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Original Research Article

Development of a reporter gene method to measure the bioactivity of anti-CD38 × CD3 bispecific antibody

Hui Xiong, Fengyan Luo, Pengfei Zhou and Jizu Yi*

Wuhan YZY Biopharma Co., Ltd, Biolake City C2-1, No. 666 Gaoxin Road, Wuhan, Hubei 430075, China

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ABSTRACT

Background: A T cell-redirecting bispecific antibody (bsAb) consisting of a tumor-binding unit and a T cell-binding unit is a large group of antibody-based biologics against death-causing cancer diseases. The anti-CD38 × anti-CD3 bsAb (Y150) is potential for treating multiple myeloma (MM). When developing a cell-based reporter gene bioassay to assess the activities of Y150, it was found that the expression of CD38 on the human T lymphocyte cells (Jurkat) caused the nonspecific activation, which interfered with the specific T cells activation of mediated by the Y150 and CD38(+) tumor cells.

Methods: Here, we first knocked-out the CD38 expression on Jurkat T cell line by CRISPR-Cas9 technology, then developed a stable monoclonal CD38(−) Jurkat T cell line with an NFAT-RE driving luciferase expressing system. Further based on the CD38(−) Jurkat cell, we developed a reporter gene method to assess the bioactivity of the anti-CD38 × anti-CD3 bsAb.

Results: Knocking out CD38 expression abolished the nonspecific self-activation of the Jurkat cells. The selected stable monoclonal CD38(−) Jurkat T cell line assured the robustness of the report genes assay for the anti-CD38 × anti-CD3 bsAb. The relative potencies of the Y150 measured by the developed reporter gene assay were correlated with those by the flow-cytometry-based cell cytotoxicity assay and by the ELISA-based binding assay.

Conclusions: The developed reporter gene assay was mechanism of action-reflective for the bioactivity of anti-CD38 × anti-CD3 antibody, and suitable for the quality control for the bsAb product.

Statement of Significance: A stable monoclonal CD38-knockout Jurkat T cell line and a reporter gene method based on the Jurkat cell with an NFAT-RE driving luciferase expressing system to assess the bioactivity of anti-CD38/CD3 bsAb were first developed. The developed assay was MOA-reflective bioactivity and suitable for the quality control of anti-CD38/CD3 bsAb.

KEYWORDS: CD3; bispecific antibody (bsAb); bioactivity; reporter gene assay; CD38 knockout

INTRODUCTION

With the advantage of highly selective targeting and low off-site side effects, monoclonal antibodies have being attracted increasing efforts in the past decades to develop unmet drug therapeutics for cancer, immune, metabolic and infectious diseases as well as other death-causing diseases. However, a typical monoclonal antibody (mAb) has its efficacy limited to a small part of population in clinical application, e.g. only ∼10–36% patients with myeloma response to Daratumumab or to Isatuximab (both are anti-CD38 mAbs) [1, 2]. It is generally accepted that a single targeted mAb cannot reach a full inhibition of multiple mechanisms of the tumor growth. Bispecific antibody (bsAb)—a class of artificial mAb that can simultaneously bind to two different epitopes or antigens—is expected to provide additional functions compared with a typical mAb for the inhibition of tumors. With the scientific efforts in development in the past a few decades, there are currently

*To whom correspondence should be addressed. Jizu Yi, Wuhan YZY Biopharma Co., Ltd, Biolake City C2-1, No. 666 Gaoxin Road, Wuhan, Hubei 430075, China. Tel: (+86-27) 82668988; Fax: (+86-27) 68788819; Email: yijizu@yzybio.com

four bsAb drugs lunched on markets, >100 academic and industry-derived molecules in clinical development and many more projects under preclinical development [3]. Among these bsAbs, over 80% of the projects are clinically indicated for cancers, and ∼50% of which are T cell-redirecting bsAbs (T-bsAbs; [4–8]). A T-bsAb is composed of a tumor-binding unit to a tumor-associated antigen on the tumor cells and a T cell-binding unit to cluster of differentiation 3 (CD3) on the T-cell surface. The simultaneous binding to T cells and tumor cells cross-links the two cells actives the T cells to release cytokines, cytotoxic granules and perforin, which further lead to kill the tumor cells [9]. Blinatumomab (Blincyto®, anti-CD19 × anti-CD3 antibody) was a single strain T-bsAb (biTE) developed by Amgen (Thousand Oak, USA) and approved by Food and Drug Administration (FDA) for the treatment of acute lymphoblastic leukemia in 2014 [10–12].

Multiple myeloma (MM) caused by the neoplastic transformation of antibody-producing plasma cells is the second most common hematological malignancy [13, 14]. Cluster of differentiation 38 (CD38) is a transmembrane glycoprotein expressed universally on lymphocytes, NK cells and dendritic cells [15] and highly expressed on multiple myeloma cells (MMC), which enables CD38 to be a unique target for therapeutic antibody development to treat MM [16–18]. Indeed, two anti-CD38 mAbs including Daratumumab and Isatuximab have been developed and marked for MM therapies [1, 2]. The anti-CD38 × anti-CD3 bsAb (Y150) developed at YZY Bio (Wuhan, China) is an asymmetric IgG-like bsAb consisting of a monovalent unit of anti-CD38 and a single-chain unit of anti-CD3 to form a ScFv-Fab-IgG1 structure [19], and binding specifically to CD38 on the MMC and to CD3 on the T cells. The Fc region of Y150 has been modified by "Salt Bridge" and "Knobs-into-Holes" techniques for favorably forming the heterodimeric bsAb [20, 21].

