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Development of a simple new flow cytometric antibody-dependent cellular cytotoxicity (ADCC) assay with excellent sensitivity

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ABSTRACT

Antibody-based therapeutic strategies have become recognized as useful clinical options in several types of cancer, often with the expectation that such therapies will trigger target cell elimination *via* antibody-dependent cellar cytotoxicity (ADCC) by natural killer cells. The successful development of therapeutic monoclonal antibodies (mAbs) requires an assay system that permits a critical evaluation of their physicochemical and biological characteristics. At present a number of ADCC assay systems have been reported, however, there is still room for improvement in terms of usability, operability and sensitivity. Here we report a novel flow cytometric ADCC assay that uses a human natural killer cell line stably transfected with mouse FcγRIII, and Fc receptor common-γ chain (FcRγ) and a reporter gene as effector cells. This assay relies on discriminating effector and target cells by their differential immunofluorescence, which allows for clear-cut gating and accurate calculation of the number of surviving cells in a target population. This assay is easy and quick to perform and provides reliable data even for low frequency target cells in assay samples and with low concentrations of mAbs. Furthermore, our approach allows us to identify synergistic ADCC activity of mAbs with different epitope specificities on the same target antigen.

1. Introduction

Monoclonal antibody (mAb)-based therapeutics are now well accepted therapies for cancer and inflammatory diseases, and new varieties of clinically applicable mAb-based approaches are continuing to undergo development (Suzuki et al., 2015). The therapeutic assessment of mAbs relies on their activity to mediate blocking of receptor mediated signaling, ligand neutralization, immune-independent direct apoptosis, complement-dependent cytotoxicity (CDC), and antibody-dependent cellular cytotoxicity (ADCC) (Suzuki et al., 2015). ADCC is a critical mechanism that leads to apoptosis of target cells. In ADCC, mAbs recognize target cells *via* their variable region and crosslink Fc receptors (FcR) present on immune effector cells by their Fc regions. The activation cascade through FcR common-γ chain (FcRγ) molecules associated with the FcR results in cellular activation (Bruhns, 2012).

In a typical ADCC assay, peripheral blood mononuclear cells

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; Calcein, calcein-acetoxymethyl; CDC, complement-dependent cytotoxicity; CFSE, carboxyfluorescein diacetate succinimidyl ester; ^{51}Cr, Chromium 51; FBS, fetal bovine serum; FcγR, Fcγ receptor; FcR, Fc receptor; FcRγ, Fc receptor common-γ chain; FSC, forward-scattered light; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LAK, Lymphokine-activated killer; LDH, lactate dehydrogenase; mAb, monoclonal antibody; M-CSFR, macrophage colony-stimulating factor receptor; MFI, median fluorescence intensity; NK, natural killer; PBMCs, peripheral blood mononuclear cells; SSC, side-scattered light

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(PBMCs) or enriched NK cells have been used for human studies and IL-2 activated NK cells for mouse studies. However, such effector preparations take time to prepare and, in addition, it has been difficult to develop a reliable readout system for target cell death when performing ADCC assays using such effectors cells.

To overcome this problem, there have been trials using transfected NK or T cell lines rather than donor derived effector cells. One group chose an NK leukemia cell line, KHYG-1, thinking that it should have strong cytotoxic activity (Suck et al., 2005). KHYG-1 cells have lost the expression of CD16 (FcγRIIIA) during their establishment (Yagita et al., 2000) and thus do not have ADCC activity by themselves (Mishima et al., 2012), however, when transfected with CD16, the cells showed stable cytotoxic activity with high reproducibility in a model analysis of rituximab-induced ADCC activity (Mishima et al., 2012). To adapt this system to measure the ADCC activity of mouse mAbs, Clémenceau et al. reconstituted the $CD16^+$ human NK-92 cell line with a chimeric cDNA that concatenated mouse FcγRIII to human FcRγ (Clemenceau et al., 2013). This mutant cell line is useful to analyze ADCC activity against human B cell lymphoma cell lines by mouse mAbs, or by a human chimera IgG CD20 mAb (rituximab).

Another important factor in ADCC assays is the read-out system. In this context, the Chromium 51 (^{51}Cr) release assay has been classically used to measure target cell death, providing highly sensitive results (Brunner et al., 1968). To avoid using radioactivity, other markers have been considered, such as the fluorescent dye, calcein-acetoxymethyl (calcein-AM), which is pre-loaded into target cells. However, it has been reported that the dye is spontaneously released, making it difficult to accurately estimate cell death induced by candidate antibodies (Somanchi et al., 2015; Gillissen et al., 2016). In addition, the release of intracellular enzymes lactate dehydrogenase (LDH) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been used as an indicator of cell death, but such assays also have several problems in interpretation of the results.

Instead of ^{51}Cr or calcein release assays, flow cytometric methods for ADCC have been proposed. In this case the target cells are labeled with calcein or carboxyfluorescein diacetate succinimidyl ester (CFSE) and mixed with effector cells, such as PBMC or an NK cell line, followed by measurement of number of surviving labeled target cells with a flow cytometer (Gillissen et al., 2016; Yamashita et al., 2016). These assays require labeling target cells with fluorescent dyes to distinguish them from effector cells, however, specific labeling of the target cells is difficult when handling clinical samples. Instead of target cell labeling, labeling of effecter cells after their isolation from heterogeneous immune cells could be considered as an alternate approach, but the execution of this type of assay is not easy.

To overcome the shortcomings of existing assays, we have developed a novel flow cytometric ADCC assay that relies on a precise quantification of the number of surviving target cells. This is possible because there is a clear-cut separation of effector and target cells due to their differential fluorescence. As effector cells, a human NK cell line was transduced with mouse FcγRIII and FcRγ (mFcγR) and a GFP reporter gene. This modified cell line has potent and stable ADCC activity mediated by mouse and human chimeric antibodies. The assay system is easy and quick to perform and provides reproducible and reliable data even with low frequency target cells in assay samples. Furthermore, our approach allows us to investigate the synergistic ADCC activity of mAbs with different epitope specificities on the same target.

2. Materials and methods

2.1. Commercial mAbs

The following monoclonal antibodies were purchased: rituximab (Chugai Pharmaceutical Co. Ltd., #136100766), PE-human M-CSFR (9-4D2-1E4; BioLegend, #347304 and 12-3A3-1B10; eBioscience, #12-1159-41), PE-mouse NKp46 (29A1.4, BD Biosciences, #560757), APC-mouse CD49b (DX5; BioLegend, #103516), APC-mouse CD16/32 (93, BioLegend, #101326), PE-human CD25 (BC96, BioLegend, #3020), APC-human CD16 (3G8, BioLegend, #302012), APC-human CD19 (H1B19; BioLegend, #302212) and APC-human CD2 (RPA-2.1010; BioLegend, #300214).

2.2. Cell lines and animals

The human KHYG-1 NK cell line and the Daudi lymphoma cell line were obtained from the JCRB Cell Bank (JCRB0156 and JCRB9071). The mouse A20 lymphoma cell line was obtained from the RIKEN-BRC Cell Bank (RCB2745). Cell lines were cultured in RPMI1640 medium supplemented with 2 mM Glutamax I, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 55 μM 2-mercaptoethanol and 10% FBS (GIBCO-Invitrogen;). KHYG-1 cells were maintained in medium additionally supplemented with recombinant human interleukin 2 (IL-2; PeproTech, #200-02).

BALB/c and SCID (C.B.-17/lcr-scid/scidJcl) mice were purchased from CLEA Japan, Inc. All experiments were in accordance with protocols approved by the RIKEN Animal Care and Use Committee.

2.3. Production and purification of recombinant antigens

A cDNA encoding an Fc-fusion protein of the extracellular domain of hM-CSFR (1–517 a.a.) and hCD25 (1–240 a.a.) was obtained by PCR using hM-CSFR and hCD25 cDNA and pFUSE-mIgG2b-Fc vector (InVivoGen, #pfuse-mg2bfc2) as templates and was cloned into the mammalian expression vector pOriP (Mukai et al., 2008). The recombinant hM-CSFR-Fc and hCD25-Fc fusion protein were transiently expressed in 293-F cells (ThermoFisher Science, #R79007) using the 293fectin Transfection Reagent (ThermoFisher Science, #12347750). The centrifuged and filtered cell culture medium was loaded on a Hi-Trap rProteinA column (GE Healthcare, # 17508001). The target protein was eluted using an acidic glycine buffer gradient. The protein was concentrated using an Amicon Ultra Centrifugal Filter (Merck KGaA, # UFC805008) and then fractionated on a HiLoad Superdex200 column (GE Healthcare, # 28989336).

2.4. Establishment of anti-hM-CSFR mAb and anti-hCD25 mAb

BALB/c mice were immunized with hM-CSFR-Fc recombinant fusion protein or hCD25 recombinant fusion protein in Titer Max Gold (TiterMax USA, # G-1). Spleen cells were obtained from the immunized mice, fused with the P3U1 plasmacytoma cell line and plated into wells of 96 well culture plates. After growing the cells in selective media, culture supernatants were screened for hM-CSFR or hCD25 reactivity by flow cytometry using the hM-CSFR or hCD25 transductants, followed by second screening by ADCC assay. The mAbs were purified from ascites or culture supernatants using Protein G–Sepharose 4 Fast Flow columns (GE Healthcare, # 17061802).

