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Research paper

Determination of human γδ T cell–mediated cytotoxicity using a non-radioactive assay system

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ABSTRACT

The adoptive transfer of immune effector cells, such as $CD8^+$ killer αβ T cells, γδ T cells, NK (natural killer) cells, and genetically–modified T cells, has been receiving increasing attention. It is essential to determine cellular cytotoxicity so as to monitor the function and quality of ex vivo–expanded immune effector cells before infusion. The most common method is the [^{51}Cr]–sodium chromate release assay. It is, however, preferable to avoid the use of radioactive materials in clinical laboratories. In order to establish a non-radioactive alternative to the standard radioactive assay, we previously synthesized a chelate–forming prodrug (BM-HT) and demonstrated that a combination of BM-HT and europium (Eu^{3+}) was useful to determine NK cell–mediated cytotoxicity. In the present study, we examined whether or not this improved assay system could be used to determine the cellular cytotoxicity exhibited by $V\gamma2V\delta2^+$ γδ T cells. In addition, we compared Eu^{3+} and terbium (Tb^{3+}) in the measurement of cellular cytotoxicity. Our assay system using BM-HT could be used successfully for the analysis of both γδ T cell receptor (TCR)– and CD16–mediated cytotoxicity. When the intensity of fluorescence was compared between Eu^{3+} and Tb^{3+}, Tb^{3+} chelate was more sensitive than Eu^{3+} chelate, suggesting that the detection system using Tb^{3+} is superior to Eu^{3+} when tumor cells are not efficiently labeled with BM-HT. The method established herein is expected to promote the development of novel adoptive cell therapies for cancer.

1. Introduction

Cancer immunotherapy has received significant attention since the success of immune checkpoint inhibitors and chimeric antigen receptor (CAR)–T cells (Leach et al., 1996; Iwai et al., 2002; Couzin-Frankel, 2013; June and Sadelain, 2018). For the adoptive transfer of immune effector cells, it is essential to determine cellular cytotoxicity in clinical laboratories. The most common method to monitor the cytotoxicity of ex vivo–expanded immune effector cells is the [^{51}Cr]-sodium chromate–release assay (Brunner et al., 1968). In this assay system, radio–labeled target cells are challenged by immune effector cells, and the amount of intracellular and extracellular [^{51}Cr]-sodium chromate is determined through a γ-counter at the end of the assay. This radioactive assay system is reliable and reproducible, and is therefore the gold

Abbreviations: ADCC, antibody–dependent cellular cytotoxicity; BM-HT, bis(butyryloxymethyl) 4′-hydroxymethyl-2,2′:6′,2″-terpyridine-6,6″-dicarboxylate; BTN3A1, butylophilin 3A1; CAR, chimeric antigen receptor; EDTA, disodium ethylenediaminetetraacetic acid; Eu, europium; FITC, fluorescein isothiocyanate; HT, 4′-hydroxymethyl-2,2′:6′,2″-terpyridine-6,6″-dicarboxylate; mAbs, monoclonal antibodies; NK cells, natural killer cells; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PD-1, programmed death-1; PE, phycoerythrin; PTA, tetrakis-pivaloyloxymethyl 2-(thiazole-2-ylamino)ethylidene-1,1-bisphosphonate; TA, 2-(thiazole-2-ylamino)ethylidene-1,1-bisphosphonate; Tb, terbium.

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standard for cell–medicated cytotoxicity measurement. The disadvantages of this method, however, include the handling and disposal of radioactive materials that are strictly regulated in clinical laboratories. A number of non-radioactive methods have been developed, including assays based on the detection of intracellular enzymes, such as alkaline phosphatase (Szekeres et al., 1981) and lactate dehydrogenase (Sepp et al., 1996), the release of fluorescent probes (Bruning et al., 1980), and single cell analysis by flow cytometry (Packard and Komoriya, 2008).

One of the most promising approaches for an alternative to the radioactive cytotoxicity assay is time–resolved fluorometry (TRF) (Kolber et al., 1988; Volgmann et al., 1989; Maley and Simon, 1990; Blomberg and Ulfstedt, 1993; Pacifici et al., 1993; Lövgren and Blomberg, 1994; Blomberg et al., 1996; von Zons et al., 1997; Wu and Zhang, 2002; Zaritskaya et al., 2010). When the target tumor cells are treated with a prodrug of a chelate–forming compound, the probe prodrug permeates the cell membrane, where the compound is hydrolyzed by intracellular esterases to give a chelate–forming compound. The nascent compound is negatively charged and no longer permeates the cell membrane freely. Upon encountering the labeled target cells, immune effector cells such as natural killer (NK) cells and $V\gamma2V\delta2^+$ γδ T cells secrete perforin and perforate the target cell membrane. The immune effector cells then deliver granzyme B through the membrane holes into the target cells. Granzyme B is a pro-apoptotic protein that causes the target cells to undergo apoptosis. During apoptosis, the chelate–forming probe is released from the disrupted membrane into the culture media. Upon the addition of europium (Eu^{3+}) to the culture media, a probe/Eu^{3+} chelate is formed. When the chelate solution is pulsed with excitation light of 340 nm, the probe/Eu^{3+} chelate emits specific fluorescence. As the decay time of the fluorescence derived from the chelate is longer than that from the fluorescent ingredients present in the media, the long–lived fluorescence can be measured using a TRF multiplate reader.

Since the efficiency of cell labeling was relatively low and the spontaneous release rate was high when previously developed TRF–probes were employed, we recently synthesized bis(butyryloxymethyl) 4′-hydroxymethyl-2,2′:6′,2″-terpyridine-6,6″-dicarboxylate (BM-HT), a prodrug of 4′-hydroxymethyl-2,2′:6′,2″-terpyridine-6,6″-dicarboxylate (HT) (Sakai et al., 2017), and demonstrated that a combination of BM-HT and Eu^{3+} could be used for the determination of NK cell–mediated cytotoxicity (Senju et al., 2018). In the present study, we examined whether the newly improved assay can be used for the determination of $V\gamma2V\delta2^+$ γδ T cell–mediated cellular cytotoxicity and further improved the assay method using terbium (Tb^{3+}).