During bsAb development, it is important to develop a quantitative method to assess the biological activity, which can reflect the MOA of the antibody product. Ag–Ab association-based potency assays, including Biacore [22], ELISA [23, 24] and flow cytometry (FCM) [25], were generally and successfully applied for the binding potency analysis. Cell-based function assays were also required along with the product development [26]. Although cytotoxicity assays, using peripheral blood mononuclear cells (PBMC) or T cells or cytokine-induced killer (CIK) cells as the effector cells [27–31], are directly measuring the biological activities of a bsAb in killing the tumor cells, these cell-based cytotoxicity assays are hardly validated for evaluation of biological activity of bsAb products for the quality control (QC) due to the unavailability and variability of the effector cells. In recent years, cell-based reporter gene assays were reported for measuring the bioactivity of monoclonal and bispecific antibodies, with advantages of robust performance and easy operation [32–34]. Promega Corporation developed a cell-based luciferase reporter gene assay to monitor the Jurkat T cell activation through binding to CD3 on the Jurkat T cells engineered with an nuclear factor of activated T cells response element (NFAT-RE) driving luciferase expression [35, 36]. However, the CD38 expressed on the Jurkat T cells interferes the assay when the reporter gene bioassay was performed to measure the bioactivity of the Y150 bsAb. In order to eliminate this interference, the clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein-9 (Cas9) (or CRISPR-Cas9) technology was applied to knockout CD38 gene in the Jurkat T cells. The generated CD38-knockout Jurkat T cells were applied to develop the bioassay for the Y150 potency measurement. The study showed that this developed cell-based luciferase reporter gene assay was MOA-reflective and stability-indicating.

MATERIALS AND METHODS

Cell lines and reagents

Jurkat-CD3-NFAT-RE-Luc cell line expressing luciferase driven by NFAT-RE was purchased from Promega Corporation (Madison, USA). Bio-lite Luciferase Assay System was purchased from Vazyme (Nanjing, China). NCI-H929 and U266B1 cell lines were purchased from CCTCC (Wuhan, China) and ARH-77 cell line from ATCC (Manassas, USA), and cultured for expansion as recommended from the inventors. CIK cells was made from PBMC at YZY Bio (Wuhan, China) with *ex vivo* activations by anti-hCD3ε, Interferon-gamma (INF-γ), Interleukin 1α (IL-1α) and IL-2. All of the cell lines in this study were stored in freezers in liquid nitrogen till usage.

Fluorescein isothiocyanate (FITC)-labeled anti-human CD38 antibody and isotype control antibody (FITC Mouse IgG1, κ Isotype Ctrl antibody) were purchased from Biolegend (San Diego, USA). CD38 antigen, CD38-HRP detection antigen and Y150 bsAb were made at YZY Bio.

CD38 knocked-out from Jurkat-CD3-NFAT-RE-Luc cell line

The CD38-knockout Jurkat T cell line was made from the Jurkat-CD3-NFAT-RE-Luc cells using CRISPR-Cas9 technology to knockout CD38 gene according to previous description [37]. Briefly, the Jurkat-CD3-NFAT-RE-Luc cell line was engineered to express luciferase under the control of NFAT-RE from the IL-2 promoter. The specific guide sequences (TCGCGGTGGTCGTCCCGAGG) was synthesized and Cas9-gRNA plasmid was constructed according to a previous study [37]. The plasmid was transfected into 2×10^7 Jurkat-CD3-NFAT-RE-Luc cells using cell electroporation (Celetrix, VI, USA; 1080 V, 30 ms, 1 pulse). Then the single clones were selected by passing the cultured cells into 96-wells plates initially at one cell per well, and cultured till the VCD at least 0.5×10^6 cells/mL for measuring the CD38 expression. FCM analysis using anti-human CD38 antibody-labeled with FITC (FITC-CD38) was performed to evaluate the CD38 expression levels of these monoclonal cells. For this analysis, the cells were harvested at about 1×10^6 cells/mL and washed with 1 × PBS (pH 7.4) by centrifugation at 300 *g* for 5 min. Five microliter FITC-CD38 was added and incubated on ice for 15–20 min in the dark. The cells were then washed twice with 1 × PBS (pH 7.4) by centrifugation at 300 *g* for 5 min. The monoclonal cells treated with isotype control antibody were used as a negative control. The cell pellets were resuspended

in 0.5 mL of 1 × PBS (pH 7.4) for cytometric analysis. Further, different passages of CD38-knockout Jurkat cells were tested for stability (cell viability assay and cell density assay during serial passage), and the cells attained 23 serial passages with negative in CD38 expression by comparing with the cells treated with the isotype control antibody were selected for method development.

Reporter gene assay

An appropriate amount of targeted tumor cells (e.g. NCI-H929) were adjusted to 2×10^6 cells/mL by assay buffer (1% FBS-RPMI 1640 medium), and followed by adding 20 μL of the cells into each of 96-wells in a white plate. Next, the assay buffer was used to serially dilute antibody samples, and 20 μL of either the diluted sample or assay buffer as the negative control was added into each well, followed by 20 μL of 3×10^6 cells/mL CD38-knockout Jurkat T cells added as the effector cells. Further, the 96-wells plates with the Y150 and the two cells were incubated at 37°C and 5% CO_2 for 6 h. Then 60 μL of Bio-Lite luciferase assay solution was added into each well of the plates, followed by further incubation at room temperature for 15 min in dark. Luminescence values in relative luminescence units (RLU) were measured using a modular multi-technology microplate reader (Varioskan™ LUX, Thermo Scientific), and plotted versus the antibody concentrations to determine the EC50 using GraphPad Prism software (version 5.0). The relative potency is equal to the ratio of EC_{50} of the reference standard to the EC_{50} of the tested sample.