2.5. Production of human chimeric antibodies

The cDNAs encoding the variable heavy (VH) and light (VL) genes of murine mAbs were obtained by PCR using single stranded cDNA libraries from each of the hybridoma cell lines. Amplification of the VH-CH1and VL-CL gene was performed using a mixture of twelve or ten forward primers designed to complement each of the N-terminal sequences of FVH- or FVL-coding regions and a reverse primer designed to complement each C-terminal sequence of the CH1 or CL region. The PCR products were cloned into the pCR2.1-TOPO vector for sequencing analyses. The gene coding sequences for VH and VL were fused to the

genes coding the constant regions of the heavy chain and the light chain of trastuzumab (GenBank accession no: APZ76730.1 and APZ76731.1), respectively. The above genes coding chimeric antibody chains were cloned into the pcDNA3.4 vector downstream of the leader sequence of immunoglobulin kappa light chain. Before transfecting into expi293F cells, all expression vectors were verified by DNA sequencing. A strategy similar to that used for recombinant antigen production was also used to obtain chimeric antibodies.

Fc variants with optimized Fc receptor affinity and specificity can provide substantial ADCC enhancement over wild-type trastuzumab across a broad range of HER2 expression level (Shields et al., 2001). We prepared mouse-human chimeric antibodies whose heavy chain constant region was replaced with the trastuzumab S242D_ A333L _ I335E variant. This designed Fc variant was expected to display > 2 orders of magnitude enhancement of *in vitro* effector function resulting in increased ADCC activity (Lazar et al., 2006).

2.6. Generation of LAK cells

LAK cells were generated from spleen cells of SCID mice by culture in the presence of IL-2 (100 ng/ml) for 3 days and further subcultured for 3 days with IL-2. LAK cells were characterized by flow cytometry with the NK cell markers NKp46 and DX5.

2.7. Vectors

A cDNA encoding human FcγRIIIA harboring the V158F single-nucleotide polymorphism was purchased from OriGene Technologies Inc. (#SC124061). Mouse FcγRIII (GenBank accession no. AK077227) and human CD25 (GenBank accession no. AF008556.1) were amplified by reverse transcription–PCR from total RNA extracted from mouse spleen cells and human PBMC, respectively. Mouse FcRγ cDNA (GenBank accession no. AK155600) was a kind gift of Dr. Takashi Saito (RIKEN-IMS) and hM-CSFR cDNA (GenBank accession no. BC047521) was provided by Dr. Issay Kitabayashi (National Cancer Center Research Institute, Tokyo, Japan). The cDNAs were ligated into the retroviral expression vector pMYs-IRES-GFP (Cell Biolabs, Inc., # RTV-021).

2.8. Transductants

The KHYG-1 NK cell line (Yagita et al., 2000) was transduced with human FcγRIIIA (hFcγR) or mouse FcγRIII and mFcRγ in retroviral expression vectors. B cell lymphoma cell lines Daudi and A20 were transduced with hM-CSFR or hCD25 by using retrovirus vectors. The retroviral expression vectors and the envelope expression vectors, p10A1 or pAmpho were co-transfected into GP2–293 cells using the Retro-X Universal Packaging system (Takara Bio Inc., #631530). At 2–3 days after transfection, the culture supernatants were filtered through 0.45 μm filters and then used to transduce the above cell lines with 10 μg/ml polybrene (Sigma-Aldrich, # P8155). One week after transduction, 40% of the cells expressed the introduced molecules on the surface. The cells were finally sorted to > 98% purity by FACS Aria III (BD Biosciences) and used for ADCC assays.

2.9. Flow cytometry

Cell staining was carried out as described previously (Kaji et al., 2012). Briefly, cells were incubated with Fc blocker for staining of human samples (Miltenyi, # 130–059-901) or 2.4G2 (BD Biosciences, # P9155) for mouse samples. Thereafter cells were stained with the indicated mAb for 20 min on ice, followed by incubation with APC-conjugated anti-mouse IgG (BioLegend, # 405308) in the case of using purified unlabeled mAb to stain. The cells were washed and resuspended in propidium iodide (PI; Sigma-Aldrich, #P4864) and then analyzed by FACS Canto II (BD Biosciences).

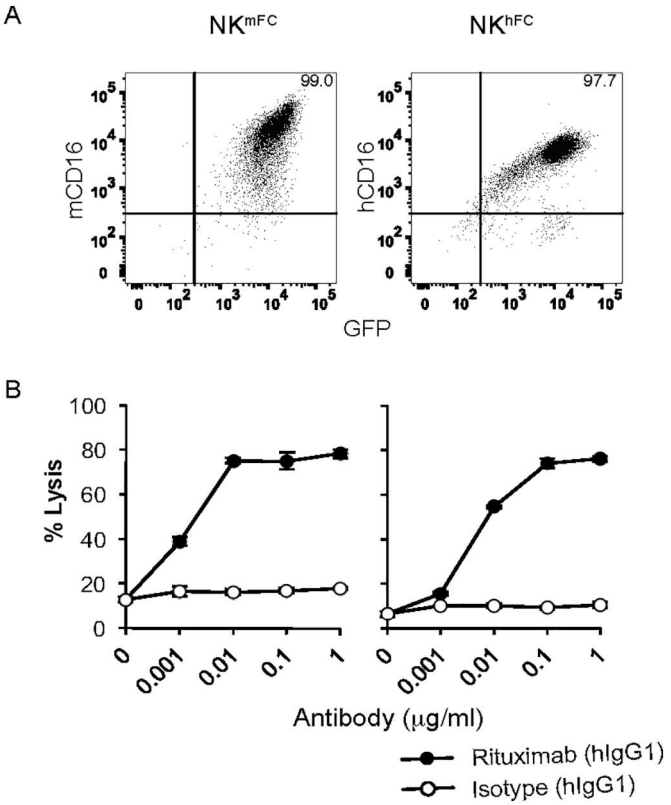

Fig. 1. Human KHYG-1 NK cells transduced with mouse FcyR efficiently mediate ADCC with a human chimeric mAb. (A) Expression of mouse FcyRIII (mouse CD16) and human CD16 with a GFP reporter by KHYG-1 cells (NK^{mFc} and NK^{hFc}, respectively) analyzed by flow cytometry. (B) ADCC activity of NK^{mFc} (left panel) and NK^{hFc} (right panel) against Daudi cell lines in the presence of mouse-human chimeric anti-CD20 lgG1 antibody, rituximab at an E:T ratio of 10:1. ADCC was measured by calcein release assay (see Materials and Methods). Shown on the Y axis is the percentage specific lysis, estimated as described in Materials and Methods. Each bar represents mean ± SD.

2.10. Flow cytometric binding inhibition assay

The M-CSFR+ or hCD25+ Daudi cell lines were incubated with serially diluted anti-hM-CSFR mAbs or anti-hCD25 mAbs, respectively, for 15 min, followed by incubation with phycoerythrin (PE)-coupled hM-CSFR or hCD25 for 15 min. When a biotin-labeled mAb was used instead of PE-conjugated mAb, the cells were stained with UltraAvidin-APC (Leinco Technologies Inc., # A107). Binding inhibition was analyzed by flow cytometry. The median fluorescence intensity (MFI) was calculated using FlowJo software (BD Biosciences).

2.11. Calcein release assay

The target cells were labeled with Calcein-AM (1 μg/ml, Dojindo Laboratories, #C396) for 1 h at 37 °C with occasional shaking, washed and plated onto 96-U bottom well plates at a density of 1×10^4 cells/well. The indicated mAbs were added at various concentrations, and effector cells were then added at an effector: target (E:T) ratio of 10:1. After a 3 h incubation at 37 °C in 5% CO_2, each supernatant was harvested and the fluorescence was analyzed using ARVO x3 (PerkinElmer, Inc.) with excitation filter: 485 ± 9 nm; band-pass filter: 530 ± 9 nm. Spontaneous release was determined by incubation of target cells in medium alone, and maximum release was obtained by suspending cells in 1% Triton X-100. The ADCC percentage for each sample (in triplicate) was calculated using the following formulas: % Lysis = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100.