2. Materials and methods

2.1. Derivation of human $V\gamma2V\delta2^+$ γδ T cells

Heparinized peripheral blood samples were obtained from ten healthy adult volunteers (HD01-HD10) after approval of the institutional review board of Nagasaki University Hospital and with written informed consent. All methods were performed in accordance with the guidelines and regulations of Nagasaki University Hospital. The blood samples (10 mL) were diluted with 10 mL of Dulbecco's phosphate–buffered saline (−) (PBS, Nissui Pharmaceutical Co., Ltd. Taito-ku, Tokyo, Japan) and loaded on Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). After gradient centrifugation at 600 x g for 30 min at ambient temperature, peripheral blood mononuclear cells (PBMCs) fraction was collected. The cells were washed two times with 35 mL of PBS (−) and resuspended in 7 mL of Yssel's medium containing human AB serum (Cosmo Bio Co., Ltd., Koto-ku, Tokyo, Japan). Of the cell suspension, 1 mL was used for flow cytometric analysis. To the rest of the cell suspension (6 mL) were added 6 μL of tetrakis-pivaloyloxymethyl 2-(thiazole-2-ylamino)ethylidene-1,1-bisphosphonate (PTA, 1 mM dimethylsulfoxide solution) (Techno Suzuta Co., Ltd., Heiwa-Machi, Nagasaki, Japan) to give a final concentration of 1 μM and the plate was incubated at 37 °C with 5% CO_2 for 24 h. The culture medium was replaced with Yssel's medium plus 100 U/mL of IL-2 (Shionogi Pharmaceutical Co., Ltd., Chuo-ku, Osaka, Japan) from day 1 through day 5 and with RPMI1640 medium (Merck & Co., Inc., Kenilworth, NJ) supplemented with 10% fetal calf serum (FCS) (Merck & Co., Inc.), 10^{-5} M 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd., Chuo-ku, Osaka, Japan), 100 U/mL of penicillin (Meiji Seika Pharma Co., Ltd., Chuo-ku, Tokyo, Japan), 100 μg/mL of streptomycin (Meiji Seika Pharma Co., Ltd.), and 100 U/mL IL-2 from day 6 through day 10. On day 11, the cells were harvested and analyzed for the expression of cell surface markers and the immune effector functions.

2.2. Tumor cell lines

K562 (erythrocytoma), U937 (monocytic leukemia), P31/FUJ (monocytic leukemia), and RAMOS-RAI (Burkitt's lymphoma) were obtained from the National Institutes of Biomedical Innovation, Health and Nutrition JCRB Cell Bank (Ibaraki, Osaka, Japan). KG-1 (monocytic cell line) and 786-0 (renal cell carcinoma) were purchased from American Type Culture Collection (Manassas, VA) and C1R (B cell lymphoma) was obtained from RIKEN BioResource Center (Tsukuba, Ibaraki, Japan). UOK111 (renal cell carcinoma) and UOK121 (renal cell carcinoma) were kind gifts from Dr. Hirohito Kobayashi (Tokyo Women's Medical University, Shinjuku-Ku, Tokyo, Japan).

2.3. Flow cytometric analysis

PBMCs or PTA–expanded cells were plated out at 2×10^5 cells/50 μL in a 96-well plate (Corning Inc.). The cells were incubated with 3 μL of mAbs, including fluorescein isothiocyanate (FITC)–conjugated T cell receptor (TCR) Vδ2 mAb (BD Biosciences, San Diego, CA) and phycoerythrin (PE)–conjugated anti-cluster of differentiation 3 (CD3) mAb and anti-CD16 mAb (BD Biosciences) on ice for 15 min. After being washed three times with 200 μL of PBS, the cells were resuspended in 400 μL of PBS and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The cell population was analyzed using FlowJo ver. 10. (FlowJo LLC, Ashland, Oregon).

2.4. Determination of spontaneous release rate of chelate-forming ligand

Renal cell carcinoma cell lines 786-0, UOK111, and UOK121 were grown in 30 mL of complete RPMI1640 medium in 75 cm^2 culture flasks at 37 °C with 5% CO_2 for 2 days. The culture medium was removed and the cells were washed once with 10 mL of PBS (−). Then, the cells were treated with 0.25% trypsin/0.5 mM disodium ethylenediaminetetraacetic acid (EDTA, Merck & Co., Inc.) at 37 °C with 5% CO_2 for 5 min, to which were added 10 mL of complete PRMI1640 medium. The cells were washed twice with the medium and resuspended in the medium at a cell concentration of 1×10^6 cells/mL. For labeling cells, 2.5 μL of 10 mM bis(butyryloxymethyl) 4′-hydroxymethyl-2,2′:6′,2″-terpyridine-6,6″-dicarboxylate (BM-HT), a non-radioactive chelate-forming proligand (Techno Suzuta Co., Ltd., Heiwa-Machi, Nagasaki, Japan), was added to the cell suspension to give a final concentration of 25 μM. After incubation for 15 min at 37 °C with 5% CO_2, the cells were washed three times with 5 mL of complete RPMI1640 medium and resuspended in 20 mL of the medium to give a cell concentration of 5×10^4 cells/mL. The cell suspensions (100 μL each) were placed in 6 wells of a 96-well round bottom plate (Merck & Co., Inc.), to three wells of which were added 100 μL of complete RPMI1640 medium for the determination of spontaneous release and to the remaining three wells of which were added 90 μL of complete RPMI1640 medium for the determination of maximum release. The plate was centrifuged at 500 rpm at room temperature for 2 min in an AX-521 low speed centrifuge (Tomy Seiko Co., Ltd.) and incubated at 37 °C with 5% CO_2 for 30 min. Then, 10 μL

