoupled KIR caused ADCC enhancement. When the levels of ADCC by whole NK cells were compared, there were substantial differences among the donors regardless of the HLA-C matching status. Subjects with HLA-C mismatch may not have an advantage when cytoimmunotherapy using allogeneic NK cells is considered in combination with rituximab.

Keywords HLA-C, Rituximab, ADCC, Allogeneic, NK cells.

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INTRODUCTION

As a treatment modality against various malignancies, monoclonal antibody-based therapy is expanding its indications. Monoclonal antibodies attack tumor cells mainly through complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC). ADCC requires Fc receptor-bearing effector cells such as natural killer (NK) cells. After the Fc receptor engagement by an antibody, NK cells kill antibody-bound target cells by secretion of cytokines such as IFN-γ, and by discharge of the contents of their granules, such as perforin and granzyme (Cartron et al., 2004). NK cells also have a device that prevent themselves from attacking normal autologous cells by recognizing "self" with receptors that transduce inhibitory signals, many of which have specificity for HLA class I or HLA class I-related molecules.

Among such inhibitory receptors, a family of killer immunoglobulin-like receptors (KIRs) has been well-characterized (Lanier, 1998; Parham and McQueen, 2003). HLA-C is the main ligand for most inhibitory KIRs. It has been shown that HLA-C is classified into two groups, C1 and C2, by the amino acid substitutions at positions 77 and 80 in the α-1 helix structure (Boyington and Sun, 2002; Farag et al., 2002; Gumperz et al., 1995).

When NK cells face allogeneic target cells that cannot be recognized by inhibitory KIRs, they sense the missing expression of "self" class I alleles and mediates alloreactions (Ruggeri et al., 1999). Allogeneic cells missing expression of KIR ligands can therefore trigger NK cell alloreactivity (Ruggeri et al., 2002). Based on this theory, a simple algorithm known as the KIR ligand mismatch model was developed, in which comparison between donor and recipient HLA class I genotype allows prediction of NK alloreactivity (Baron et al., 2009).

The benefit of KIR ligand mismatch is well analyzed in allogeneic transplantation, especially T-cell-depleted HLA-mismatched transplantation (Giebel et al., 2003; Ruggeri et al., 1999; Ruggeri et al., 2002). Although the contribution of KIR ligand incompatibility to NK cell cytotoxic activity was reported, there is only a single study that documents the role for KIR ligand incompatibility in ADCC thus far (Stein et al., 2006).

In the current study, we focused on ADCC via a chimeric mAb, rituximab (Reff et al., 1994) that binds specifically to CD20 and induces apoptosis in a subset of CD20-expressing lymphoma cells through ADCC. We hypothesized that ADCC against lymphoma cells expressing HLA class I would be suppressed by KIRs on autologous NK cells. In contrast, ADCC would be enhanced and expected to be effective to refractory B-cell lymphomas if we use allogeneic NK cells with KIR ligand mismatch.

MATERIAL AND METHODS

Study Approval

This study was approved by the institutional review board in University of Tsukuba and all subjects were given written informed consent.

Cell Lines

The CD20-positive human Burkitt lymphoma cell line RAJI was provided by RIKEN BRC CELL BANK (Ibaraki, Japan). Epstein-Barr virus-transformed lymphoblastoid cell line, 103-LCL was provided by Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). HLA-C*0304 and HLA-C*0401 bearing B-lymphoblastoid cell lines (LBL), which were produced by retroviral transduction of HLA class I-deficient 721.221 LBL with each HLA-C molecule, were described previously (Akatsuka et al., 2002). All cell lines were cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated at 37°C with 5% CO_2.

Antibodies

For flow cytometric analysis, NK cell sorting, and ADCC assay, the following mouse monoclonal antibodies were used: FITC-conjugated anti-human CD20 (B-Ly1; DAKO), PE-conjugated anti-human HLA-A, -B, and -C (W6/32; BioLegend, San Diego, CA), FITC-conjugated anti-human CD56 (MEM188; eBioscience), PE-conjugated anti-human CD158b1/b2,j (GL183; Beckman Coulter), and APC-conjugated anti-human CD158a,h (EB6.B; Beckman Coulter). Anti-human CD20 mAb rituximab was kindly provided by Zenyaku Kogyo Co. Ltd (Tokyo, Japan). Human polyclonal immunoglobulin and human IgG1γ isotype control antibody were purchased from Baxter Japan (Tokyo, Japan) and Beckman Coulter (Fullerton, CA, USA), respectively.

HLA Typing

HLA-C typing of target cells and NK donors were performed at Human Leukocyte Antigen (HLA) Laboratory, Nonprofit Organization (Kyoto, Japan) by a reverse sequence-specific oligonucleotide-probe method using the Luminex (Austin, TX, USA) platform.

NK Cells

Heparinized blood was obtained from normal healthy subjects, and NK cells were isolated from whole blood by density gradient centrifugation using

RosetteSep NK cell enrichment cocktail (Stemcell Technologies, BC, Canada) according to the manufacturer's instructions. Isolated NK cells were stained with anti-CD158a (KIR2DL1) and anti-CD158b1/b2 (KIR2DL2/3) antibodies, and then sorted by BD FACSAria (BD Biosciences) into populations positive for either KIR. We used NK cells or the sorted NK subgroup populations only when the purity was >90% (data not shown).