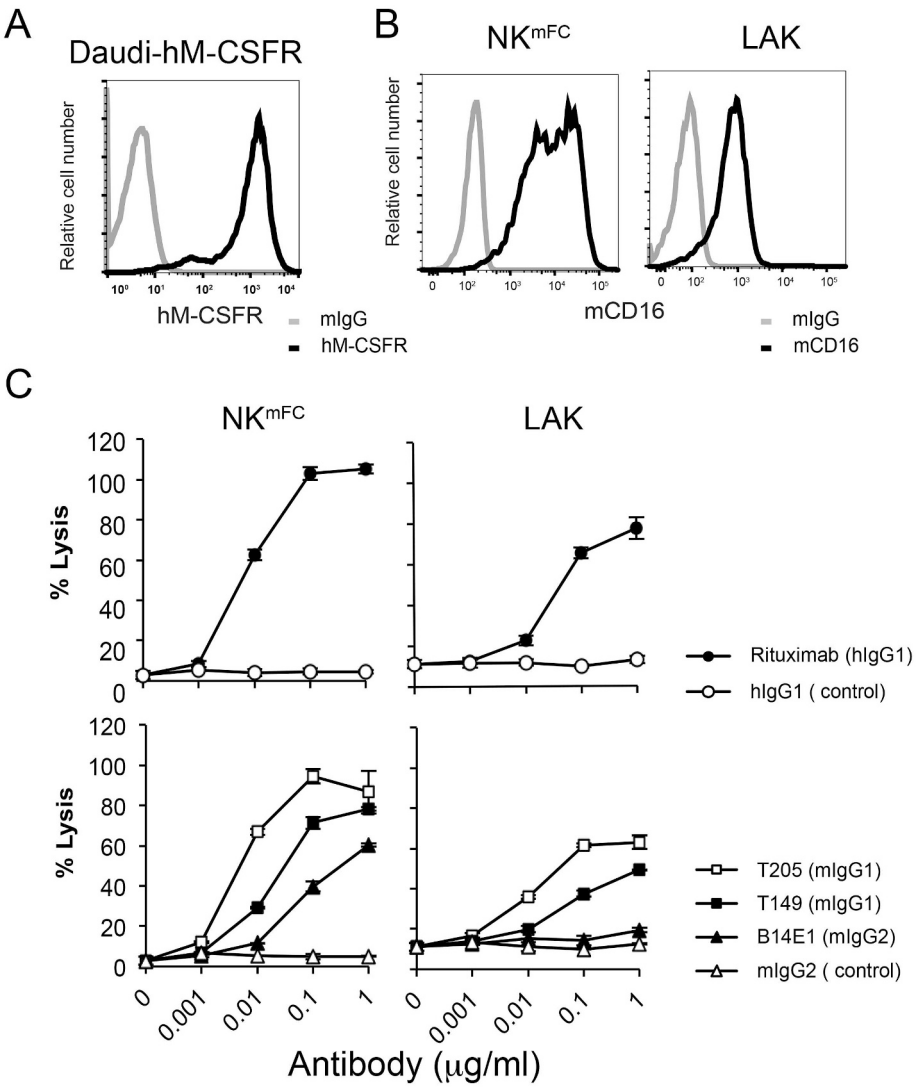

Fig. 2. More potent ADCC activity of NK^{mFc} cells than mouse LAK cells with mouse mAbs. (A) Daudi cells were transduced with an hM-CSFR expression retrovirus vector and used as target cells. Shown is the level of cell surface hM-CSFR expression analyzed by flow cytometry. (B) Expression level of mouse CD16 on NK^{mFc} cells and mouse LAK cells analyzed by flow cytometry. LAK cells were prepared from SCID mouse spleen cells by culture in the presence of IL-2. (C) ADCC activity by NK^{mFc} cells (left panel) and LAK cells (right panel) for the hM-CSFR+ Daudi cell line in the presence of anti-hM-CSFR mAbs (T205, T149 and T236) at different antibody concentrations. Rituximab was used in the assay as a positive control antibody. The assays were repeated twice with similar results.

2.12. Purification of NK cells and ADCC assay

All experiments has been approved by the Ethics Committee of RIKEN, and conformed to the provision of the Declaration of Helsinki. Peripheral blood was obtained from healthy donors after obtaining their informed consent. Purified NK cells were obtained from peripheral blood after centrifugation on a Ficoll/Hypaque gradient, followed by negative selection using by MACS NK Cell Isolation Kit (Miltenyi Biotec, #130-092-657) as enriched NK cells (> 80%) on a FACS LSRFortessa X-20 (BD Biosciences). ADCC assays were performed with purified NK cells and Daudi cells which expressed either hM-CSFR or hCD25 at an effector-target (E:T) ratio of 10:1. Experiments were performed in the presence of humanized chimeric IgG against hM-CSFR or hCD25 and rituximab.

3. Results

3.1. Establishment of KHYG-1 FcγR transduced cell lines as effector cells for the ADCC assay

NK cells that are used in ADCC assays are usually obtained from blood, lymphoid tissue or spleen or prepared as Lymphokine-activated killer (LAK) cells (Nelson et al., 2001). However, the cytotoxic activity of the cells varies depending on their source, and even with the same source of human or mouse prepared at different times, which makes it difficult to compare the results between assays. To develop an ADCC assay that allows stable measurement with as high sensitivity as possible, an NK leukemic cell line was transfected with a human FcγRIIIA gene and used as effector cells in ADCC assays with human antibodies (Mishima et al., 2012).

To extend the system to allow measurement ADCC with mouse antibodies, we established stable NK transductants with mouse FcγR (NK^{mFC}, Fig. 1A). As mFcγRIII binds both mouse and human IgG Abs of different isotypes (Dekkers et al., 2017), NK^{mFC} should mediate ADCC by both human and mouse antibodies (Overdijk et al., 2012). In fact, we observed in a calcein release assay that NK^{mFC} mediated ADCC in the presence of chimeric mouse-human CD20 mAb (rituximab) (Clemenceau et al., 2013) with the Daudi B cell lymphoma cell line as a target as efficiently as did an NK cell line transfected with human FcγR (NK^{hFC}) (Fig. 1B). We compared the calcein and LDH assays and observed that the calcein assay is more sensitive than LDH assay (Supplemental Fig. 1).

3.2. NK^{mFC} is useful for detection of mAbs with ADCC activity

We next compared the activity of NK^{mFC} and LAK cells in an ADCC

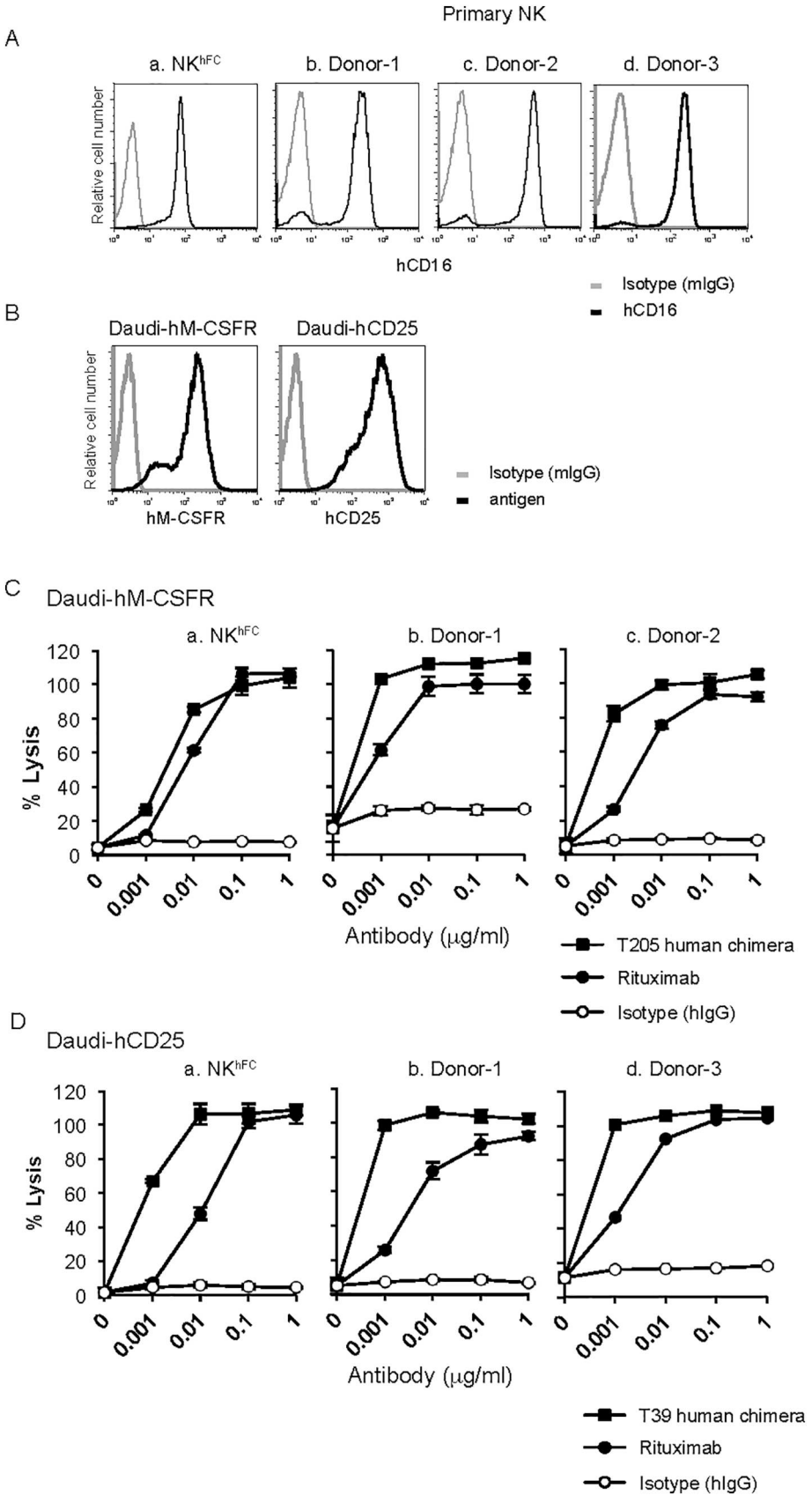

Fig. 3. Comparison of the ADCC activity obtained with the NK^{hFc} cell line and with NK cells derived from normal donors. (A) Expression of human FcγRIIIA (CD16) on effector cells. (B) Expression level of hM-CSFR (left panel) and hCD25 (right panel) on the surface of the Daudi cell line analyzed by flow cytometry. (C) ADCC activity of NK^{hFc} (a) and human primary NK cells (band c) at an E:T ratio of 10:1. ADCC was performed against hM-CSFR + Daudi cell lines in the presence of the T205M mouse-human chimeric antibody by calcein release assay. (D) ADCC activity of NK^{hFc} (a) and human primary NK cells (b and d) at an E:T ratio of 10:1 against hCD25 + Daudi cell lines in the presence of anti-CD25 mouse-human chimeric antibody (T39-11-7M). ADCC activity was measured by calcein release assay.