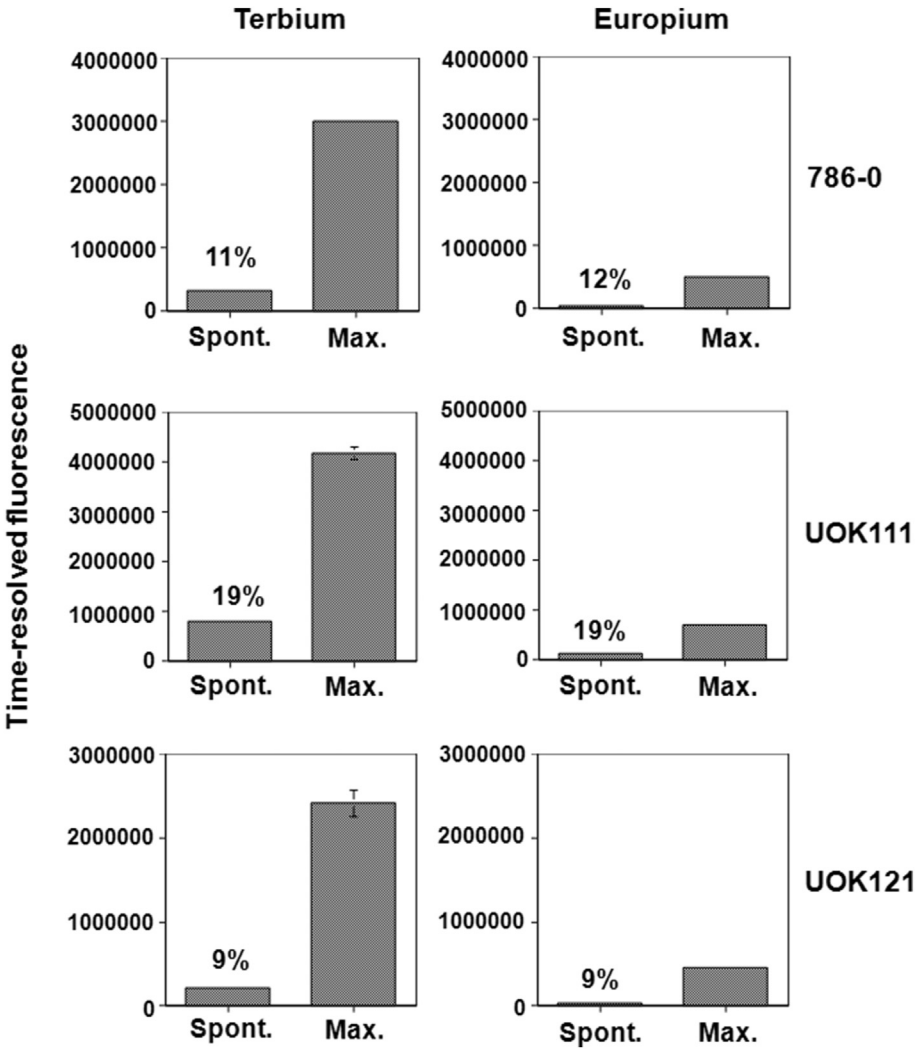

Fig. 1. Comparison of terbium and europium in the measurement of spontaneous release rates of HT from tumor cells. The effect of Tb^{3+} and Eu^{3+} on the spontaneous release rate of HT from human renal cell carcinoma cell lines 786-0, UOK111, and UOK121 were determined. Data show mean ± SD and are representative of five independent experiments.

of 0.125% digitonin (Wako Pure Chemical Industries Ltd.) in 19% dimethylsulfoxide (Wako Pure Chemical Industries Ltd.) were added to three wells for the determination of maximum release. The plates were incubated at 37 °C with 5% CO_2 for 30 more min and centrifuged at 600 x g for 2 min at 4 °C. The supernatants (25 μL each) were removed into wells of 96-well round bottom plates (Merck & Co., Inc.) containing 250 μL each of Tb^{3+} or Eu^{3+} solution (50 μM Tb^{3+} or Eu^{3+} (Merck & Co., Inc.) in 0.3 M sodium acetate buffer, pH 4.0 (Wako Pure Chemical Industries Ltd.)). The samples were mixed well and 200 μL each of them were removed into a 96-well optical bottom plate with polymer base (Thermo Fisher Scientific Inc., Waltham, MA). Then, TRF was determined through a PHERAstar FS multiplate reader (BMG Labtech Ltd., Allmendgruen, Ortenberg, Germany). All experiments were performed in triplicate. The maximum release was calculated as [maximum release (counts) – background (counts)] and the spontaneous release as [spontaneous release (counts) – background (counts)]. Then, the spontaneous release rate (%) was calculated as [(100 x spontaneous release)/maximum release].

2.5. Cellular cytotoxicity assay

Tumor cells were grown in 30 mL of complete RPMI1640 medium in 75 cm^2 culture flasks at 37 °C with 5% CO_2 for 2 days and resuspended in the medium at a cell concentration of 1×10^6 cells/mL. To 1 mL of the tumor cell suspension was added 2.5 μL of BM-HT and the tumor cells/BM-HT were incubated for 15 min at 37 °C with 5% CO_2. After being washed three times with 5 mL complete RPMI1640 medium, the labeled tumor cells were washed three times with the medium and resuspended in 20 mL of the medium to give a cell concentration of 5×10^5 cells/mL. The cell suspension was plated out in a round bottom 96-well plate (5×10^4 cells/100 μL) (Corning Inc.) and challenged by 100 μL of $V\gamma2V\delta2^+$ γδ T cells at effector to target ratios of 0, 0.625, 1.25, 2.5, 5, 10, 20 and 40: 1 for 60 min at 37 °C with 5% CO_2. After being centrifuged at 600 x g for 2 min at room temperature, the supernatants (25 μL each) were removed to a new round bottom 96-well plate containing 250 μL of Tb^{3+} or Eu^{3+} solution, from which 200 μL samples were transferred to a 96-well optical plate (Thermo Fisher Scientific Inc.). TRF was measured through a PHERAstar FS multiplate reader (BMG Labtech Ltd.). All experiments were performed in triplicate. Specific lysis (%) was calculated as 100 x [experimental release (counts) – spontaneous release (counts)] / [maximum release (counts) – spontaneous release (counts)] and spontaneous release (%) was calculated as100 x [spontaneous release (counts) – background (counts)] / [maximum release (counts) – background (counts)].