Preparation of CIK cells

CIK cells were prepared from fresh PBMC according to the previous report [30] with modifications. Briefly, the PBMC was expanded with X-VIVO™ 15 medium (Lonza, Basel, Switzerland). At the commencement of the culture, 1000 IU/mL interferon (IFN)-γ (PeproTech, NJ, USA) and 2% FBS (Biological Industries, Kibbutz Beit-Haemek, Israel) were added into the medium and the cells were incubated at 37°C and 5% CO_2 for 24 h, followed by additions of 100 ng/mL anti-hCD3ε (R&D Systems, MN, USA), 1.0 ng/mL IL-1α (PeproTech, NJ, USA) and 1000 IU/mL IL-2 (Four Rings Bio., Beijing, China). The culture was continued with the supplementary media containing 2% FBS and 1000 IU/mL IL-2 every 2–3 days and the cell density was controlled to be about 1.5×10^6 cells/mL. On day 14, cells were pooled and cryopreserved in 2-mL cryovials for further usages.

Cytotoxicity assay by FCM

The cytotoxicity assay was performed using CIK cells as effector cells and NCI-H929 as target cells. The target cells (2.0×10^4 cells/well) labeled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, CA, USA) were placed on 96-wells micro plates. After 2-h culture, different concentrations of the Y150 bsAb and effector cells (1.0×10^5 cells/well) at an effector-to-target ratio (E:T) of 5:1 were added, followed by incubation for 24 h. The cells were treated with propidium iodide (PI; Sigma, San Francisco, USA) and then the florescence was measured on flow-cell cytometery (FC500, Beckman Coulter, CA, USA), and the collected data were analyzed using software FlowJo. Negative florescence represents florescence released from target cells in the presence of effector cells but in the absence of antibodies. Cytotoxicity curves were fitted to calculate the half maximal effective concentration (EC_{50}) using GraphPad Prism software with 4-parameter equation fitting.

Sandwich ELISA assay

Briefly, 96-wells Maxisorp immunoplates were coated with CD38 antigen and incubated overnight at 4°C. On the next day, the coated wells were blocked with 3% BSA in PBS and followed by adding different concentrations of the Y150 bsAb. After incubation for 1 h at 37°C, the plates were washed and then added with CD3 antigen labeled with Horseradish peroxidase (HRP) for detection. The absorbance was measured at 450 nm using SpectraMax® Plus 384 Absorbance Plate Reader (Molecular Devices, San Jose, USA) and plotted against the antibody concentrations using a four-parameter curving fitting program with SoftMax Pro 5.4.1 software. The relative binding activity is equal to the percentage of EC_{50} of the reference standard to the EC_{50} of the tested sample.

SEC-HPLC

Sample purity and its high and low molecular weight species were determined by a size exclusion chromatography (SEC) method with a TSKgel G3000SWXL column (7.8 × 300 mm, TOSOH, Tokyo, Japan) adapted on a high performance liquid chromatography (HPLC) system (Waters, Milford, USA). The relative percentage of the main peak was determined as the SEC-purity.

CEX-HPLC

Charge variants of a tested sample were profiled by a cation exchange chromatography (CEX) method using MabPac SCX-10 column (Thermo Scientific, Waltham, USA) connected on a HPLC system with a UV–VIS detector (Waters, Milford, USA). 3 μL of 10 mg/mL protein sample was loaded in 95% Buffer A (20 mM MES, 60 mM NaCl, pH = 6.0) and 5% Buffer B (20 mM MES, 500 mM NaCl, pH = 6.0), and eluted at a flow rate of 0.5 mL/min of a linear gradient from 30 to 100% Buffer B in 10 min.

Validation of the reporter gene assay

The specificity, linearity, accuracy, intermediate precision and robustness of the reporter gene assay were conducted. For specificity assessment, intact or stressed Y150 samples after oxidization with 4% hydrogen peroxide, or a nonspecific antibody as the negative control, were incubated with both NCI-H929 cells and CD38-knockout Jurkat T cell, and the RLU signals were measured. For linearity, accuracy and intermediate precision of the assay, 50, 75, 100, 150 and 200% (expected potency) in concentration of Y150 samples were tested three times on three different days by each of

two analysts with total of six independent measurements for each sample. Another 100% sample was added as the reference. The linearity (R^2) was analyzed by plotting the measured potency versus the expected potency. The recovery (the ratio of measured potency to expected potency) and the RSD value of each sample were determined. For robustness, both reference and test samples were measured under the same conditions. The incubation time of the Y150 samples and cells was varied from 6 to 4 h or 8 h, and the detection incubation time of Bio-lite reagent with the cells was varied from 15 to 10 min or 20 min. The relative potency of the test sample to the reference after each of the changed conditions was compared with that prior to the change.