RNA Interference

Double-stranded, short (21-mer) interfering RNA (siRNA) corresponding to enhanced green fluorescent protein (EGFP) [sense: 5'-CGUAAACGGCCACAAG UUCTT-3', antisense: 5'-GAACUUGUGGCCGUUUACGTT-3', starting from nucleotide 66 of the EGFP coding sequence (Matin et al., 2004)] and β_2-microglobulin were synthesized by Invitrogen. Two types of siRNA for β_2-microglobulin (accession number AB021288) were used: β_2-microglobulin A, 5'-GAUUCAGGUU UACUCACGUTT-3' (sense) and 5'-ACGUGAGUAAACCUGAAUCTT-3' (antisense), starting from nucleotide 91 of β_2-microglobulin sequence (Matin et al., 2004); and β_2-microglobulin B, 5'-GGUUUCAUCCAUCCGACAUTT-3' (sense) and 5'-AUGUCGGAUGGAUGAAACCTT-3' (antisense), starting from nucleotide 76. siRNA for EGFP was used as a control siRNA for those of β_2-microglobulin. We transferred these siRNAs by electroporation using the amaXa Nucleofector system (Koeln, Germany) according to the manufacturer's instructions.

Briefly, 2×10^6 cells, 150 pmol siRNA, and the 100 μl Nucleofector Solution V were combined, and then transferred to a cuvette. The cells were electroporated using the cell-type specific program. Cells were rinsed with medium and cultured. The cells were used as the targets in ADCC assay after 36 hours, at the time of maximal decrease of HLA class I expression determined by serial flow cytometry analyses (data not shown).

Flow Cytometry Assays for ADCC

NK cell ADCC against RAJI, 721.221LCL HLA-C*0304, 721.221LCL HLA-C*0401, and 103-LCL cells was measured using the LIVE/DEAD®. Cell-Mediated Cytotoxicity Kit (Invitrogen) based on procedures described previously (Kroesen et al., 1992). In brief, target B-cell lines (5×10^4 cells) were incubated for 20 min at 37°C, 5% CO_2 with the green fluorescent dye 3,3'-dioctadecyloxacarbocyanine (DiO) according to the manufacturer's instructions. Then the cells were washed twice with phosphate buffered saline.

Target cells were seeded in triplicates in 96-well microplates. Either whole or sorted NK cells were used as an effector, which were mixed with target cells at desired effector:target (E:T) ratios in the presence of rituximab or control antibody (human polyclonal immunoglobulin or human IgG1γ isotype control). The ideal concentration of rituximab for each target was determined by measuring ADCC

with various concentrations of rituximab (data not shown). After incubation at 37°C for 2 hours, propidium iodide (PI) was added and the cultures were analyzed by flow cytometry (BD FACSCalibur and CellQuestPro software, BD Bioscience). Specific cytotoxicity against target cells was calculated as: Dead target cells (DiO^+, PI^+)/Total (live and dead) target cells (DiO^+, PI^+ or $^-$) (Reff et al., 1994)

Statistical Analysis

Differences between variables were evaluated using the Student *t*-test using the Statcel software (OMS, Saitama, Japan). In this study, $P < 0.05$ was considered significant.

RESULTS

Knockdown of HLA Class I on Target Cells Enhances ADCC

NK cells prepared from peripheral blood of healthy subjects contained large granular lymphocytes and were >95% CD56-positive and CD3-negative (data not shown). Rituximab-dependent ADCC against the Burkitt lymphoma cell line, RAJI, was observed only when NK cells were present (Fig. 1). Cytotoxicity was saturated at approximately 60% with 1 μg/ml rituximab. As expected, rituximab alone induced almost no cytotoxicity (Fig. 1). Of note, RAJI cells express both HLA-C groups (C1/C2) (Table 1), indicating that NK cells from any donors are not alloreactive to RAJI cells.

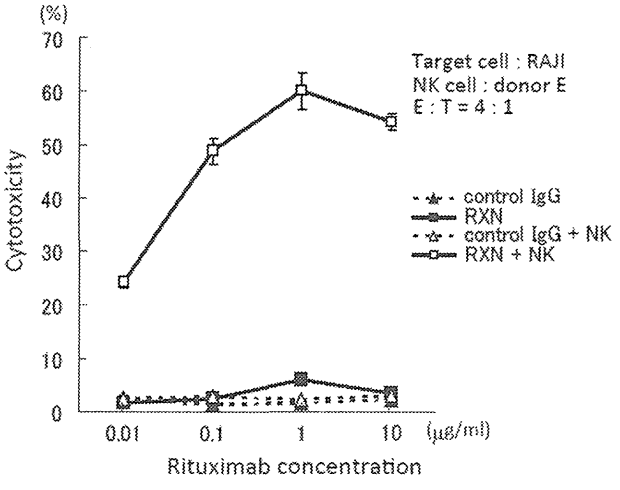

Figure 1: NK cell ADCC on lymphoma cells in the presence of rituximab.A CD20-positive human Burkitt lymphoma cell line RAJI was incubated with or without peripheral blood NK cells from a healthy volunteer donor with various concentration of control polyclonal IgG or rituximab (RXN). Control IgG alone (▲), control IgG with NK cells (△), RXN alone (■), RXN with NK cells (□). After 2-hour incubation, RAJI cells were stained by propidium iodide (PI) and analyzed by flow cytometry. Cytotoxicity was calculated by percentage of PI-positive RAJI cells in the total RAJI cell population. Mean cytotoxicity ± standard deviation (SD) of triplicate samples are shown at various antibody concentrations. The results were reproducible in three independent experiments.