2.6. ADCC assay

To determine the antigen–dependent cellular cytotoxicity (ADCC),

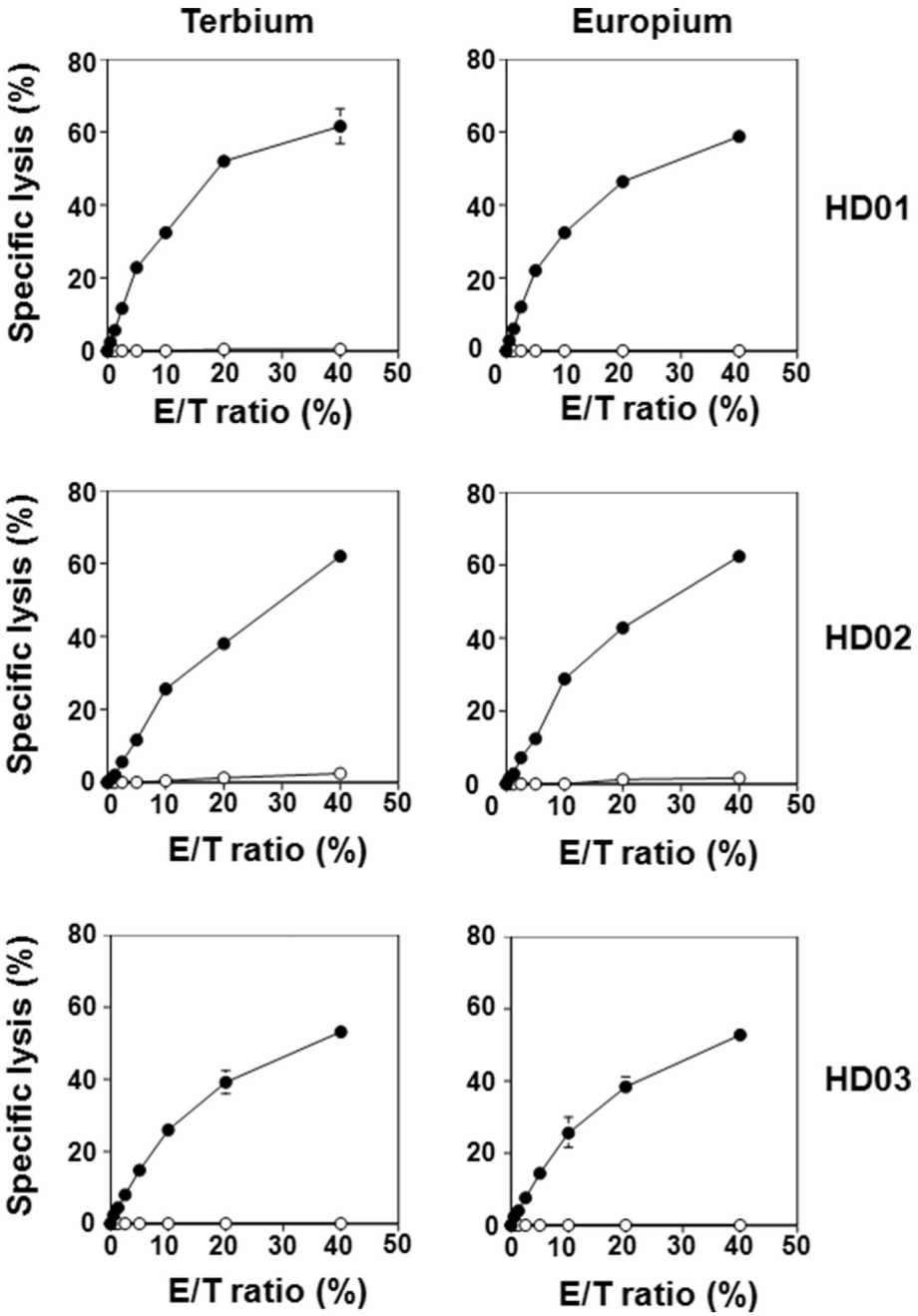

Fig. 2. Comparison of terbium and europium in the determination of Vγ2Vδ2$^+$ γδ T cell-mediated cytotoxicity. The effect of Tb^{3+} and Eu^{3+} on the specific lysis by Vγ2Vδ2$^+$ γδ T cells (HD01, HD02, and HD03) of HT-labeled and PTA-sensitized 786-0 cells were determined (○, 0 nM PTA; ●, 200 nM PTA). Data show mean ± SD and are representative of three independent experiments.

RAMOS-RAI Burkitt's lymphoma cells were pre–incubated with anti-CD20 mAb (Rituximab, Zenyaku Kogyo Co., Ltd., Bunkyo, Tokyo, Japan) at concentrations of 0, 0.5 or 2 μg/mL for 15 min at 37 °C with 5% CO_2. The opsonized tumor cells were then challenged by CD16-positive and negative Vγ2Vδ2$^+$ γδ T cells. ADCC was determined as described in the "Cellular cytotoxicity assay" section. All experiments were performed in triplicate.

2.7. Average and standard deviation

Average and standard deviation were calculated using CA-Cricket Graph III (version 1.5.3).