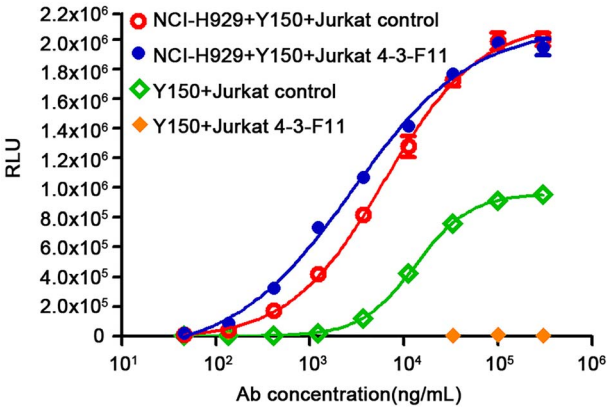

Figure 1. The Y150-dependence of gene activation of Jurkat cells: in presence of Jurkat-CD3-NFAT-RE-Luc cells only (in Green), or in presence of Jurkat-CD38$^-$-CD3-NFAT-RE-Luc cells only (Jurkat-4-3-F11, in Brown), or in presence of both Jurkat-CD3-NFAT-RE-Luc cells and NCI-H929 cells (in Red), or in presence of both Jurkat- CD38$^-$-CD3-NFAT-RE-Luc cells and NCI-H929 cells (in Blue). In the reporter gene assay, Jurkat cells (effector cells) and NCI-H929 (target cells) were in a ratio of 1.5:1 (E:T).

RESULTS

Generation of CD38-knockout Jurkat T cell lines

To measure the T cell activation activity of a CD3-targeted bsAb, Jurkat-CD3-NFAT-RE-Luc cells as effector cells and tumor cells as target cells were co-cultured. In the presence of the Y150 bsAb, the Jurkat T cell would be activated by a targeted tumor cell through the linkage of the two cells by the bsAb. However, due to the expressions of both CD38 and CD3 molecules on the surface of the Jurkat-CD3-NFAT-RE-Luc cells, the Jurkat T cells can be also self-activated by the anti-CD38 × anti-CD3 bsAb through the cross-linkage of the two Jurkat T cells, which causes non-specific signal in this reporter gene assay (Fig. 1). In order to eliminate this non-specific interference, we used CRISPR-Cas9 technology to knockout CD38 gene expression on Jurkat T cells as described in Material and Method, whereas the stable expression of the luciferase gene was remained under the control of the NFAT-RE. Two monoclonal cell clones, clonal #Jurkat 4-3-F11 (The Top Stable Clone) and Jurkat 4-3-D6 showed no CD38 expression (Fig. 2A and B, and Jurkat 4-3-F11 was selected for method development for Y150 bioactivity. The results showed that the non-specific signal was not detectable when Jurkat-4-3-F11 cells were used as effect cells (Fig. 1), demonstrating that the CD38 expression knocked-out fully abolished the nonspecific activation of the Jurkat T cells.

Considering that the stability of this novel CD38-knockout Jurkat cell was important to the assay accurate and robustness with long-term cultures, Jurkat-4-3-F11 cell line was passaged up to 23 generations and the cell viability, viable cell density (VCD) were measured before and after each cell passage, and the cells were tested in the reporter gene assay at Passages 4 and 22. The results showed a high stability of the CD38 knockout Jurkat T cells at these different passages (Fig. 3A), and a high consistent activities of the Y150 in a concentration dependent manner with EC_{50} of 919.5 and 1088 ng/mL for Passage 4 and 22, respectively (Fig. 3B).

CD38-dependent reporter bioassay

After eliminating the expression of CD38, the Jurkat-4-3-F11 cells were evaluated for the assay in different CD38 expression levels on the tumor cells including NCI-H929, U266B1 and ARH77cells as the targets. The CD38 expressions were measured by FCM, and the results confirmed that the CD38 expression levels were in order: NCI-H929 > ARH77 > U266B1 (Fig. 4A). A positive correlation of bioluminescence signal generated by Y150-mediating the Jurkat activation with the CD38 expression levels of the NCI-H929, ARH77 and U266B1 cells was observed with EC_{50} of 1131, 2338 and 4509 ng/mL, respectively (Fig. 4B). The results demonstrate that the gene reporter intensity, which impacts the assay sensitivity, is a dependence of the CD38 levels on the target tumor cells. Therefore, NCI-H929 with the highest CD38 expression among the three tested tumor cells was chosen as the target cell for Y150 bioassay development.

In order to confirm if the reporter gene assay with the CD38 knockout Jurkat T cells provided a feasible assay for QC of Y150 product development, the specificity, linearity, accuracy, intermediate precision and robustness of the assay were validated. The results demonstrated that the Y150 samples stressed with oxidation (4% hydrogen peroxide) provided a lower response than the intact samples (Fig. 5A). The non-specific antibody could not stimulate Jurkat T cells and did not show any activities (Fig. 5A). The Y150 activation signal showed a linear dependent response to Y150 concentrations with R^2 of 0.9973 (Fig. 5B). The accuracy (recovery) were between 80 and 120% and the RSD values were below 15% (Table 1), demonstrating the acceptable accuracy and precision of the assay.

For robustness of the assay, the relative potencies of Y150 sample under the changed conditions were within 88–113%, which were consistent with 97–111% among six repeats under the determined condition. All these results assured that the validated reporter gene assay is highly specific to the Y150 bsAb and feasible for its bioactivity analyses in QC.