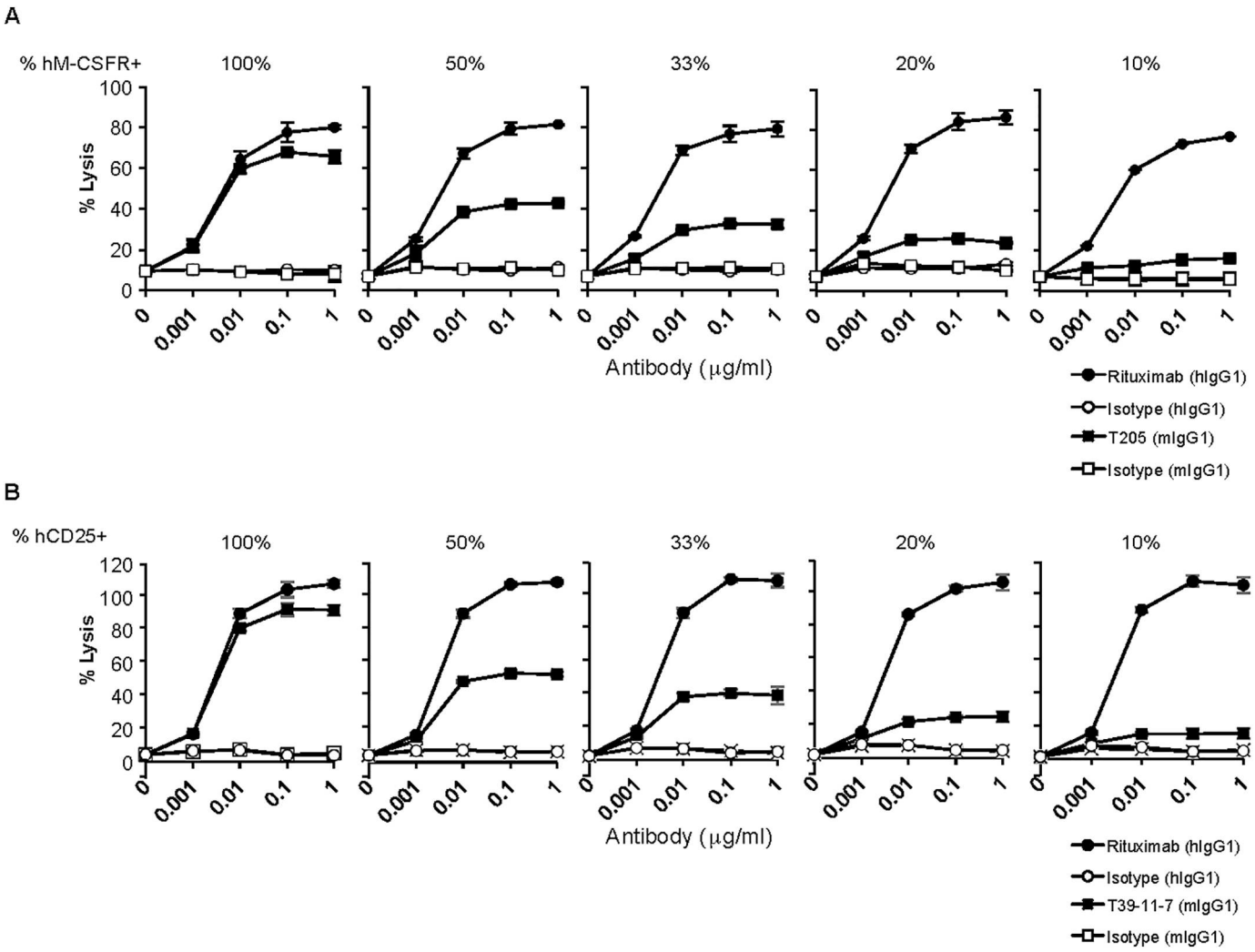

Fig. 4. Limit of ADCC function of NK^{mFc} cells in a calcein release assay with low frequency target cells. hM-CSFR+ Daudi transfectants (A) or hCD25+ Daudi transfectants (B) were mixed with parental cells at different ratios and used for target cells. ADCC was measured in the presence of NK^{mFc} cells and mouse anti-hM-CSFR mAb, clone T-205 (A) or mouse anti-hCD25 mAb, clone T39-11-7 (B). ADCC activity was also measured in the presence of rituximab as a positive control and hIgG1 isotype as a negative control. The E:T cell ratio was 10:1.

model system. We prepared hybridoma cell lines from mice immunized with human macrophage colony-stimulating factor receptor (hM-CSFR), which is a known target candidate for human lymphoma and leukemia (Patel and Player, 2009; Aikawa et al., 2010; Lamprecht et al., 2010). We established Daudi B cell lymphoma transductants expressing hM-CSFR (Aikawa et al., 2010) (Fig. 2A) as target cells and screened individual culture supernatants for reactivity to hM-CSFR, followed by an ADCC assay using NK^{mFC} and LAK (Fig. 2B). We observed that ADCC activity in mAb culture supernatants of different Ig isotypes was reported more reliably by NK^{mFC} than by LAK cells (Supplemental Fig. 2).

We confirmed the results in Supplemental Fig. 2 using purified mAbs (Fig. 2C). NK^{mFC} mediated potent ADCC activity against the hM-CSFR transductant cells even in the presence of a low dose of mouse hM-CSFR mAbs, as low as 0.01 μg/ml. By contrast, LAK mediated ADCC but it was much less efficient compared to NK^{mFC}. The difference in activities may reflect the level of FcR expressed on the surface of the two cell types (Fig. 2B).

To know whether the human NK transfectant has the same level of ADCC ability as NK cells in PBMC, we performed an ADCC assay by using NK transductants with human FcγRIII (NK^{hFC}) and NK cells enriched from PBMC of healthy donors, in the presence of mouse-human chimeric anti-hM-CSFR (T205 M) and, in addition, anti-hCD25 (T39–11–7 M) mAbs, whose heavy chain constant region had been replaced with human IgG1Fc region carrying amino acid substitutions that are expected to enhance *in vitro* effector function (Shields et al., 2001). Fig. 3 showed that NK^{hFC} mediated ADCC activity for either hM-CSFR+ or hCD25+ target cells as effectively as did NK cells enriched from PBMC of healthy donors. These results suggested that human NK^{hFC} cell lines and human NK cells are mostly comparable in their activity to mediate ADCC by chimeric mAbs with potent activity.

3.3. Limitations in the calcein release assay with low frequency target cells

The target cells obtained from clinical samples are usually a mixture of many types of cells, such as disease causing cells and normal cells. It is also known that phenotypic and functional heterogeneity arises among cancer cells within the same tumor (Marusyk and Polyak, 2010; Meacham and Morrison, 2013). Therefore, an ADCC assay that is robust enough to measure activity even against low-frequency target cells in assay samples is highly desirable. To determine the lower limit of target cell frequency in the NK^{mFC} ADCC assay, hM-CSFR+ Daudi transductants were mixed with parental cell lines at different ratios and tested for ADCC mediated by the T205 mAb (Fig. 4A). The ADCC susceptibility was high in a mAb-dose dependent manner when the frequency of the hM-CSFR+ Daudi target cell line was 100%; however, it became difficult to interpret the data when the frequency of target cells was below