3. Results

3.1. Comparison of Tb^{3+} and Eu^{3+} in tumor cell labeling

We previously synthesized bis(butyryloxymethyl) 4'hydroxymethyl-2,2′:6′,2″-terpyridine-6,6″-dicarboxylate (BM-HT), a prodrug of 4′-hydroxymethyl2,2′:6′,2″-terpyridine-6,6″-dicarboxylate (HT), and improved a non-radioactive cellular cytotoxicity assay (Sakai et al., 2017). We initially used Eu^{3+}, one of the lantanoid metals, to form a fluorescent chelate with HT, because the Stokes shift (a difference between positions of the band maxima of the absorption and emission spectra) of the HT/Eu^{3+} chelate is relatively large. Since Tb^{3+}, another lantanoid metal, is known to form a complex with terpyridine dicarboxylate derivatives, we first compared the emission fluorescence properties

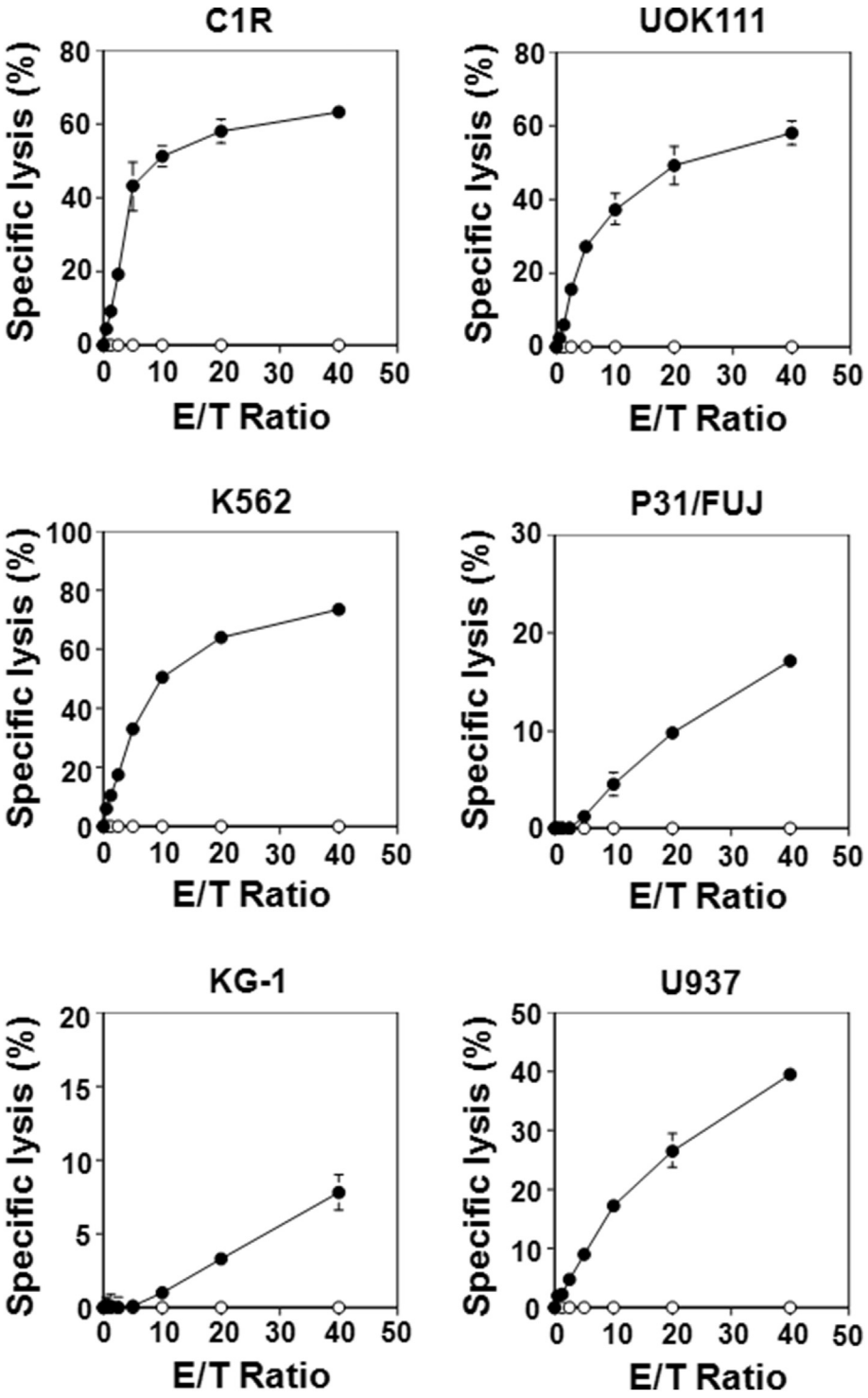

Fig. 3. Vγ2Vδ2^{+} γδ T cell-mediated cytotoxicity against various tumor cell lines. The specific lysis by Vγ2Vδ2^{+} γδ T cells (HD04) of HT-labeled and PTA-sensitized human tumor cell lines C1R, UOK111, K562, P31/FUJ, KG-1, and U937 were determined (○, 0 nM PTA; ●, 200 nM PTA). Data show mean ± SD and are representative of three independent experiments.

between HT/Tb^{3+} and HT/Eu^{3+}.

When the emission spectra of HT/Tb^{3+} and HT/Eu^{3+} after excitation at 340 nm were compared at the same molar concentration (0.5 μM), specific fluorescence was observed at 545 nm for HT/Tb^{3+} and 615 nm for HT/Eu^{3+}, with the maximum fluorescence values being 769.481 for HT/Tb^{3+} and 52.161 for HT/Eu^{3+}. These results indicate that HT/Tb^{3+} chelate was more sensitive than HT/Eu^{3+} chelate whereas the Stokes shift of HT/Tb^{3+} was smaller than that of HT/Eu^{3+} (Supplementary Fig. S1).