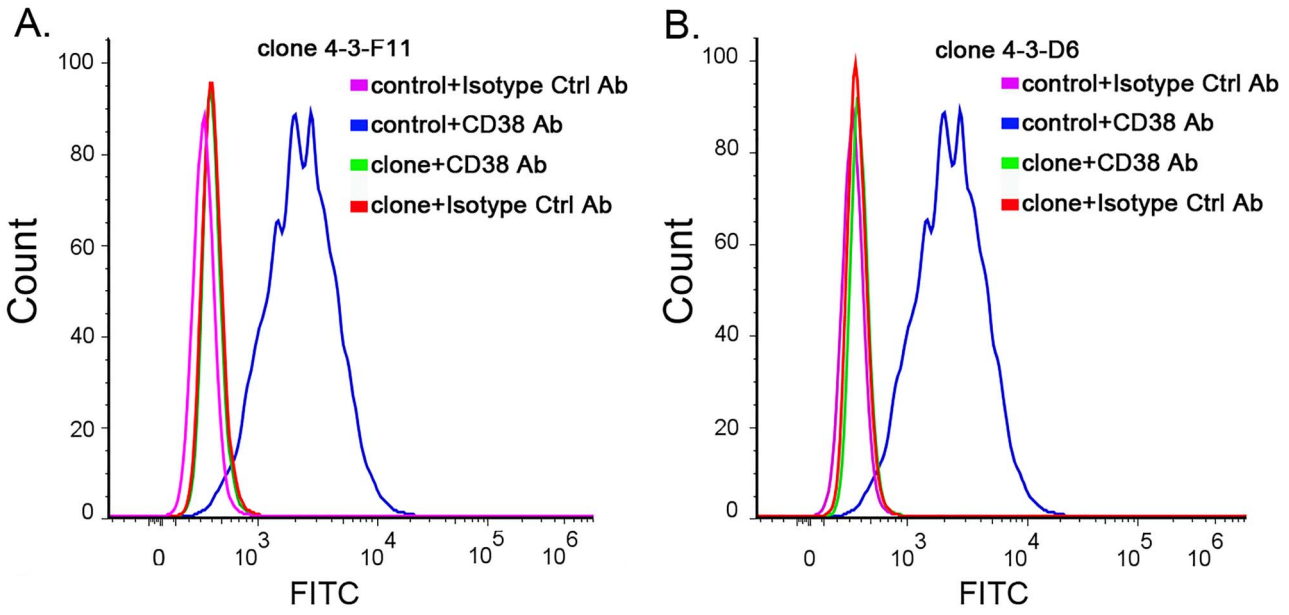

Figure 2. CD38 expression levels on the knockout Jurkat T cells clone 4-3-F11 (A) and clone 4-3-D6 (B). Anti-human CD38 antibody-labeled with FITC (or Isotype control antibody-labeled with FITC) were incubated with the cells, followed by the FCM analysis of the fluorescence intensity. The results of the monoclonal cells treated with anti-CD38 antibody are showed in Green, and monoclonal cells treated with isotype control antibody in Red, the original Jurkat-CD3-NFAT-RE-Luc cells treated with anti-CD38 antibody in Blue, and the original Jurkat-CD3-NFAT-RE-Luc cells treated with isotype control antibody in purple.

Table 1. The evaluation of accuracy and precision for reporter gene assay

Expected potency (%)	50	75	100	150	200
Measured potency (%)	46.0	69.0	98.3	143.8	204.3
Recovery* (%)	92.0	92.0	98.3	95.9	102.2
90% confidence interval of Recovery (%)	87.3–96.7	86.1–97.9	95.2–101.5	90.9–100.9	93.5–110.9
RSD (%)	7.7	9.5	4.8	7.8	12.7

*The recovery was calculated as the percentage of mean of measured potency to expected potency.

The correlations of reporter gene activity with cytotoxicity activity and binding activity

To evaluate the correlations of biological activities measured by the established reporter gene assay, cell-based cytotoxicity assay and molecule-based binding assay, the Y150 samples stressed under high temperature (25 ± 2°C for 0.5, 1 and 3 months) were tested and compared with the Y150 reference sample (intact sample). Together with the purity by SEC-HPLC and charge variants by CEX-HPLC (Fig. 6A), the results of the relative potencies by these three assays were summarized (Table 2). As expected, the purity of Y150 slightly decreased after the stress under 25°C, and the relative potency by each of these three assays decreased with the increase of the acidic variants by CEX-HPLC (Fig. 6B), suggesting that these potency assays were stability indicating. Importantly, the trends of these results among three potency assays were strongly correlated with a high consistency ($P > 0.05$) by two-way analysis of variance (ANOVA), Tukey's multiple comparison test (GraphPad Prism 8.0). These results demonstrate that reporter gene assay developed to reflect the Y150 mediating activation of T cells are linearly correlated with the cell-based cytotoxicity activities and/or the molecule-based binding activity. Therefore, the developed reporter gene assay is applicable for the biological potency analysis of anti-CD38 × anti-CD3 bsAb.

DISCUSSIONS AND CONCLUSIONS

Bioassays play an important role in the QC for biotherapeutics. To determine the biological activities of biopharmaceuticals, biochemical assays, cell-based and animal-based bioassays are often applied. Bioactivity determination methods for biotherapeutics should be developed according to the following principles: MOA-based, QC suitable (small variability, simple operation and transferability) and compliance with good manufacturing practices validation [26]. Animal-based bioassays are rarely used in the QC due to their high variability, high cost, labor intensiveness and the global trend of the application of 3R