Table 1: HLA-C typing and corresponding KIRs of cell lines and donors.

Cells	HLA-C	HLA-C group	Corresponding KIRs	License status	
				KIR2DL2,3$^+$ NK (C1-specific NK)	KIR2DL1$^+$ NK (C2-specific NK)
Target cells					
RAJI	Cw3 / Cw10	C1 / C2	KIR2DL2, 3 / KIR2DL1		
721.221LCL C*0304	Cw3 / -	C1 / -	KIR2DL2,3		
721.221LCL C*0401	Cw4 / -	C2 / -	KIR2DL1		
103-LCL	Cw8 / -	C1 / -	KIR2DL2, 3		
NK cell Donor					
A	Cw14 / Cw15	C1 / C2	KIR2DL2, 3 / KIR2DL1	Licensed	Licensed
B	Cw1 / Cw15	C1 / C2	KIR2DL2, 3 / KIR2DL1	Licensed	Licensed
C	Cw12 / Cw2	C1 / C2	KIR2DL2, 3 / KIR2DL1	Licensed	Licensed
D	Cw1 / Cw8	C1 / C1	KIR2DL2, 3	Licensed	*Unlicensed*
E	Cw1 / Cw7	C1 / C1	KIR2DL2, 3	Licensed	*Unlicensed*
F	Cw12 / Cw14	C1 / C1	KIR2DL2,3	Licensed	*Unlicensed*

Thus, to mimic the effects of allogeneic recognition by NK cells, we down-regulated the surface expression of HLA class I with siRNA targeting β_2-microglobulin (β2MG). Serial flow cytometric analyses demonstrated maximal downregulation of HLA class I at 36 hours after electroporation of the siRNA (data not shown). The mean fluorescence intensity of HLA class I expression was decreased to approximately 20% and 50% with β2MG-KO-A and β2MG-KO-B relative to that of untreated Raji cells, respectively, while the control siRNA (siRNA for EGFP, which is not relevant to this cytotoxicity assay system) had minimal effect (Fig. 2a). In contrast, these siRNA treatments did not affect the expression levels of CD20 (Fig. 2b). Allogeneic NK activity was readily enhanced in inverse correlation with the decreased expression level of HLA class I with the individual siRNAs when NK cells from two donors were tested in the presence of control IgG (Fig. 2c).

Finally, we evaluated the effect of HLA class I down-regulation with siRNAs in combination with rituximab-mediated ADCC. The target cell lysis was significantly enhanced according to the level of HLA class I downregulation. This phenomenon is explained as the increase of natural killing as a result of "missing-self." However, the effect became less clear when the concentration of rituximab was higher (Fig. 2d), suggesting that rituximab-dependent ADCC might be a major component of cytotoxic activity even in the NK cell-mediated allogeneic settings. In this experiment, both donor D and E have homozygous in HLA-C group (C1/C1) but the level of cytotoxicity was different between the donors. ADCC variation among individuals or FcγRIII polymorphisms might have resulted in this difference (see Discussion).

HLA-C-uncoupled KIR Enhances ADCC by Allogeneic NK Cells

We next examined whether ADCC is enhanced by NK cells carrying HLA-C-uncoupled KIR. HLA-C phenotypes were examined in six healthy subjects (Table 1). Three were heterozygous (C1/C2) and the other three were homozygous (C1/C1) in HLA-C groups. We sorted NK cells from these subjects into either KIR2DL2/3-positive (C1-specific) or KIR2DL1-positive (C2-specific) populations (Fig. 3a).

First, we used 721.221LCL HLA-C*0304 and 721.221LCL HLA-C*0401 as the target cells, which express exclusively C1 and C2, respectively. These cells do not express any other HLA class I molecules and expression levels of HLA-C were similar to each other (Fig. 3b). In this setting, KIR2DL1+ NK cells showed higher natural killing than KIR2DL2/3+ NK cells to the 721.221LCL HLA-C*0304 (C1) target, and conversely, KIR2DL2/3+ NK cells showed higher natural killing than KIR2DL1+ NK cells to 721.221LCL HLA-C*0401 (C2), regardless of whether NK cells were prepared from donors having C1/C1 or C1/C2 (Figs. 4a and b).