Next, we compared HT/Tb^{3+} and HT/Eu^{3+} in tumor cell labeling (Fig. 1). After human renal cell carcinoma cell lines 786-0, UOK111, and UOK121 were treated with 25 μM BM-HT for 15 min at 37 °C, the spontaneous release and maximum release in 60 min were determined. The spontaneous release and maximum release from 786-0 for HT/Tb^{3+} were 323,616 ± 5722 and 3,017,735 ± 56,323; and those for HT/Eu^{3+} were 58,981 ± 1113 and 504,034 ± 16,253, respectively.

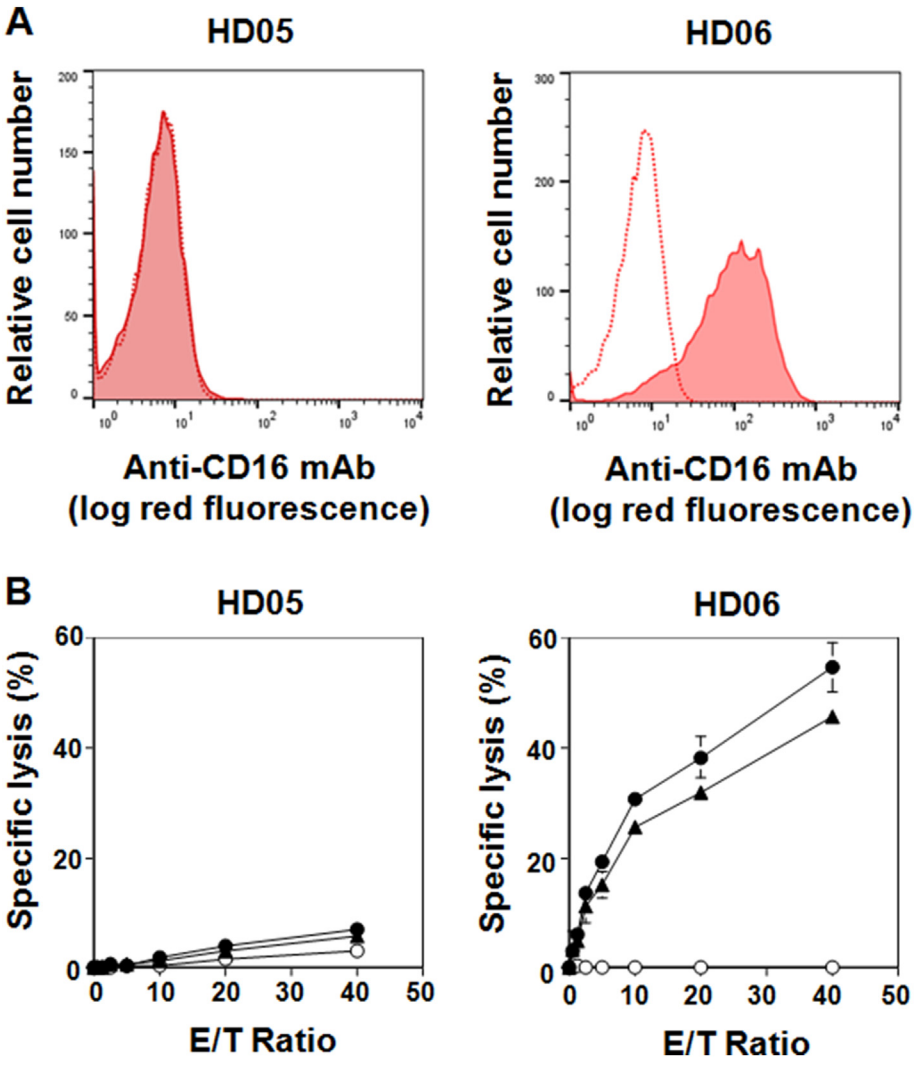

Fig. 4. Vγ2Vδ2^{+} γδ T cell–mediated ADCC against RAMOS-RAI. (A) Flow cytometric analysis of Vγ2Vδ2^{+} γδ T cells. The expression of CD16 on PTA/IL-2–expanded Vγ2Vδ2^{+} γδ T cells (HD05 and HD06) were examined by flow cytometry (dotted line: open area, isotype–matched control Ab; solid line: filled area, anti-CD16 mAb). A representative result of three independent experiments is shown. (B) Determination of ADCC exhibited by Vγ2Vδ2^{+} γδ T cells against RAMOS-RAI. The effect of anti-CD20 mAb (○, 0; ▲, 0.5; ●, 2 μg/mL) on the specific lysis by Vγ2Vδ2^{+} γδ T cells (HD05 and HD06) of the HT-labeled RAMOS-RAI cell line. Data show mean ± SD and are representative of three independent experiments.

Similarly, the spontaneous release and maximum release from UOK111 for HT/Tb^{3+} were 816,349 ± 16,390 and 4,193,751 ± 119,587; those for HT/Eu^{3+} were 133,918 ± 3129 and 700,497 ± 121,991; those from UOK121 for HT/Tb^{3+} were 210,204 ± 23,755 and 2,415,422 ± 155,314; and those for HT/Eu^{3+} were 42,388 ± 3098 and 457,706 ± 12,797, respectively. The spontaneous release rates (= 100 x spontaneous release/maximum release) of HT/Tb^{3+} and HT/Eu^{3+} for 780-6 were 11% and 12%, those for UOK111 were 19% and 19%, and those for UOK121 were 9% and 9%, respectively. The results indicated that the sensitivity of HT/Tb^{3+} was 5-fold to 6-fold higher than that of HT/Eu^{3+}, although the spontaneous release rate (%) was essentially the same.