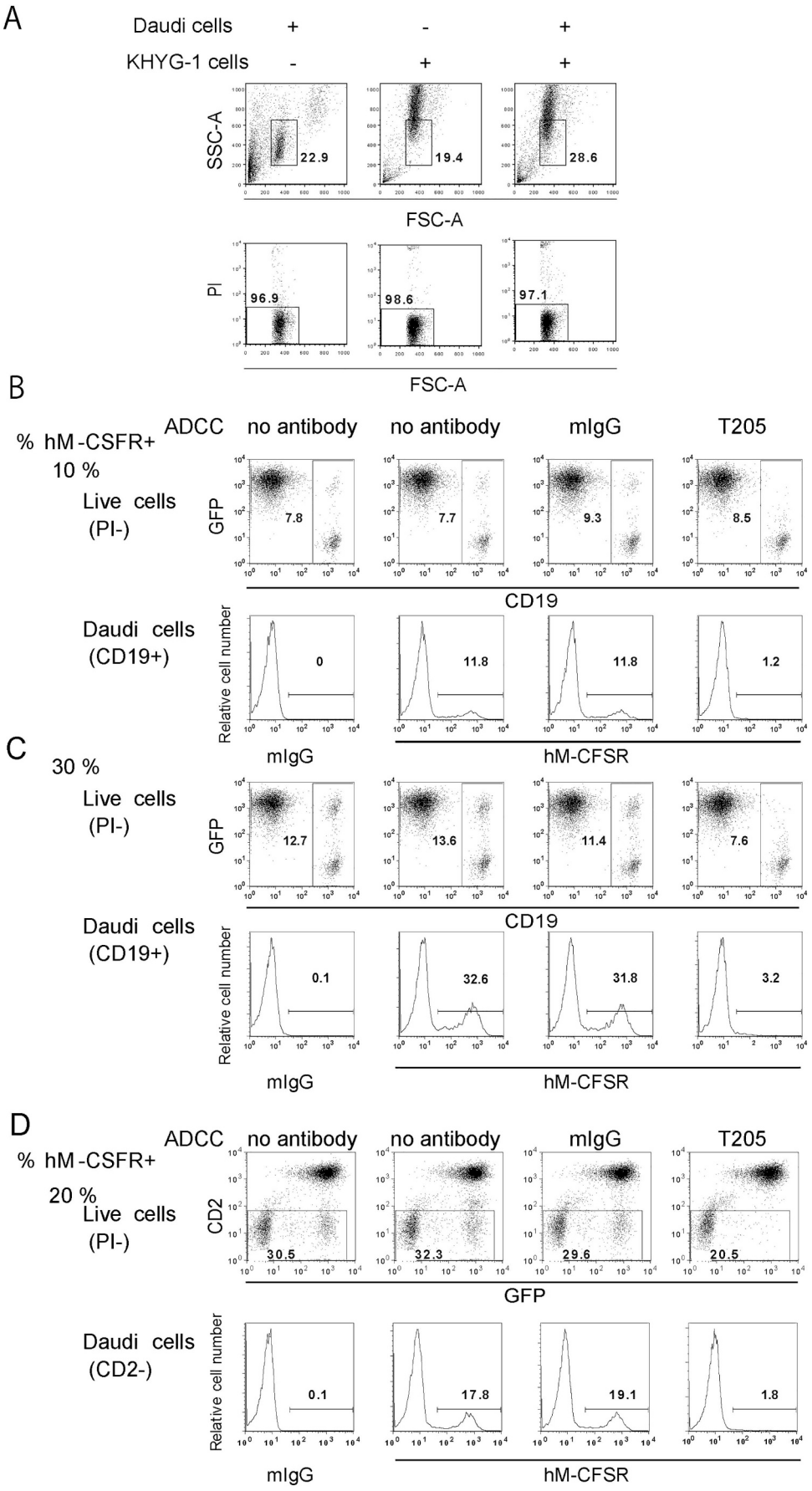

Fig. 5. Flow cytometric ADCC assay enables measurement of killing of low frequency target cells by anti-hM-CSFR mAb. (A) Gating strategy for Daudi cells mixed with NK^{mFc} in ADCC assay using forward scatter and side light scatter (FSC/SSC). Upper panel shows dot plots for Daudi cells (left panel), NK cells (middle panel) and a mixture of both in the ADCC assay (right panel). Mixed cells were gated based on the FSC/SSC gate for Daudi cells (upper panel). After gating out doublet cells, the mixed cells enriched for Daudi cells were identified as PI negative cells (lower panel). (B and C) Discrimination of NK^{mFc} (GFP+), CD19+ hM-CSFR+ Daudi (GFP+) and Daudi parental cell lines (GFP-) in enriched cell populations. hM-CSFR+ Daudi cells mixed with parental cells at 10% (B, upper panel) and 30% (C, upper panel). CD19+ cells were gated to estimate the number of target cells. Lower panels shown histogram of hM-CSFR+ Daudi and Daudi parental lines. (D) Discrimination of CD2+ NK^{mFc}, hM-CSFR+ Daudi (GFP+) and Daudi parental cell lines (GFP-). hM-CSFR+ Daudi cell lines mixed with parental cell lines at 20%. Lower panels shown histogram of hM-CSFR+ Daudi and parental cell lines. Specific killing of hM-CSFR+ Daudi target cells is indicated by their decreased frequency only in the presence of both hM-CFSR and T205 mAbs. Numbers in the histogram indicate % of hM-CSFR+ Daudi cells in target cells.

Table 1
Summary of FACS ADCC assays for anti-hM-CSFR and anti-hCD25 mAbs[a].

		ADCC[d]		
Target cells[b]	Staining mAbs[c]	no antibody	mIgG	T205
10% hM-CSFR+	CD19 (target cells)	11.8%[e]	11.8%	1.2%
30% hM-CSFR+	CD19 (target cells)	32.6%	31.8%	3.2%
20% hM-CSFR+	CD2 (effector cells)	17.8%	19.1%	1.8%
Target cells[f]	Staining mAbs[g]	no antibody	mIgG	T39-11-7
Ex1				
10% hCD25+	CD2 (effector cells)	9.4%	8.6%	1.1%
20% hCD25+	CD2 (effector cells)	17.8%	17.1%	2.5%
30% hCD25+	CD2 (effector cells)	26.9%	25.2%	3.2%
Ex2				
10% hCD25+	CD2 (effector cells)	10.0%	10.4%	2.0%
20% hCD25+	CD2 (effector cells)	22.7%	21.6%	4.5%
30% hCD25+	CD2 (effector cells)	31.1%	32.4%	8.0%

[a] Data based on the results in Figs. 5 and 6.
[b] hM-CSFR+ Daudi cells mixed with parental cells at 10%, 20%, and 30%.
[c] CD2+ NK^{mFC} effector cells in the ADCC assay were discriminated from hM-CSFR+ Daudi cells mixed with parental cells (CD19+) by either anti-CD19 mAb or anti-CD2 mAb staining.
[d] ADCC by NK^{mFC} was performed for hM-CSFR+ or hCD25+ Daudi cell lines in the absence (no antibody) or presence of mouse mAb with specificity for the target antigen or isotype control (mIgG).
[e] The % cell numbers was calculated as 100 × hM-CSFR+ or hCD25+ Daudi cells / PI- Daudi parental cells.
[f] hCD25+ Daudi cells mixed with parental cells at 10%, 20%, and 30%.
[g] CD2+ NK^{mFC} effector cells in the ADCC assay were discriminated from hCD25+ Daudi cells mixed with parental cells (CD19+) by anti-CD2 mAb staining.

20%. Likewise, ADCC mediated by mouse anti-hCD25 mAb, T39-11-7 was effective against a hCD25+ Daudi target cell line when the cells were present at high frequency, but became difficult to evaluate when the frequency of target cells was low (Fig. 4B).

3.4. Establishment of a flow cytometry-based ADCC assay

We established an experimental model to determine whether a flow cytometric ADCC assay would enable measurement of a low frequency of target cells. hM-CSFR+ Daudi cell lines expressing a GFP reporter were added to the parental Daudi cell line at a frequency of 10% and 30% and then incubated with the NK^{mFC} cell line in the presence of the hM-CSFR mAb (Fig. 5). After a 3 h incubation, cells were washed and stained with phycoerythrin (PE)-labeled hM-CSFR mAb, 9-4D2-1E4 (Breton et al., 2015; Lee et al., 2015). Importantly, 9-4D2-1E4 recognizes an epitope different from that of mAb T205, which was used in the ADCC assay (Supplemental Fig. 3). As shown in Fig. 5A, a mixture of the hM-CSFR+ Daudi target and the parental Daudi cell line could be distinguished from NK^{mFC} cells in ADCC cultures. This was based on cell shape, which is proportional to cell-surface size as measured by forward-scattered light, FSC, and cell granularity (side-scattered light, SSC). After exclusion of doublets and dead cells (data not shown), Daudi cells were distinguished from NK^{mFC} cells based on expression of the CD19 B cell marker (Fig. 5B and C, upper panels) and the frequency of GFP+ Daudi cells in the mixture with parental cells was determined (Fig. 5B and C, lower panels). The results shown as histograms indicated that FACS analysis made it possible to measure ADCC activity with 10% target cells, which was not feasible in the calcein release assay (Fig. 5B and Table 1).

In an attempt to make the flow cytometric ADCC analysis even more sensitive, NK^{mFC} effector cells were distinguished in the co-cultures using CD2 as a marker of KHYG-1 effector cell lines instead of target cell staining (Fig. 5D, upper panel). In this protocol, ADCC activity against M-CSFR+ Daudi target cells at a 20% mixture of parental cell lines could be measured by flow cytometry (Fig. 5D, lower panel and Table 1).