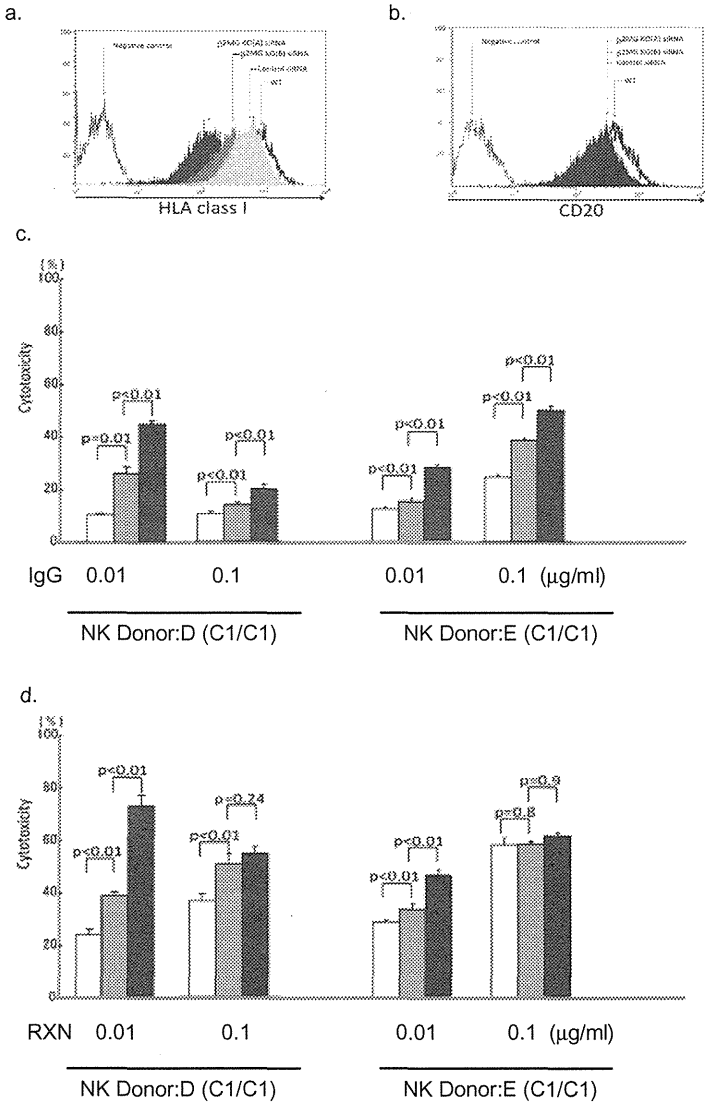

Figure 2: HLA class I knockdown enhances ADCC. (a) Flow cytometric analysis of HLA class I expression in cells treated with two different β2MG siRNAs, A (filled with black) and B (filled with dark gray), or control siRNA (filled with light gray). Isotype control, thin line; HLA class I expression of untreated cells, thick line. Analysis was performed 36 hours after siRNA treatment. (b) CD20 expression in the same cells as in (a). (c, d) RAJI cells, 36 hours after treatment with siRNAs (β2MG-KO-A in black, β2MG-KO-B in gray and control siRNA in white) incubated in the presence of peripheral blood NK cells from healthy volunteer donor D and E with 0.01 μg/ml or 0.1 μg/ml of control IgG (c) or RXN (d) for 5 hours at 37°C, 5% CO_2. E:T ratio was 4:1. Mean cytotoxicity ± SD of triplicate samples was measured. Data are representative of at least three independent experiments.

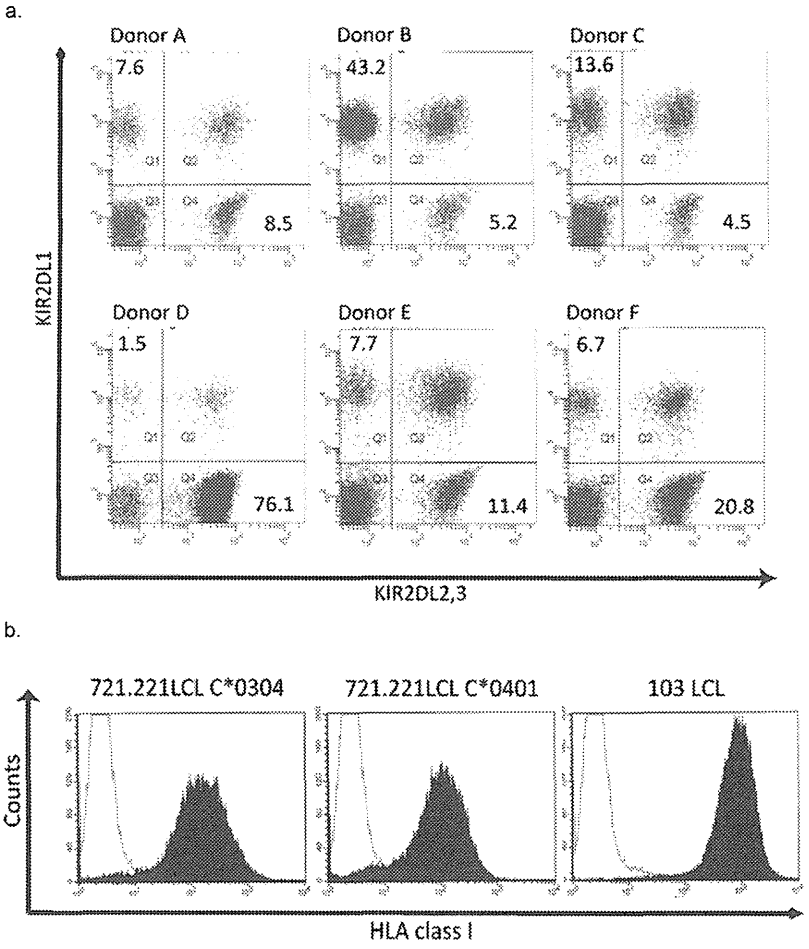

Figure 3: KIR2DL and HLA class I expression in NK cells and target cells. (a) NK cells were purified from a whole blood sample from a healthy donor as described in Materials and Methods. The cells were separated into either KIR2DL2/3+ / KIR2DL1- (KIR2DL2/3+ NK cells) or KIR2DL2/3- / KIR2DL1+ (KIR2DL1+ NK cells) population by FACS sorting. The representative staining of NK cells before sorting in each subject is shown. The value in each quadrant represents the percentages of NK cell subpopulations. (b) Expression of HLA class I on target cells, 721.221LCL HLA-C*0304, 721.221LCL HLA-C*0401, and 103-LCL is shown.