3.2. Comparison of Tb^{3+} and Eu^{3+} in cellular cytotoxicity assay

Next, we compared HT/Tb^{3+} and HT/Eu^{3+} in the determination of the cellular cytotoxicity exhibited by Vγ2Vδ2^{+} γδ T cells. In order to validate the non-radioactive cellular cytotoxicity assay system for the measurement of tumoricidal activity exhibited by human Vγ2Vδ2^{+} γδ T cells, peripheral blood samples were obtained from healthy adult volunteers and peripheral blood mononuclear cells (PBMCs) were purified. As previously described, tetrakis-pivaloyloxymethyl 2-(thiazole-2-ylamino)ethylidene-1,1-bisphosphonate (PTA) was used for the expansion of Vγ2Vδ2^{+} γδ T cells (Matsumoto et al., 2016; Tanaka et al., 2017; Tanaka et al., 2018). After stimulation of PBMCs with PTA and IL-2 for 11 days, Vδ2^{+} cells occupied 98% or more of the lymphocyte gate in three healthy adult PBMCs (HD01: 99.7%, HD02: 99.3% and HD3: 98.4%).

PTA (0 nM or 200 nM)-sensitized 786-0 cells were then labeled with BM-HT and challenged by PTA-expanded Vγ2Vδ2^{+} γδ T cells derived from three healthy adult volunteers (HD01, HD02, and HD03). As shown in Fig. 2, Vγ2Vδ2^{+} γδ T cells failed to kill 786-0 cells that had not been sensitized by PTA whereas effector to target ratio–dependent cytotoxicity was observed when 786-0 cells were pretreated with 200 nM PTA. It is of note that essentially the same specific lysis (%) was obtained in HT/Tb^{3+} and HT/Eu^{3+} detection systems for all three donors.

Although the intensity of emission fluorescence from HT/Eu^{3+} was weaker than that from HT/Tb^{3+}, the intensity increased with the increasing concentration of HT/Eu^{3+}, as shown in Supplementary Fig. S2. This demonstrates that the HT/Eu^{3+} assay system is as good as the HT/Tb^{3+} assay system when the efficiency of tumor cell labeling is high.

3.3. Measurement of Vγ2Vδ2^{+} γδ T cell–mediated cytotoxicity against tumor cells

It was previously demonstrated that lymphoma and myeloid leukemia cells were poorly sensitized by zoledronic acid at sub-micromolar concentrations. We therefore set out to determine Vγ2Vδ2^{+} γδ T cell–mediated cytotoxicity against PTA (200 nM)–pretreated lymphoma and myeloid leukemia cells using the non-radioactive BM-HT–based assay system. Although the HT/Tb^{3+} assay system was more sensitive

than the HT/Eu^{3+} system, lymphoma and myeloid leukemia cells were efficiently labeled with BM-HT, and the HT/Eu^{3+} assay system was thus employed to measure cytotoxicity for further study. In addition, the TRF multiplate reader for the Eu^{3+} chelate is more common in standard biomedical laboratories.

PTA (0 nM or 200 nM)-sensitized C1R B cell lymphoma cells were labeled with BM-HT and challenged by Vγ2Vδ2$^+$ $\gamma\delta$ T cells derived from a healthy adult volunteer (HD04). Although PTA–untreated C1R cells were resistant to Vγ2Vδ2$^+$ $\gamma\delta$ T cells, PTA (200 nM)–sensitized C1R cells were efficiently killed by Vγ2Vδ2$^+$ $\gamma\delta$ T cells in an effector to target ratio–dependent manner (Fig. 3A, upper left panel). The specific lysis against PTA–sensitized C1R was higher than that against PTA–-sensitized UOK111 renal cell carcinoma cell lines (Fig. 3A, upper right panel). Similarly, PTA (200 nM)–sensitized K562 erythrocytoma cells, and P31/FUJ, KG-1, and U937 monocytic cell lines, were also efficiently killed by Vγ2Vδ2$^+$ $\gamma\delta$ T cells in an effector to target ratio–dependent manner (Fig. 3A, middle and lower panels). This demonstrates that a newly developed BM-HT–based assay system can be used for the determination of Vγ2Vδ2$^+$ $\gamma\delta$ T cell–mediated cellular cytotoxicity against adherent and non-adherent tumor cells. Essentially the same results were obtained in two other healthy donors, as shown in Supplementary Figs. S3 and S4.

3.4. Measurement of Vγ2Vδ2$^+$ $\gamma\delta$ T cell–mediated antibody–dependent cellular cytotoxicity

Next, we examined whether the assay system could be used for the determination of Vγ2Vδ2$^+$ $\gamma\delta$ T cell–mediated antibody–dependent cellular cytotoxicity (ADCC). Because ADCC is mediated by CD16, we first screened several healthy adult volunteers for the expression of CD16 on Vγ2Vδ2$^+$ $\gamma\delta$ T cells. As shown in Fig. 4A, Vγ2Vδ2$^+$ $\gamma\delta$ T cells derived from a healthy adult volunteer (HD05) did not express CD16 whereas those from HD06 expressed a high level of CD16.

We thus compared ADCC between HD05 and HD06's Vγ2Vδ2$^+$ $\gamma\delta$ T cells. RAMOS-RAI Burkitt's lymphoma cells were pretreated with 0, 0.5, or 2 μg/mL of anti-CD20 mAbs and BM-HT for 15 min at 37 °C and challenged by Vγ2Vδ2$^+$ $\gamma\delta$ T cells derived from HD05 and HD06. As shown in Fig. 4B, mAb–untreated RAMOS-RAI cells were not killed by HD05 Vγ2Vδ2$^+$ $\gamma\delta$ T cells whereas CD16–expressing HD06 Vγ2Vδ2$^+$ $\gamma\delta$ T cells exhibited ADCC in an mAb concentration–dependent manner. Essentially the same result was obtained in another combination of healthy donors, as shown in Supplementary Fig. S5. This observation clearly demonstrated that the newly developed assay system using BM-HT could be used for the measurement of Vγ2Vδ2$^+$ $\gamma\delta$ T cell–mediated ADCC.