The characteristics of this method was also demonstrated in ADCC by using mAb other than anti-hCD25 mAb. As shown in Fig. 6, we analyzed ADCC activity by anti-hCD25 mAb, T39-11-7, for hCD25+ Daudi transfectants (GFP+) mixed with parental cell lines (GFP-) at different frequencies. As shown in the histograms, after exclusion of effector cells by anti-CD2 mAb, % of CD25+ target cells was monitored by staining with anti-CD25 mAb, BC96, which has specificity for a CD25 epitope different from T39-11-7 (Supplemental Fig. 4A). Fig. 6A suggest that flow cytometric ADCC quantified killing of $hCD25^{+}$ target cells at a 10% mixture of parental cell lines (lower panel). The results are summarized in Table 1. Taken together, these results indicate that this new flow cytometric assay is more robust than other existing assays in accurately evaluating ADCC activity in a clinically relevant situation where there is a low frequency target cells.

3.5. ADCC flow assays allowed for identification of synergistic ADCC by using two mAbs recognizing distinct epitopes

We analyzed ADCC on transductants of the A20 cell line that express a low level of cell surface hM-CSFR, as evaluated by staining with a PE-labeled M-CSFR mAb, 12-3A3–1B10 (Breton et al., 2015), approximately 10-fold less than that of the hM-CSFR+ Daudi cells (Fig. 7A). Consistent with reports that antigen expression level on target cells is particularly relevant to ADCC susceptibility (Jazirehi et al., 2007; Johnson et al., 2009; Horvat et al., 2010), hM-CSFR-dependent ADCC in the flow cytometric assay was low for A20 as compared to Daudi cells, even at a high dose of mAbs recognizing different epitopes on hM-CSFR (Fig. 7B and C, upper panels). In agreement, a calcein release assay showed that A20 transductants with a lower density of hM-CSFR antigen were less sensitive to ADCC than hM-CSFR+ Daudi transductants, even at a high dose of antibodies (Fig. 8, left panel). Thus, these results confirmed that the limitation of ADCC activity is proportional to the level of target antigen.

To compensate for this problem of small amounts of antibody binding in ADCC due to low antigen density, we treated A20 transductant targets with a combination of two mAbs recognizing distinct epitopes on hM-CSFR. These mAb, by themselves, had little if any ADCC activity even at a high concentration. However, in a combination ADCC assay the B14E1 (mIgG2a) mAb had enhanced killing activity when mixed with either T205 (mIgG1) or T149 (mIgG1) mAbs, which recognize an epitope distinct from B14E1 (Fig. 7B, lower panel and Supplemental Fig. 5). However, a mixture of T205 and T149, which recognize the same epitope, did not show any synergy. A synergistic effect of mAbs recognizing different hM-CSFR epitopes was also apparent in ADCC against hM-CSFR+ Daudi cell lines (Fig. 7C, lower panel). The T149 and B14E1 mAbs had no significant ADCC activity at a low concentration, however, B14E1 acquired significant activity when mixed with the same low dose of T205 or T149. In addition, a high concentration of T205 and B14E1 eliminated the small number of remaining cells expressing a low density of antigens (Fig. 7C, lower panel).

A synergistic effect of mAbs recognizing different epitopes on the same molecule was also apparent in ADCC assay with anti-hCD25 mAbs, T39-11-7 and T32-6-16. T39-11-7, T32-6-16 and BC96 used for flow cytometric ADCC assay have different epitope specificities (Supplemental Fig. 4B). Each mAb had little ADCC activity on A20 transfectants, which express low level of hCD25 on the surface (Fig. 9A), However, flow cytometric ADCC assay suggests that a mixture of low dose of T39-11-7 and T32-6-16 efficiently killed the targets (Fig. 9A), The results were also supported by calcein release assays (Fig. 9A, left panel). Each mAb had potent ADCC activity for Daudi cell lines, which expressed a high level of hCD25 and a mixture of both mAbs at low doses eliminated most of target cell lines in the presence of NK^{mFC} (Fig. 9B).

Based on these results, we speculate that a combination mAb therapy could be an effective approach for ADCC against resistant

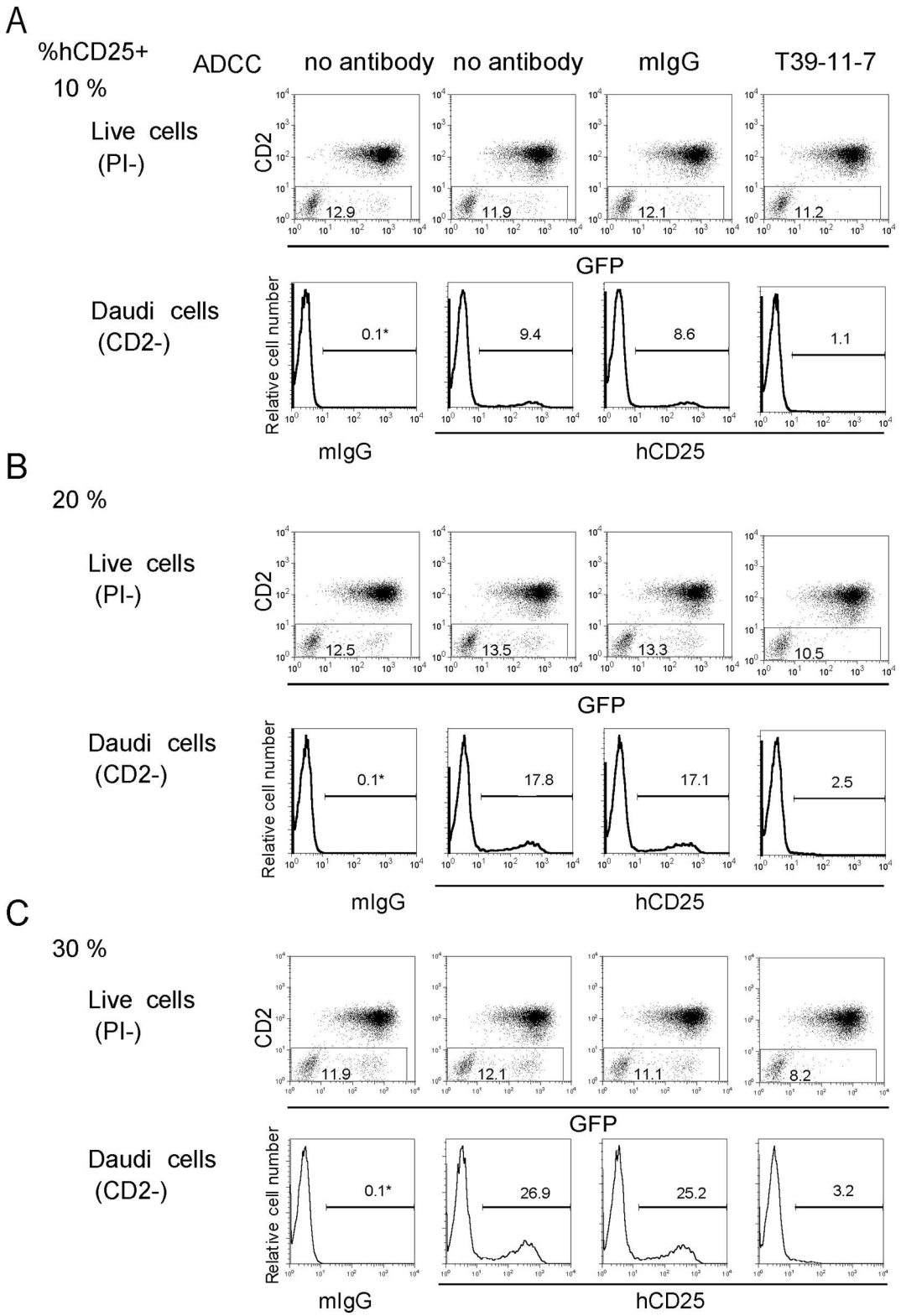

Fig. 6. Flow cytometric ADCC assay quantifies killing of low frequency target cells by anti-hCD25 mAb. The NK^{mFc} cell line was discriminated by CD2 staining from hCD25+ Daudi (GFP+) and Daudi parental cell lines (GFP-). hCD25+ Daudi cell lines mixed with parental cell lines at 10% (A), 20% (B) and 30% (C). Specific killing of hCD25+ Daudi target cells is indicated by their decreased frequency only in the presence of both hCD25 and T39-11-7 mAbs. Numbers in histogram indicate % of hCD25+ target cells. 0.1* indicates that the % of living cells was below 0.1%.