ADCC by rituximab in addition to the natural killing was observed in a significant manner, and HLA-C-uncoupled KIR consistently enhanced the total killing activity consisting of natural killing and rituximab-mediated ADCC. In Figure 4d, the data from all donors were pooled and compared the NK cell killing of HLA-C-coupled KIR with uncoupled (Fig. 4d upper two panels), which shows higher cytotoxicity in HLA-C-uncoupled KIR in both natural killing (IgG) or rituximab-mediated ADCC. Next, 103-LCL cells, which have a homozygous group of HLA-C (C1/-), were used as another target (Fig. 4c).

In this experiment, enhancement of natural killing by NK cells with C1 group-uncoupled KIR was not observed, whereas rituximab-mediated ADCC was enhanced by HLA-C-uncoupled KIR-bearing NK cells from three out of six donors. Interestingly, all the three donors whose NK cells showed enhancement by HLA-C-uncoupled KIR had C1/C2 groups of HLA-C (Fig. 4c, donor A, B and C).

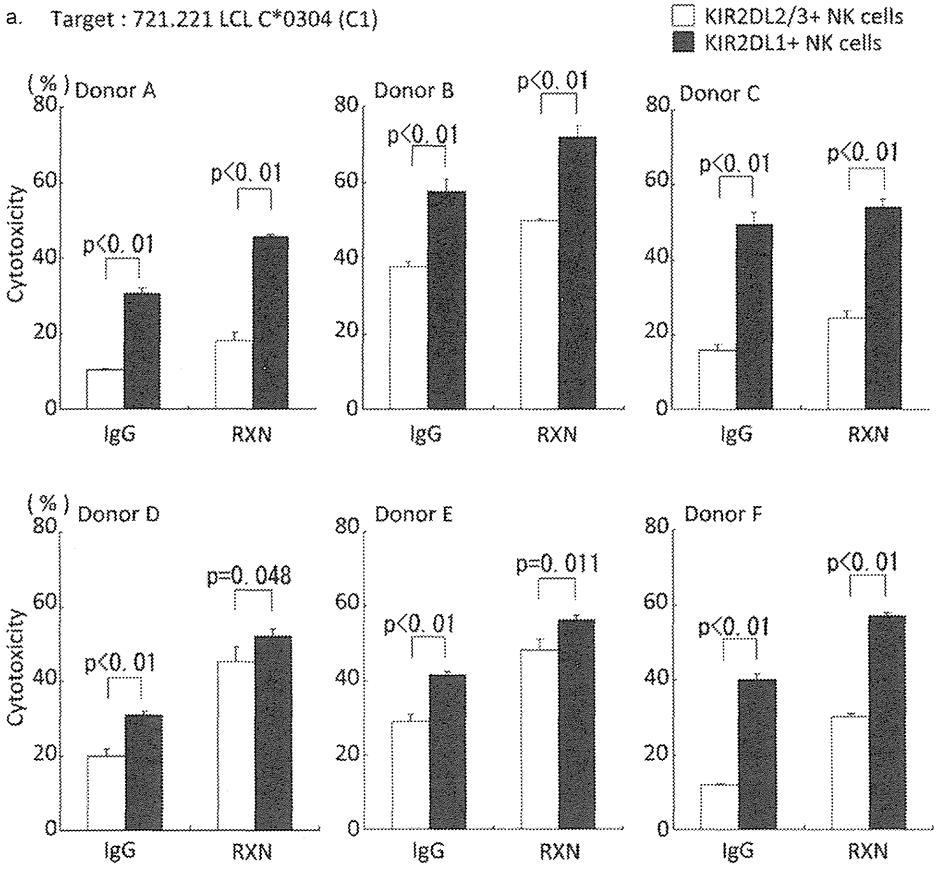

Figure 4: The effect of HLA-C-uncoupled KIR on rituximab-mediated ADCC using purified NK cells. KIR2DL2/3+ and KIR2DL1+ NK cells from healthy volunteers were separately incubated with 721.221LCL HLA-C*0304 (a), 721.221LCL HLA-C*0401 (b), and 103-LCL (c) target cells with 10 μg/ml RXN or control IgG1γ antibody for 5 hours at 37°C, 5% CO_2. The E:T ratio was 5:1. Mean cytotoxicity ± SD of triplicate samples is shown. Data are representative of at least two independent experiments. (d) Comparison of NK cell killing between HLA-C-coupled KIR and HLA-C uncoupled KIR. The data in Figures 4a, b, or c were pooled in every target cell and reanalyzed. In 721.221LCL HLA-C*0304 target cell, HLA-C-coupled KIR and -uncoupled KIR are KIR2DL2/3 and KIR2DL1, respectively; in 721.221LCL HLA-C*0401, HLA-C-coupled KIR and -uncoupled KIR are KIR2DL1 and KIR2DL2/3; and in 103-LCL, HLA-C-coupled KIR and -uncoupled KIR are KIR2DL2/3 and KIR2DL1. (e) The effect of license status on NK cell killing. The results of 721.221LCL HLA-C*0304 target cell in Figure 4a, and 103-LCL in Figure 4c, were reanalyzed, merging the killing of KIR2DL1+ NK cell into licensed or unlicensed categories. "Licensed NK cells" are KIR2DL1+ NK cells from donors A, B and C; "Unlicensed NK cells" are KIR2DL1+ NK cells from donors D, E and F.