4. Discussion

Cell–mediated cytotoxicity is commonly determined using a [^{51}Cr]-sodium chromate release assay in immunology laboratories (Brunner et al., 1968). Although this radioactive assay system is reproducible and reliable, it is challenging to use γ-rays in clinical laboratories for cancer cell therapies. A number of non-radioactive methods have thus been developed to measure cellular cytotoxicity, including the detection of enzyme activity and flow cytometry. The [^{51}Cr]-sodium chromate release assay has not been replaced by these alternatives that have a relatively low level of sensitivity and a poor labeling of some target cells.

One of the most promising methods is the assay using TRF, in which the target cells are pre-labeled with a chelate–forming non-radioactive probe and the amount of intracellular and extracellular probes are compared by mixing with Eu^{3+} after a challenge by immune effector cells (Blomberg et al., 1996). For the labeling of tumor cells with [^{51}Cr]-sodium chromate, the probe is passively internalized into tumor cells and binds to intracellular proteins. The resulting probe/protein complex is retained inside the cells, leading to a relatively slow spontaneous rate. In the case of a non-radioactive chelate-forming probe prodrug, the hydrophobic compound permeates target cells where it is hydrolyzed to give a functional probe. Since the resulting chelate–forming probe is negatively charged, it is retained in the cytoplasm for some time. It does not, however, interact with intracellular proteins and is eventually released from the cells. Thus, the spontaneous release rate of non-radioactive probes is generally greater than that of [^{51}Cr]-sodium chromate.

We previously synthesized BM-HT that was improved in the intracellular retention and labeling efficiency, compared to conventional bis(acetoxymethyl)2.2′:6′,2″-terpyridine-6.6″-dicarboxylate (BATDA) (Blomberg et al., 1996). We demonstrated that BM-HT could be used as a probe for the measurement of NK cell–mediated cytotoxicity (Senju et al., 2018). It is known that innate immune cells like NK cells can kill tumor cells and virus–infected cells rapidly. NK cells have been called large granular lymphocytes because they have a large amount of intracellular granules containing perforin and granzyme B that are responsible for cellular cytotoxicity. In contrast, adaptive immune cells like CD8$^+$ αβ T cells kill target cells rather slowly as they have to be primed and activated before killing. γδ T cells are considered to be immune cells that have properties of both innate and adaptive immune cells since they express rearranged TCRs and kill tumor cells and virus-infected cells rapidly (Constant et al., 1994; Tanaka et al., 1994; Tanaka et al., 1995; Hintz et al., 2001; Rohdich et al., 2002; Harly et al., 2012; Wang et al., 2013). We thus examined whether or not the specific lysis of tumor cells by γδ T cells could be determined with the improved assay system using BM-HT.

We first attempted to increase the sensitivity of the non-radioactive cellular cytotoxicity assay using BM-HT. To detect chelate-forming probes, Eu^{3+} has often been used as a chelate partner, and the probe/Eu^{3+} chelate has a relatively large Stokes shift. When the Stokes shift is large, the difference between the excitation wavelength and the emission wavelength is large, and the superimposition of the excitation pulse onto the emission fluorescence is limited. Compared to Eu^{3+} chelate, Tb^{3+} chelate has a rather small Stokes shift upon excitation with 340 nm pulse. The fluorescence intensity of HT/Tb^{3+} chelate is, however, 5- to 6-fold stronger than that of HT/Eu^{3+} chelate, and no emission interference due to the excitation pulse is noted. This indicates that the assay system using HT/Tb^{3+} is more sensitive than that using HT/Eu^{3+}.

Even though the sensitivity of HT/Tb^{3+} chelate is higher than that of HT/Eu^{3+} chelate, the availability of the TRF multiplate reader for Tb^{3+} chelate is limited in biomedical laboratories compared to that for Eu^{3+} chelate. We thus examined the HT/Eu^{3+} dose–dependency for emission fluorescence intensity. When the dose of HT/Eu^{3+} chelate was increased, the emission intensity reached the level of HT/Tb^{3+} chelate, indicating that the system using a high concentration of HT/Eu^{3+} is as sensitive as that using HT/Tb^{3+} chelate. Next, we sought to identify tumor cell lines that could be labeled with BM-HT efficiently, since such lines produce a high concentration of intracellular HT. As demonstrated in this study, lymphoma and myeloid leukemia cells could be efficiently labeled with BM-HT. We then examined Vγ2Vδ2$^+$ $\gamma\delta$ T cell–mediated cytotoxicity against lymphoma and myeloid leukemia cells. As expected, the tumoricidal activity of Vγ2Vδ2$^+$ $\gamma\delta$ T cells against PTA–treated and anti-CD16 mAb–treated tumor cells could be successfully measured. In the future, we plan to synthesize a different series of prodrugs of chelate-forming probes that could be used to determine αβ T cell–mediated cytotoxicity.

Recently, significant attention has been focused on combination therapies using programmed death-1 (PD-1) immune checkpoint inhibitors and other therapeutics (Forde et al., 2018; Gandhi et al., 2018; Hellmann et al., 2018), because the efficacy of PD-1 immune checkpoint monotherapy is limited (Motzer et al., 2015; Weber et al., 2017). One of the most attractive combination partners are ex vivo–expanded immune effector cells, including NK cells, Vγ2Vδ2$^+$ $\gamma\delta$ T cells, and genetically–modified T cells (Hiasa et al., 2009). The measurement of

cellular cytotoxicity is critical, and the non-radioactive assay established in this study is expected to play an important role in the development of such combination therapies.

Ethics committee approval and informed consent

Peripheral blood samples were obtained from adult volunteer after approval of the institutional review board of Nagasaki University Hospital and with written informed consent.

Competing interests

YT is a co-inventor of Japanese Patent 2014-257451 on the development of the method to expand γδ T cells using PTA, a novel bisphosphonate prodrug. YT and SM are co-inventors of Japanese Patent 2014-73475 on the development of a non-radioactive cellular cytotoxicity assay using BM-HT, a precursor of a novel Eu^{3+} chelate-forming compound. The other authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.01.003.

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