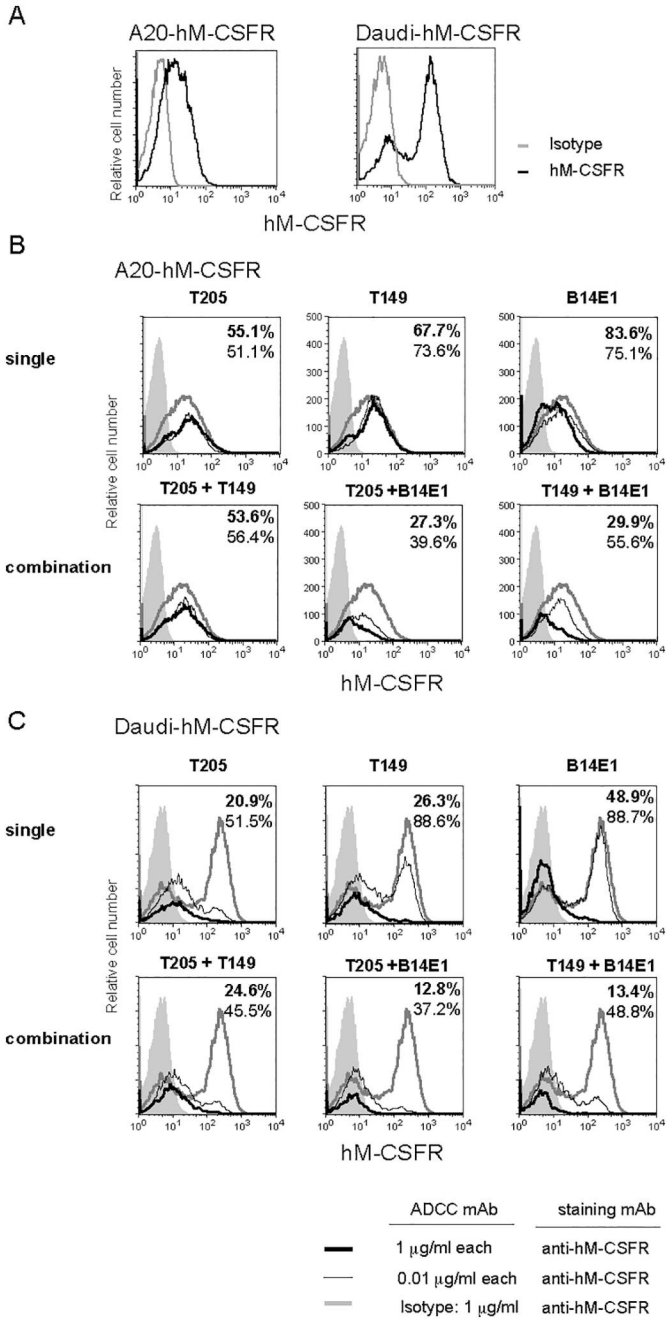

Fig. 7. A combination of two hM-CSFR mAbs recognizing different epitopes enhances ADCC activity. A combination of two hM-CSFR mAbs recognizing different epitopes enhances ADCC activity. (A) Expression of hM-CSFR on the target cell surface. hM-CSFR+ A20 cells (left) and Daudi cells (right) were stained with a PE-labeled hM-CSFR mAb, 12-3A3-1B10. (Band C) ADCC assay for hM-CSFR+ A20 cells (B) and Daudi cells (C) in the presence of hM-CSFR mAbs, T205, T149 or B14E1, or isotype control (upper panel). A combination of two mAbs with distinct epitope specificities (B14E1 plus T205 or B14E1 plus T149) exerted strong ADCC activity when the antigen expression level on target cells was low, but if the two mAb recognized the same epitope (T205 plus T149) there was no synergy (lower panel). The gray filled histograms represent background staining with a corresponding PE-Iabeled isotype control mAb. The number of viable target cells was evaluated as described in Fig. 5, and normalized relative to 1×10^5 NK^{mFc} cells. The percentage of viable target cells was calculated using the number of normalized target cells with the isotype control treatment in ADCC and is indicated for the two hM-CSFR mAb concentrations (1 μg/ml and 0.01 μg/ml; 1 μg/ml is in bold). Flow cytometric analysis utilized a PE-Iabeled 12-3A3-1B10 mAb for detection of hM-CSFR+ target cells. 12-3A3-1B10 does not react with the same epitope as T205, T149, or B14E1 mAbs as defined by flow cytometric inhibition assays (see Supplemental Fig. 5B.

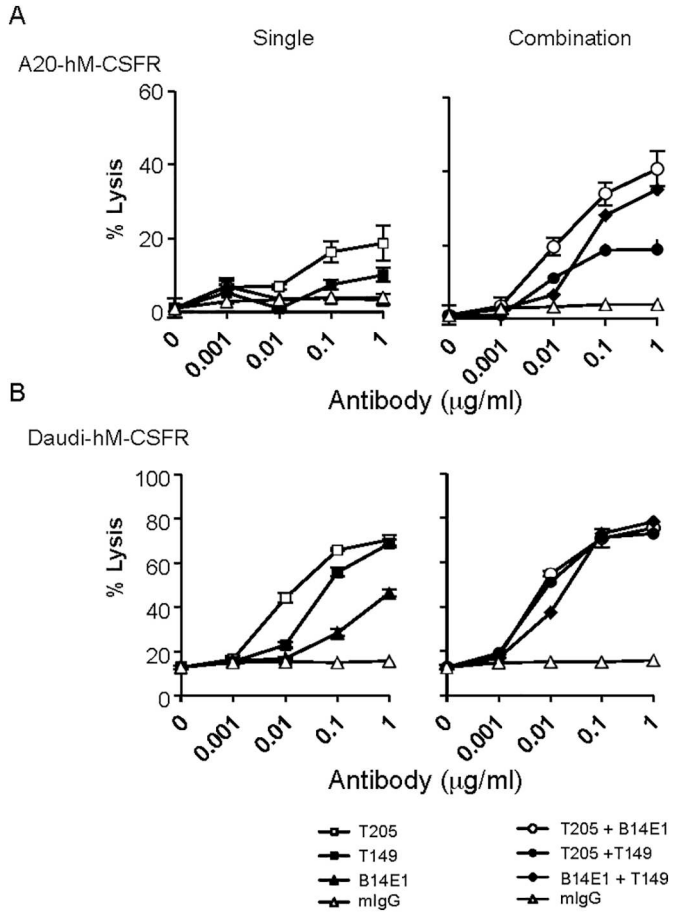

Fig. 8. Synergistic effect in ADCC by a combination of two mAbs against hM-CSFR in a calcein release assay. (A) ADCC activity for hM-CSFR+ A20 cells and (B) Daudi cells. The ADCC assay was performed in the presence of single mAbs (left panel) and with a combination of two mAbs (right panel). The treatment with a combination of mAbs exerted strong ADCC activity at low expression levels of antigen on A20 cells (see Fig. 7 A) compared to the single mAb (A, right and left panels respectively).

cancer cells expressing a low density of target antigens (Jazirehi et al., 2007; Johnson et al., 2009; Horvat et al., 2010).

4. Discussion

In the present study, we developed a new flow cytometry-based ADCC assay using an NK effector cell line transfected with mouse and human FcγR. Following binding of the mAb to the target antigen, the Fc portion of the mAb interacts with the FcR on the surface of the effector cells, leading to granule-mediated cytotoxicity. The human NK leukemia cell line, KHYG-1, was reported to have strong cytotoxicity compared with other NK cell lines such as NK-92 and YT through delivery of cytotoxic enzymes (Yagita et al., 2000; Suck et al., 2005). However, KHYG-1 cells lost the expression of human FcγRIIIA during their establishment and thereby lost their ADCC activity (Yagita et al., 2000), but it could be restored by transduction of hFcγR (Mishima et al., 2012). To optimize the screening system for use with mouse mAbs, mouse-human chimeric mAbs, and human Ig transgenic mouse-derived mAbs, we established a KHYG-1 cell line transfected with mouse FcγRIII and FcRγ and with human FcγRIIIA. As expected, KHYG-1-mFcγR cells exhibited potent ADCC activity with mouse mAbs and furthermore, were as efficient as the KHYG-1 cell line transfected with human FcγR when tested with mouse-human chimeric mAbs.

Development of potent mAbs optimized for ADCC activity is essential to improve the clinical response (de Romeuf et al., 2008; Le Garff-