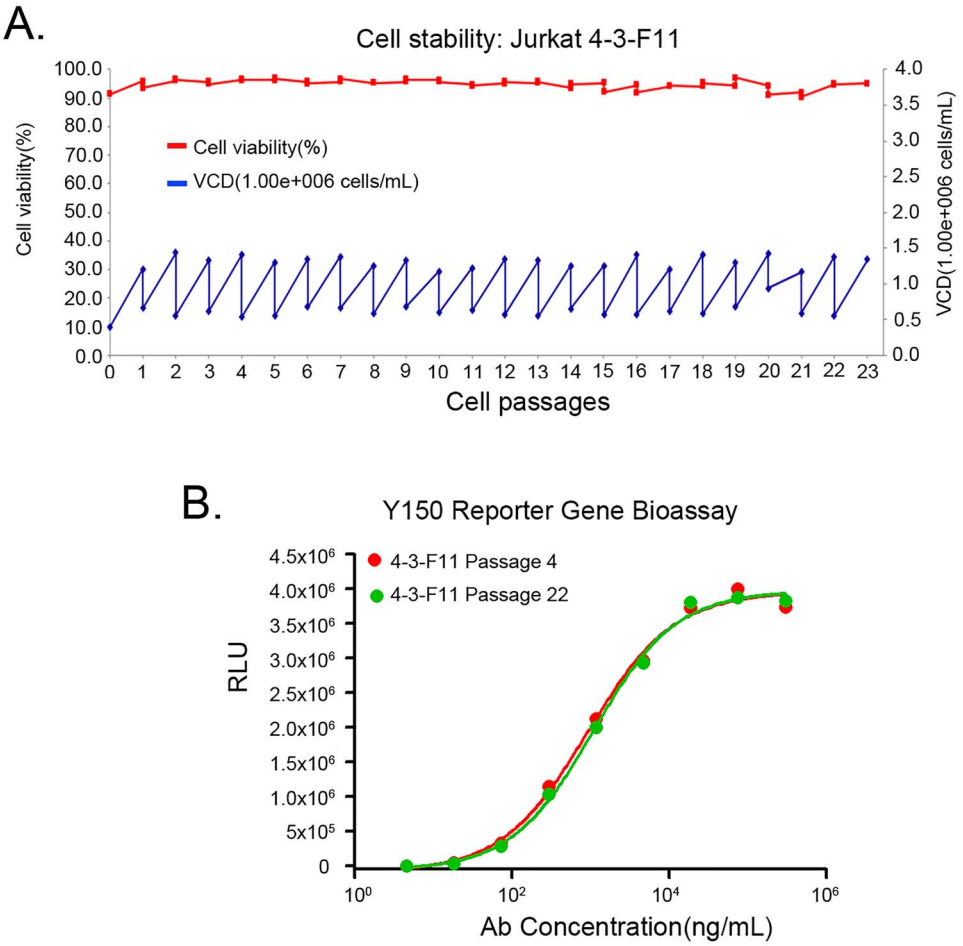

Figure 3. Stability of the CD38 knockout Jurkat 4-3-F11 cells. (A) The cell viability (red) and VCD (blue) are presented before and after each cell passage. (B) Jurkat-4-3-F11 cells at passages 4 and 22 were applied for Y150 bioactivity assay with the EC_{50} of 919.5 and 1088 ng/mL, respectively. The each data point was the average of three replicates.

Table 2. The correlations of reporter gene assay results with those from cytotoxicity assay and sandwich ELISA assay

Sample[a]	Purity (%)	Acidic Peaks%	Relative potency[b] (%)		
	SEC-HPLC	CEX-HPLC	Reporter gene	Cytotoxicity	Sandwich ELISA
Unstressed	99.6	20.0	93 ± 4.4	103 ± 6.0	94 ± 8.7
S1	99.3	32.0	81 ± 4.9	92 ± 5.7	94 ± 1.4
S2	99.1	41.8	82 ± 3.5	85 ± 4.9	89 ± 9.5
S3	98.8	68.1	71 ± 4.4	63 ± 11.2	58 ± 7.6

[a]After treated under high temperature (25 ± 2°C) for 0.5 month (S1), 1 month (S2) and 3 months (S3), the samples were supplied for SEC-HPLC, CEX-HPLC, reporter gene, cytotoxicity assay and sandwich ELISA assay.
[b]The relative potency by reporter gene assay, cytotoxicity assay and sandwich ELISA assay for each sample was tested in three independent experiments.

principles (replacement, reduction and refinement) in the use of animals. For biochemical assays, binding assays such as ELISA are the mostly common used. However, as the binding only involves the initial step of a biological event and is often far away from the end point of a biological response, it cannot completely represent the MOA of biopharmaceuticals. Many cell-based biological assays have been developed and widely used for biological activity determination [26].

A notable class of bsAbs are the T-bsAb. Simultaneous ligation of target and effector cells induces T-cell activation, followed by the killing of target cells. Although therapeutically effective, their multiple MoA, including simultaneous target and effector-cell engagement, T-cell activation and