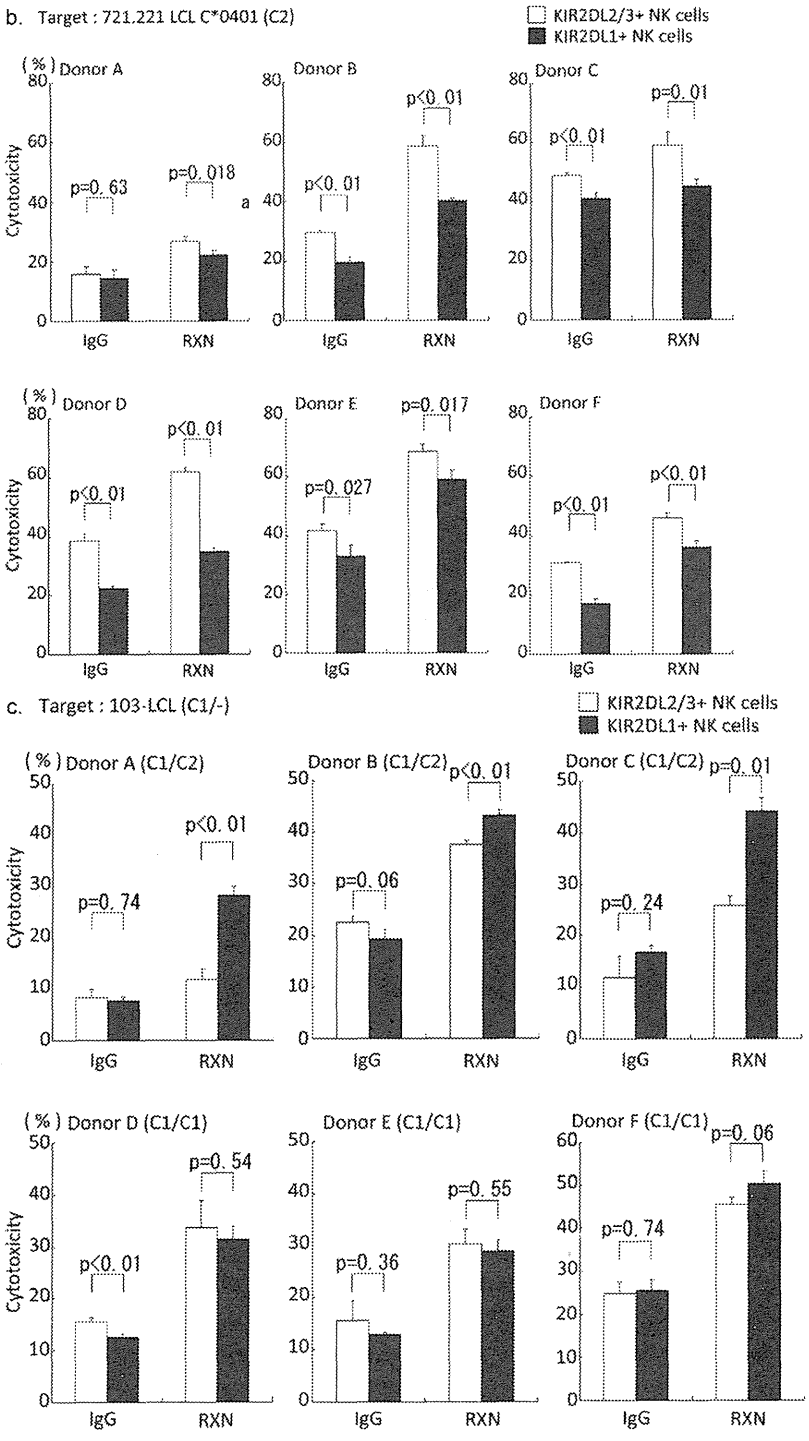

Figure 4: (*Continued*).