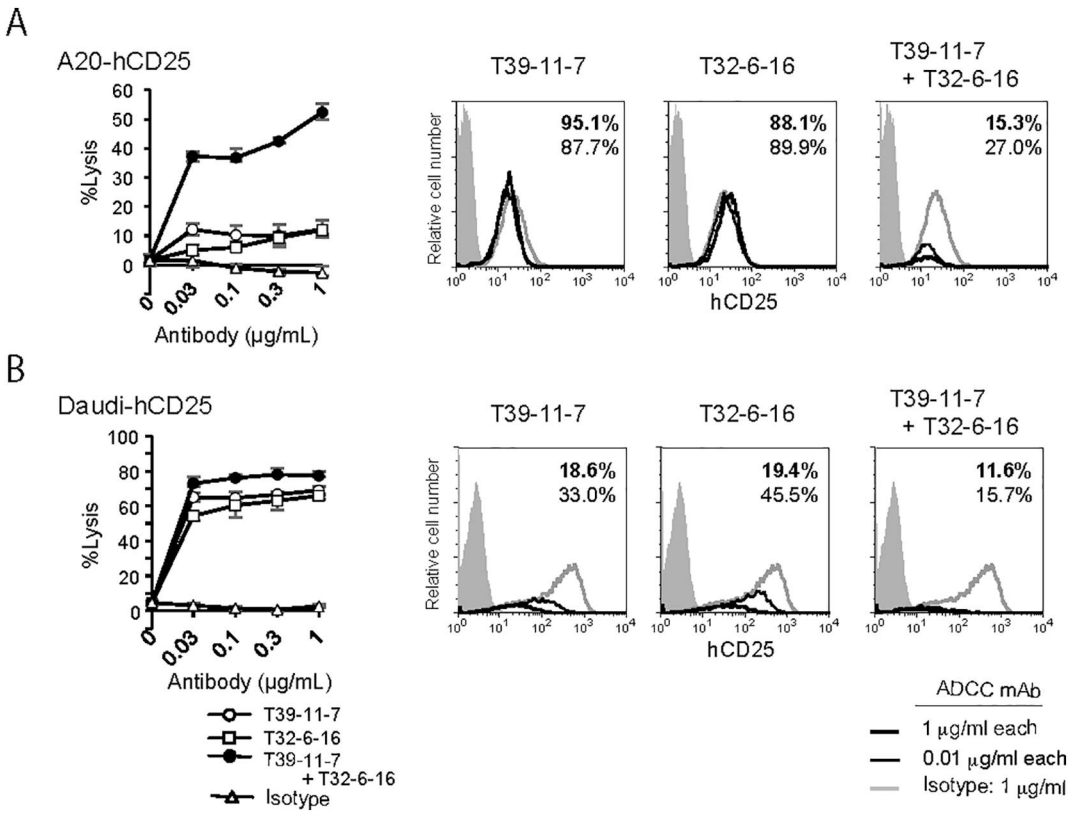

Fig. 9. A combination of two anti-CD25 mAbs recognizing different epitopes enhances ADCC activity. ADCC with hCD25+ A20 cells (A) and hCD25+ Daudi cells (B) in the presence of mouse anti-hCD25 mAbs, T39-11-7 and T32-6-16, and isotype control in a calcein release assay (dose-response curve, left panel) and flow cytometric ADCC assay (histgrams). Flow cytometric analysis utilized a PE-Iabeled BC96 mAb for detection of hCD25+ target cells. BC96, T39-11-7 and T32-6-16 mAbs recognize different epitopes on the hCD25 molecule as defined by flow cytometric inhibition assays (see Supplemental Fig. 4). The gray filled histograms represent background staining with a corresponding PE-Iabeled isotype control mAb. The number of viable target cells was evaluated as described in Fig. 5, and normalized relative to 1×10^5 NK^{mFc} cells. The percentage of viable target cells was calculated using the number of normalized target cells with the isotype control treatment in ADCC and is indicated for the two anti-hCD25 mAb concentrations (1 μg/ml and 0.01 μg/ml; 1 μg/ml is in bold). Numbers in the histogram represent % living target cells.

Tavernier et al., 2011). The present method enables selection of mAbs with potent ADCC from a large number of target recognizing mAbs. Selected mAbs have the *in vitro* activity mostly comparable to NK cell lines and NK cells in PBMC. As it has been observed that activity of functional NK cells in patients and in healthy controls are almost comparable (de Romeuf et al., 2008; Kanakaraj et al., 1994; Le Garff-Tavernier et al., 2011), antibodies receiving an evaluation of "excellent: ADCC function by the present method could be listed as therapeutic candidates for further clinical examinations. Further analysis is needed to clarify this issue.

FcR stimulation recruits Syk *via* tyrosine phosphorylation of the ITAM motif by Src kinases. This process results in the phosphorylation and recruitment of a variety of intracellular substrates, leading to a defense response, including ADCC activity (Kanakaraj et al., 1994; Johnson et al., 1995). Human FcRγ has significant homology with mouse FcRγ (88.4%) and the ITAM motif is also very well conserved (Supplemental Fig. 6). Therefore, we assumed that Src family kinases might be associated with the mFcRγ in an inactive form in a human NK cell line. Kinases become activated upon sustained mFcR receptor aggregation and rapidly tyrosine-phosphorylate the ITAM sequences, forming SH2 sites for the docking and activation of Syk kinases.

NK cells participate in xenograft rejection *via* perforin and granzyme B dependent ADCC (Clynes et al., 2000; Rieben and Seebach, 2005). The KHYG-1 transductant delivered ADCC against mouse target cells, indicating that effector molecules mediating ADCC are not species-specific. Considering these results, we conclude that mFcγR KHYG-1 effector cells are appropriate for evaluating Fc-mediated ADCC activity against human and mouse target cells.

Mouse IgG antibodies of γ1, γ2a, and γ2b isotypes bind to mFcγRIII on mouse NK cells (Rosales and Uribe-Querol, 2013), so we assumed that NK^{mFC} would mediate ADCC activity without being influenced by IgG antibody isotypes. In fact, anti-hM-CSFR mAbs T205 and T149 (IgG1), B14E1 (IgG2a) and T191 and N42 (IgG2b) were all capable of inducing ADCC with the NK^{mFC} effector cell line against hM-CSFR+ Daudi target cells. The NK^{mFC} ADCC activity is more potent than that of LAK cells, probably owing to a higher level of FcR on NK^{mFC}. Thus, our system should be useful for primary screening of hybridomas for mAbs with ADCC activity regardless of the amount of antibodies present in different culture supernatants.

We found that multiple parameter flow cytometric analysis permits more clear measurement of ADCC for less frequent target cells than the calcein assay. A number of ADCC-based flow cytometric methods have been previously proposed as alternatives to the ^{51}Cr or calcein release assays (Richard et al., 2014; Salinas-Jazmin et al., 2014; Gillissen et al., 2016; Yamashita et al., 2016). These assays used target cells labeled with fluorescent dyes to distinguish them from effector cells (Richard et al., 2014; Salinas-Jazmin et al., 2014; Gillissen et al., 2016; Yamashita et al., 2016), however, specific labeling of the target cells is difficult when dealing with heterogeneous clinical samples. It is also difficult to label effector cells derived from heterologous peripheral lymphocytes, resident NK cells or LAK cells, without cell damage. The present assay system can distinguish target cells from the KHYG-1 cell line by recognition of the target antigen with antibody of a different specificity from those used in the ADCC assay. In addition, the effector cells are recognized their stable expression of GFP, together with the

specific surface marker CD2.

The NK^{hFC} ADCC activity for chimeric mAbs is mostly well-matched to that of NK cells enriched from PBMC of healthy donors. In order to select a promising therapeutic candidates for clinical applications, antibody-dependent cellular cytotoxicity assays are usually performed in a combination of patient NK cells and autologous target cells (Kline et al., 2012; Xie et al., 2017) (de Romeuf et al., 2008) or patients NK cells and transfectant (Le Garff-Tavernier et al., 2011).

The present method by flow cytometer can be applicable to the clinical ADCC examinations by discriminating patient NK cells from autologous target cells by use of antibodies for target cells and for NK cells (Vivier et al., 2011). This method has the advantage of being able to measure ADCC activity for low frequency targets in blood and tissue samples and it also shortens the time of performance without having to label target cells with ^{51}Cr or calcein acetoxymethyl ester. Further analysis is needed to clarify this possibility.

A clinical study using rituximab indicated that the level of expression of the CD20 target antigen is an important predictive factor in the ADCC response (Johnson et al., 2009; Horvat et al., 2010). Consistently, cell surface CD20 expression was low on a rituximab-resistant clone established from a lymphoma cell line (Jazirehi et al., 2007), and our flow cytometric analysis clearly showed that target cells bearing a lower density of the target antigen escaped from the ADCC.

However, by using flow cytometric analysis it became apparent that two mAbs recognizing different antigen epitopes could synergistically increase ADCC activity against target cells that express a low density of the target antigen. The activity mediated by a low concentration of each of the mAbs was greater than that observed with the highest concentration of each mAb when used individually. If such a synergistic effect is a general rule, ADCC mediated by different antibodies recognizing the same antigen can be expected to improve cancer treatment when the cancer cells have a low antigen density. Further analysis is required to clarify the mechanism.

ADCC is thought to be an essential mechanism of the antitumor activity of mAbs but is associated with the development of resistant target cells (Reslan et al., 2009). It is known that the majority of cancer cells are heterogeneous in terms of morphological and physiological features (Marusyk and Polyak, 2010). We speculate that this new assay system could be broadly applicable to analyze the properties of target cells resistant to cell death from within heterogeneous populations containing different cell subtypes and cell states.

5. Conclusions

In this study, we developed flow cytometric ADCC using an NK cell line stably transduced with mouse FcγR or human FcγR and a GFP reporter for use as effector cells. The NK cell line mediates ADCC by both human and mouse antibodies, and this method showed that quantitative cytotoxic analysis can be performed against even minor populations of target cells expressing antigen. The findings obtained in the experiments and expanded in the Discussion have shown that cancer cells expressing a lower density of antigens are substantially resistant to ADCC activity. However, combination mAbs recognizing different antigen epitopes could significantly increase ADCC activity against resistant cancer cells. Furthermore, this method could be useful for the evaluation of therapeutic candidate mAbs in ADCC activity for clinical samples expected to contain a small and heterogeneous population of target cells.

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Declarations of interest

None.

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