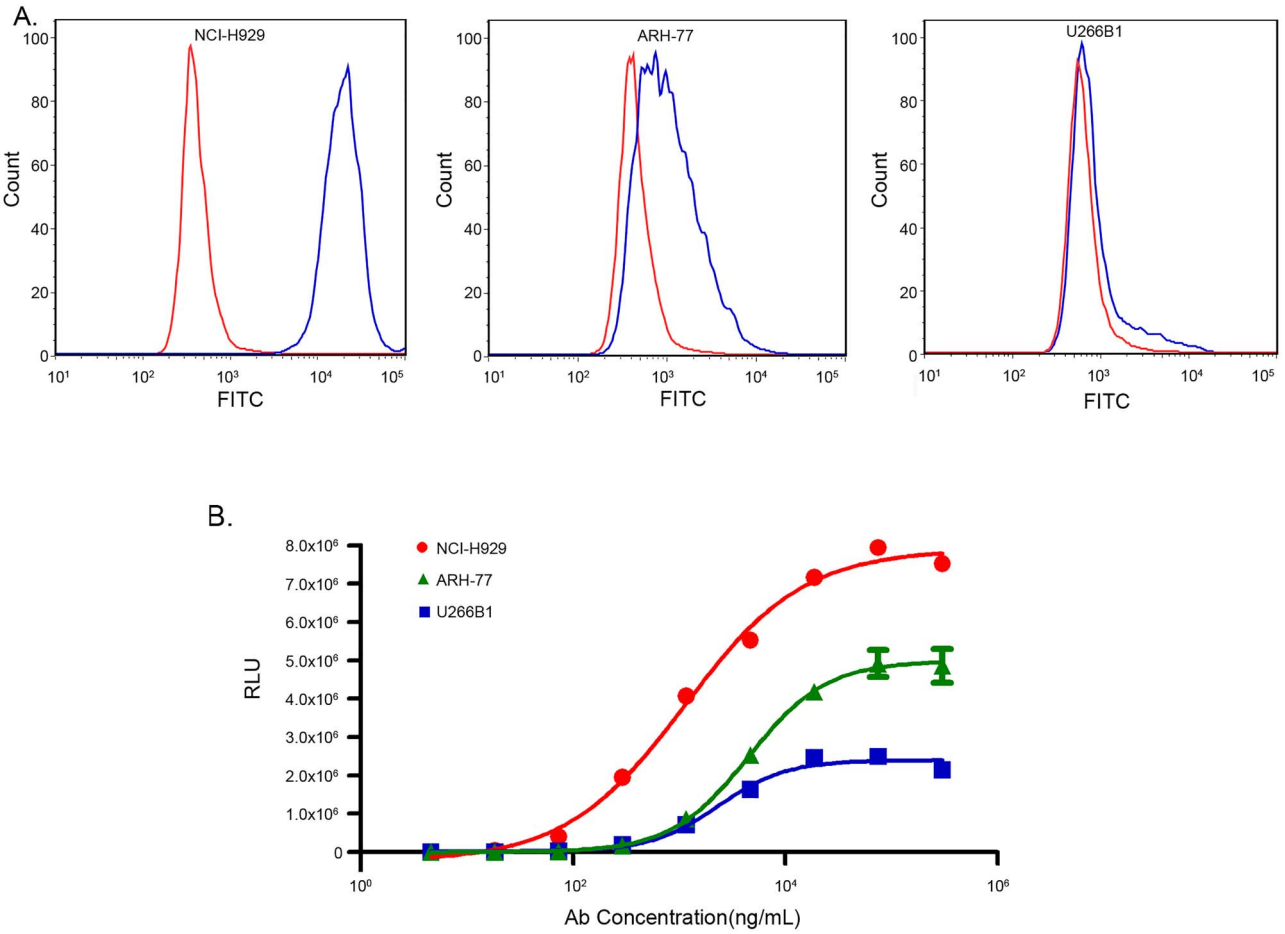

Figure 4. The Y150-mediating luminescence signal is dependent on the CD38 expression levels on the target cells. (A) The CD38 expression levels of three tumor cells (NCI-H929, ARH-77 and U266B1) were measured by FCM. The NCI-H929, ARH-77 and U266B1cells treated with isotype control antibody were used as negative controls. The CD38 expression of NCI-H929 was the highest among these tested three targets, followed by ARH-77 and U266B1. (B) The RLU signal value and EC_{50} value was positively correlated to the CD38 levels on the target cells.

target-cell killing, shows challenges for the development and selection of a cell-based potency bioassay [38]. A single potency assay that measures all key aspects of the MoA is desirable.

Cell-based killing assays such as cell counting kit-8, MTS, MTT [39–41] or lactate dehydrogenase assays [30, 42–44] have been well developed for directly measuring tumor cell growth and viability. However, in addition to reflecting the MOA, a potency assay must be also able to track the changes in product quality that have the potential to affect the molecule's biological activity. FCM-based cell-killing assay with double fluorescent labeling [45, 46] or standard chromium release assays [47, 48] were quantified for the death of tumor cell more accurately. However, because of their high assay variability and longtime consume of the procedure, which makes them difficult to sustain over a product's lifetime from development through commercialization, cell-killing assays are not suitable for the QC purpose [38].

Reporter-gene assays for T-bsAbs have emerged as an attractive alternative to cell-killing assays for QC purposes. Typically they use cell lines engineered to express luciferase under the control of a biologically relevant response element for T-cell activation, such as NFAT or nuclear factor kappa B, which allows the measurement of events upstream of cell killing [35, 49–51]. Reporter-gene assays are faster, easier to perform and more reproducible than cell killing assays, making them more QC suitable [38]. These advantages of reporter-gene assay enabled the development of a fast, robust potency assay with low assay-to-assay variability relative to most cell-killing assays. Reporter-gene assay is sensitive to product quality and is stability indicating, as demonstrated by measuring potencies of samples subjected to various stress conditions [38]. Our studies demonstrated that the two methods are similarly stability indicating and sensitive to changes in product quality (Fig. 6 and Table 2). The FDA has approved some biopharmaceuticals whose bioactivity determinations, as one of critical quality attributes, are dependent on reporter-gene assays [52–54], indicating their wide acceptance both in industry and regulatory authority [26].