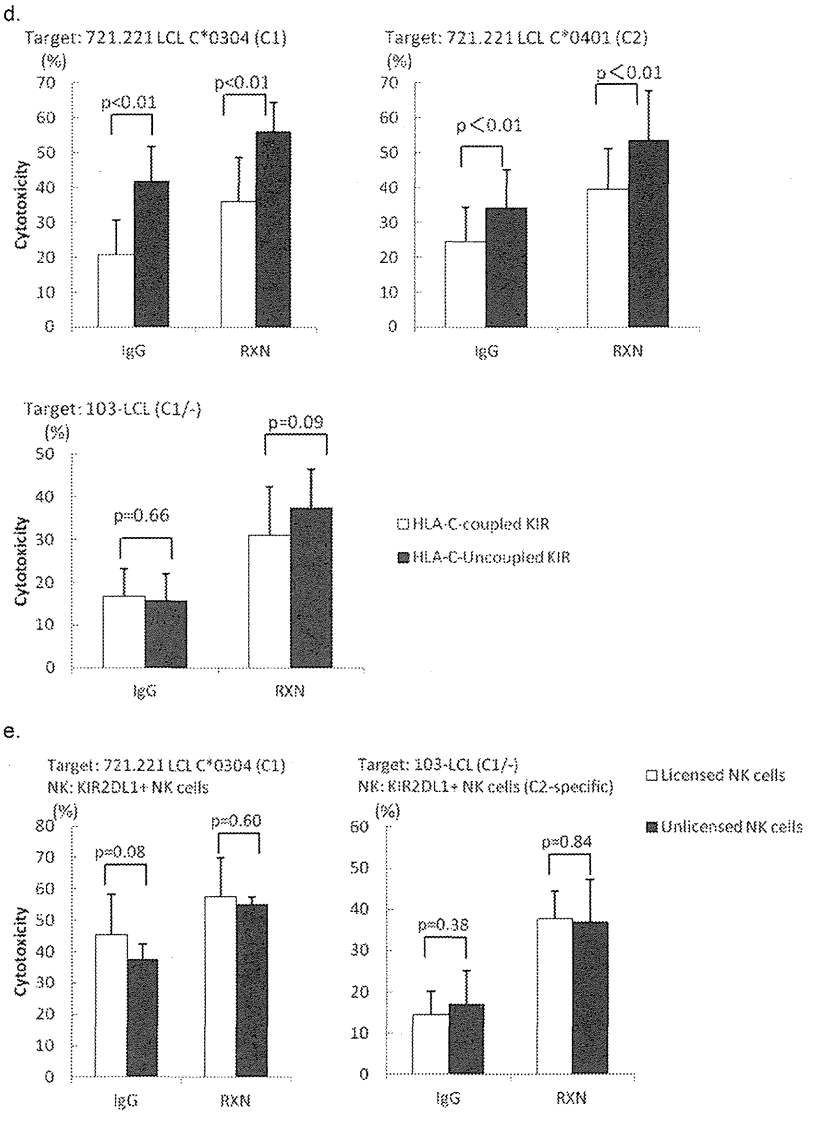

Figure 4: (*Continued*).

In contrast, such ADCC enhancement was not observed with NK cells prepared from all the three subjects having C1/C1 group of HLA-C (HLA-C-matched donors) (Fig. 4c, donor D, E and F). When the data were pooled, the natural killing and ADCC of 103-LCL cells was not different significantly between HLA-C-coupling statuses (Fig. 4d lower panel).

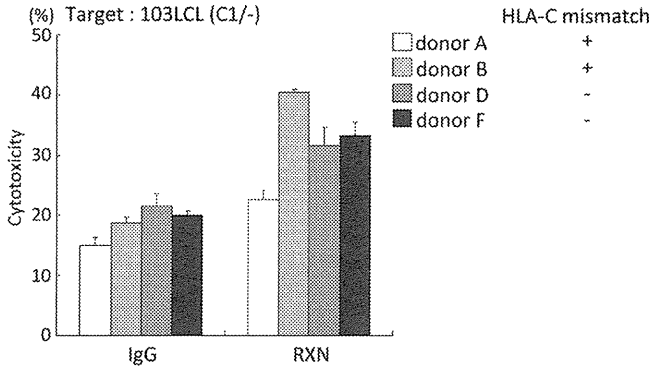

Figure 5: The effect of HLA-C mismatch between donors and target cells on rituximab-mediated ADCC using unsorted NK cells. Unsorted NK cells from healthy donors (donors A and B; HLA-C heterozygous for C1 and C2, donors D and F; homozygous for C1) were incubated with 103-LCL target cells in the presence of 10 μg/ml RXN or control IgG1γ antibody for 5 hours at 37°C, 5% CO_2. The E:T ratio was 5:1. Mean cytotoxicity ± S.D. of triplicate samples was measured. Data are representative of two independent experiments.

Although the two target cells, i.e., 721.221LCL and 103-LCL showed some inconsistent results, experiments using both target cells commonly led to the conclusion that HLA-C-uncoupled KIR enhances rituximab-mediated ADCC by NK cells, at least if prepared from HLA-C-mismatched donors. The impact of HLA-C matching status on the rituximab-mediated ADCC enhancement by HLA-C-uncoupled KIR might depend on the target cells.

Recently, it has become clear that active engagement of inhibitory receptors on NK cells by self-MHC class I molecules is the key event that determines whether an NK cell will be functionally capable of mediating "missing-self" recognition (education). Several models have been proposed to describe various aspects of NK cell education by MHC class I molecules (Hoglund and Brodin). In the "licensing model," NK cells acquire functional competence after ligation of inhibitory receptors by self-MHC class I molecules (Kim et al., 2005). According to this model, NK cells that express KIRs which does not have corresponding self-HLA will not be licensed to response the target cells which does not have a KIR-corresponding HLA (hyporesponsive).

In our system, KIR2DL1-positive (C2-specific) NK cells from donors D, E and F, are considered to be "unlicensed" because these donors lack C2 group HLA-C which corresponds to KIR2DL1 (Table 1). These NK cells are expected to be hyporesponsive to the target cells that express no C2 group of HLA-C, such as 721.221LCL HLA-C*0304 (C1) and 103-LCL (C1/-) cells. According to this, we reanalyzed the data used for Figures 4a and c, merging the results from the donors into licensed or unlicensed categories. As shown in Figure 4e, however, we found no enhancement of natural killing (IgG) or rituximab-mediated ADCC with or without license.

ADCC Enhancement by HLA-C Mismatch Does Not Occur When Whole NK Cells Were Used

Next, rituximab-mediated ADCC was measured using 103-LCL cells and whole NK cells prepared from four donors (donor A, B, D and F), because rituximab-mediated ADCC enhancement by HLA-C-uncoupled KIR was influenced by HLA-C-matching status with this target.

When whole NK cells were used as an effector, the ADCC levels were highly variable among individuals, irrespectively of whether HLA-C mismatch existed between NK cell donors and target cells, and significant differences were not observed between NK cells prepared from HLA-C-matched and HLA-C-mismatched donors (Fig. 5).

These results consequently indicate that ADCC enhancement is not expected if choosing HLA-C-mismatched donors, as far as whole NK cells are used as a source of allogeneic NK cell infusion in clinical settings.

DISCUSSION

In our analysis, ADCC enhancement by HLA-C-uncoupled KIR was observed in 721.221LCL transfectants, which may support our hypothesis that rituximab-mediated ADCC is not inhibited by KIRs when the target cells do not express the matched HLA-C ligands (Figs. 4a and b). This finding was also observed in 103-LCL cell as a target when HLA-C mismatch exists between NK cell donors and target cells (Fig. 4c donor A, B and C). Furthermore, when HLA-C mismatch does not exist between NK cell donors and target cells, ADCC enhancement by HLA-C-uncoupled KIR was not seen with 103-LCL (Figs. 4c donor D, E and F), as we expected.

This result raises a suggestion that subjects carrying HLA-C mismatch are better as a donor of allogeneic NK cells. However, the ADCC levels were highly variable among individuals when whole NK cells were used (Fig. 5), and we could not find any evidence that donors with HLA-C mismatch have an advantage. FcγRIII is known to have polymorphism with either a phenylalanine or a valine (V) at amino acid position 158. FcγRIII-158V has higher affinity for human IgG1(Koene et al., 1997), which results in increased ADCC mediated by rituximab (Hatjiharissi et al., 2007). Polymorphisms like this in NK cell donors might have resulted in the variation of ADCC among NK cell donors.

In conclusion, we demonstrated the enhancement of ADCC by HLA class I knockdown of the target cells or by purified NK cells with HLA-C-uncoupled KIR. When whole NK cells were used, however, variation of rituximab-mediated ADCC was substantial among individuals, and HLA-C mismatch between NK cell donors and target cells did not provide significant impact on the level of ADCC. Thus, donors with HLA-C mismatch may not necessarily have an

advantage when immunotherapy using allogeneic NK cells is considered in combination with rituximab for the treatment of lymphoma.

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Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Notch2 and Immune Function

Mamiko Sakata-Yanagimoto and Shigeru Chiba

Abstract Notch2 is expressed in many cell types of most lineages in the hematolymphoid compartment and has specific roles in differentiation and function of various immune cells. Notch2 is required for development of splenic marginal zone B cells and regulates differentiation of dendritic cells (DCs) in the spleen. Notch2 appears to play some specific roles in the intestinal immunity, given that the fate of mast cells and a subset of DCs is regulated by Notch2 in the intestine. Notch2 also has important roles in helper T cell divergence from na CD4 T cells and activation of cytotoxic T cells. Moreover, recent genetic evidence suggests that both gain-and loss-of-function abnormalities of Notch2 cause transformation of immune cells. Inactivating mutations are found in Notch2 signaling pathways in chronic myelomonocytic leukemia, while activating mutations are found in mature B cell lymphomas, which reflects the role of Notch2 in the developmental process of these cells.

Contents

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1 Introduction

The expression pattern of Notch1, Notch2, Notch3, and Notch4 varies from one cell type to another. Notch2 plays specific roles in the immune compartment independently of and cooperatively with Notch1 and Notch3. In this chapter, we mostly focus on evidence based on mouse genetic studies regarding Notch2 functions in immune cells. In the last part, we discuss the involvement of Notch2 in neoplastic transformation in conjunction with human hematologicmalignancies.

2 Notch2 Signaling in Marginal Zone B Cell Development

Mature splenic B cells are mainly divided into 2 types of B cells, follicular B (FOB) cells and marginal zone B (MZBs) cells (Martin and Kearney 2002). FOB cells are one of the main effectors of acquired immunity, able to respond to a large variety of antigens, while MZB cells can only elicit an immune response to a limited number of antigens. Immature B cells, developing from hematopoietic stem cells (HSCs) in the bone marrow, migrate to the spleen, and differentiate first into T1 (type1) transitional B cells (characterized by: IgM^{hi} IgD^{lo} $CD21^{lo}$), before differentiating into T2 (type2) transitional B cells (IgM^{hi} IgD^{hi} $CD21^{int}$ $CD23^{hi}$) (Loder et al. 1999). These progenitors further differentiate into the two types of B cells, MZB cells (IgM^{hi} IgD^{lo} $CD21^{hi}$ $CD23^{lo}$) and FOB cells (IgM^{lo} IgD^{hi} $CD21^{int}$ $CD23^{hi}$) (Martin and Kearney 2002). Conditional inactivation of *Notch2* in the B cell lineage results in defective MZB cell development, while FOB cell development is unaffected (Saito et al. 2003), which is basically consistent with the phenotype of *RBP-J* conditional knockout mice (Tanigaki et al. 2002). The cleaved Notch-RBP-J activator complex contains at least one out of three family members of the mastermind-like proteins (MAML1-3). Mastermind-like1 (MAML1) plays an essential role in MZB cell development, which is why MZB cells are lacking in *MAML1*-null mice (Wu et al. 2007; Oyama et al. 2007). Among Notch ligands, Delta-like 1 (Dll1) is responsible for MZB cell development, based on the fact that *Dll1* deletion using the Mx-Cre *loxP* system leads to loss of MZB cells (Hozumi et al. 2004; Sheng et al. 2008). Several lines of evidence indicate that loss of *Dll1* expression on nonhematopoietic cells causes MZB cell defects (Hozumi et al. 2004; Sheng et al. 2008; Tan et al. 2009). However, the exact cell types through which Dll1-induced Notch2 signaling triggers MZB development remains to be elucidated. The essential role of Notch signaling in MZB cell development is further proven by a number of other gene-targeted mice in which