In this study, we used CRISPR-Cas9 technology to knockout the CD38 expression in Jurkat T cells, and

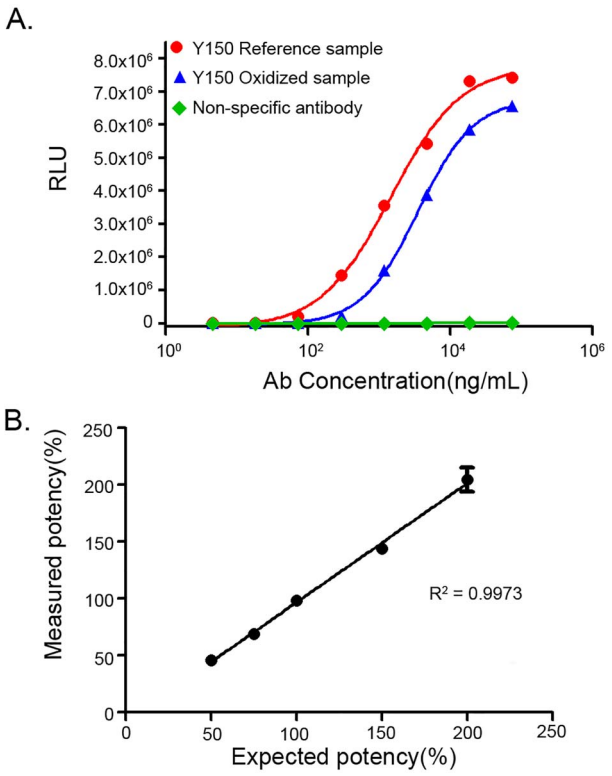

Figure 5. Validation of reporter gene assay. (A) Specificity assessment of the assay. Y150 or a nonspecific antibody was incubated with both NCI-H929 cells and Jurkat T cell line 4-3-F11, and the RLU signals were measured. Y150 sample stressed under oxidization with 4% hydrogen peroxide was tested and compared with Y150 reference sample. (B) Analysis of linearity of the assay was conducted by comparing the measured potency and expected potency. Each point represents the mean of six independent experiments.

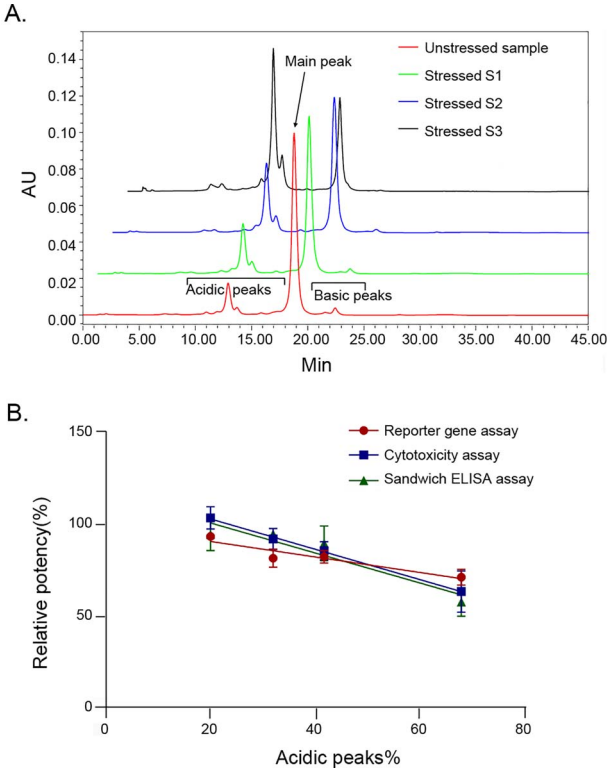

Figure 6. Correlation of relative potencies by three bioassays to the acidic variants of the Y150 samples. The Y150 samples were stressed under high temperature (25°C) for 0.5 month (S1), 1 month (S2) and 3 months (S3). (A) The chromatograms of CEX-HPLC of Y150 reference (intact) sample was in Red, stressed S1 in Green, stressed S2 in Blue and stressed S3 in Black. Acidic, main and basic peaks were marked. (B) The relative potencies of the stressed samples were measured by reporter gene, cell cytotoxicity and ELISA binding assays. The trends of three potency assays were plotted and compared with $P > 0.05$ by two-way ANOVA method using Tukey's multiple comparison test (GraphPad Prism 8.0).

further, the stable monoclonal cell with CD38-negative expression was selected for developing the reporter gene assay of Y150. After eliminating the non-specific signal, this method was shown to provide a good accuracy, precision and linearity in the range of 50–200%. In addition, this assay is easy to operate with a shorter assay time and easier data analysis procedures than those of the cell-based killing assay, making it suitable for QC analysis. In a high temperature stability study of Y150 bsAb, the purity by SEC-HPLC, the charge variants by CEX-HPLC and the potency results by reporter gene assay, cell killing assay and ELISA, were highly related with each other. The results demonstrate that the decrease in relative potencies was linearly related to the increase of acidic variants of the Y150. The trends of potency percentages by the three functional assays with ELISA reflecting the binding activity, report gene assay for the T-cell activation, and FCM for cytoxicity activity were strongly consistent, indicating that the report gene assay can reflect the biological activities of Y150 for its cell-based potency analysis.

Above all, by knocking out the CD38 expression on the Jurkat T cell a new reporter gene assay was developed for the bioactivity analysis of anti-CD3 × anti-CD38 bsAb. This new MOA reflective assay was validated for the bispecific bioactivity analysis during antibody development and for QC.

DATA AVAILABILITY

Data contained in the article can be available online.

CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interest.

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