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A novel CD2 staining–based flow cytometric assay for assessment of natural killer cell cytotoxicity

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Abstract

Background: Assessing cytotoxicity is fundamental to studying natural killer (NK) cell function. Various radioactive and non-radioactive cytotoxicity assays measuring target cell death have been developed. Among these methods, the most commonly used ^{51}Chromium-release assay (CRA) and flow cytometry–based cytotoxicity assays (FCCs) are the major representatives. Nonetheless, several drawbacks, including dye leakage and the potential effects of prior labeling on cells, curb the broad applicability of the FCCs.

Methods: Here, we report a rapid FCC for quantifying target cell death after co-incubation with NK cells. In this assay, after 4 hours of NK cell-target cell co-incubation, fluorochrome-conjugated CD2 antibody was used to identify NK cells, and SYTOX Green and Annexin V-FITC were further used to detect target cell death in CD2-negative population. In parallel, both CRA and FCC assay using CFSE/ 7-AAD were performed to validate the reproducibility and replicability.

Results: We observed that CD2 is exclusively positive on NK cells other than the most common hematological target tumor cells, such as K562, HL60, MOLM13, Raji, NCI-H929, rpmi8226, MM.1S, and KMS11. Assessment of target cell death using the CD2-based FCC shows a significantly higher percent specific lysis of the target cells compared to the standard CRA and the FCC assay using CFSE and 7-AAD.

Conclusions: We demonstrated that this CD2-based FCC is a fast, simple, and reliable method for evaluating NK cell cytotoxicity.

KEYWORDS
CD2, cytotoxicity, flow cytometry, natural killer cell

Dan Zhang and Rui Teng contributed equally to this work.

1 | INTRODUCTION

Natural killer (NK) cells are crucial innate lymphocyte subsets that mediate host rejection of both tumors and virally infected cells.[1,2] For this purpose, cytotoxic proteins including granzyme and perforin are stored within the secretory lysosomes of NK cells, and the recognition of an aberrant target cell induces the formation of a lytic immunological synapse between the NK cell and its target, finally triggering the release of cytotoxic proteins to kill the target cell.[3]

Evaluation of NK cell cytotoxicity is important for understanding NK cell biology and for adoptive immunotherapy applications.[4,5] To achieve this aim, several different assays have been used for assessing the cytotoxicity of NK cells, including radioactive and non-radioactive ones. For radioactive cytotoxicity assays, the ^{51}Cr-release assay (CRA) is the most commonly used for assessing the cytotoxicity of NK cells.[6] Given the concerns of handling and disposing of the radioactive compound, non-radioactive alternatives, a colorimetric cytotoxicity assay to measure the release of lactate dehydrogenase (LDH) by dead or dying target cells, and flow cytometry–based cytotoxicity assays (FCCs) have been developed to overcome the disadvantages and limitations of the CRA.[6-8] However, although several FCCs have been reported, there are still disadvantages in precisely distinguishing effector and target cells, caused by the prior labeling and the high rate of spontaneous leakage of the labeled fluorochromes.

Here, we described a novel FCC based on the staining using fluorochrome-labeled CD2 antibody in combination with nucleic acid dye SYTOX Green and early apoptotic marker Annexin V. In this cytotoxicity assay, CD2 antibody was used to discriminate NK cells from target cells, and SYTOX Green and Annexin V-FITC were then used to identify the necrotic and apoptotic target cells.

2 | MATERIALS AND METHODS

2.1 | Regents and antibodies

Recombinant human IL-2 (#200-02) was bought from PeproTech. CD2-APC (#341022), CD3-Horizon™ V450 (#560351), CD56-PE (#555516), Annexin V-FITC, and Annexin V-binding buffer (#556547) were bought from BD Biosciences. SYTOX Green nucleic acid stain (#S7020), carboxyfluorescein succinimidyl ester (CFSE, #C34554), and 7-Aminoactinomycin D (7AAD, #A1310) were purchased from Thermo Fisher Scientific. Ficoll-Paque PLUS (#17-1440) was bought from GE Healthcare.

2.2 | Cell lines and cell culture

NK cell lines KHYG-1 (DSMZ, #AC-725) and NK92 (ATCC, #CRL-2408) were cultured in RPMI1640 supplemented with 10% FBS, 1% penicillin/streptomycin, and 10 ng/mL IL-2. Myelogenous leukemia cell line K562 and HL60, T-cell leukemia cell line Jurkat, Burkitt lymphoma cell line Raji, acute monocytic leukemia cell line MOLM13, multiple myeloma cell line NCI-H929, RPMI8226, MM.1S, and KMS11 were kept in our laboratory and cultured in RPMI1640 supplemented with 10% FBS and 1% penicillin/streptomycin.

2.3 | Flow cytometry–based cytotoxicity assay using CD2 antibody in combination with Annexin V-FITC and SYTOX Green

NK (effector) and tumor (target) cells were resuspended in complete medium RPMI1640 supplemented with 10% FBS, 1% penicillin/streptomycin, and 10 ng/mL IL-2 at a concentration of 1×10^6 cells/mL. Effector cells and target cells were co-incubated at different E:T ratios in 24-well flat-bottom plates for 4 h at 37°C in a humidified atmosphere containing 5% CO_2. Target cells alone in culture medium were used as negative controls. After incubation, cell samples were collected and washed with ice-cold staining buffer (PBS containing 0.1% NaN_3 and 0.1% BSA) once, then stained with 5 μL of CD2 antibody in 100 μL of staining buffer for 30 minutes to distinguish the effector from target cells. After washing twice with ice-cold staining buffer, the samples were further resuspended in 100 μL binding buffer and labeled with 5 μL of Annexin V-FITC and 1 μL of SYTOX Green (1 mg/mL) for 15 minutes. Before running flow cytometric analysis, 300 μL of binding buffer was added to each sample placed on ice. The samples were finally analyzed using a flow cytometer CytoFLEX (Beckman Coulter Life Sciences). A minimum of 10 000 events was collected per sample, and the results were further analyzed using Flowjo v7.6.2 (Tree Star Inc). The percentage of specific lysis (PSL) was calculated as positive for both Annexin V and SYTOX Green in CD2-negative cells/ total CD2-negative cells, after subtracting the spontaneous lysis (%) in the negative control.

2.4 | Flow cytometry–based cytotoxicity assay using 7-AAD/CFSE

The flow cytometry–based cytotoxicity assay using 7-AAD/CFSE was performed as previously reported.[9] Briefly, 5×10^6 target cells were labeled with 2 μmol/L CFSE in PBS for 15 minutes at 37°C in a volume of 1 mL. After washing twice with complete medium, CFSE-labeled target cells were then incubated with NK cells at different E:T ratios in 24-well flat-bottom plates for 4 hours. In parallel, target cells cultured alone in the medium were used as a negative control to measure basal cell death. After coculture for 4 hours at 37°C, 5% CO_2, the cell mixture was stained with 5 μL of 7-AAD for 15 minutes in the dark. The samples were analyzed on the flow cytometer and FlowJo software, as described above. NK cytotoxicity (%) was calculated as cells positive for both CFSE and 7-AAD/total CFSE-positive cells, after subtracting the spontaneous lysis (%) in the negative control.
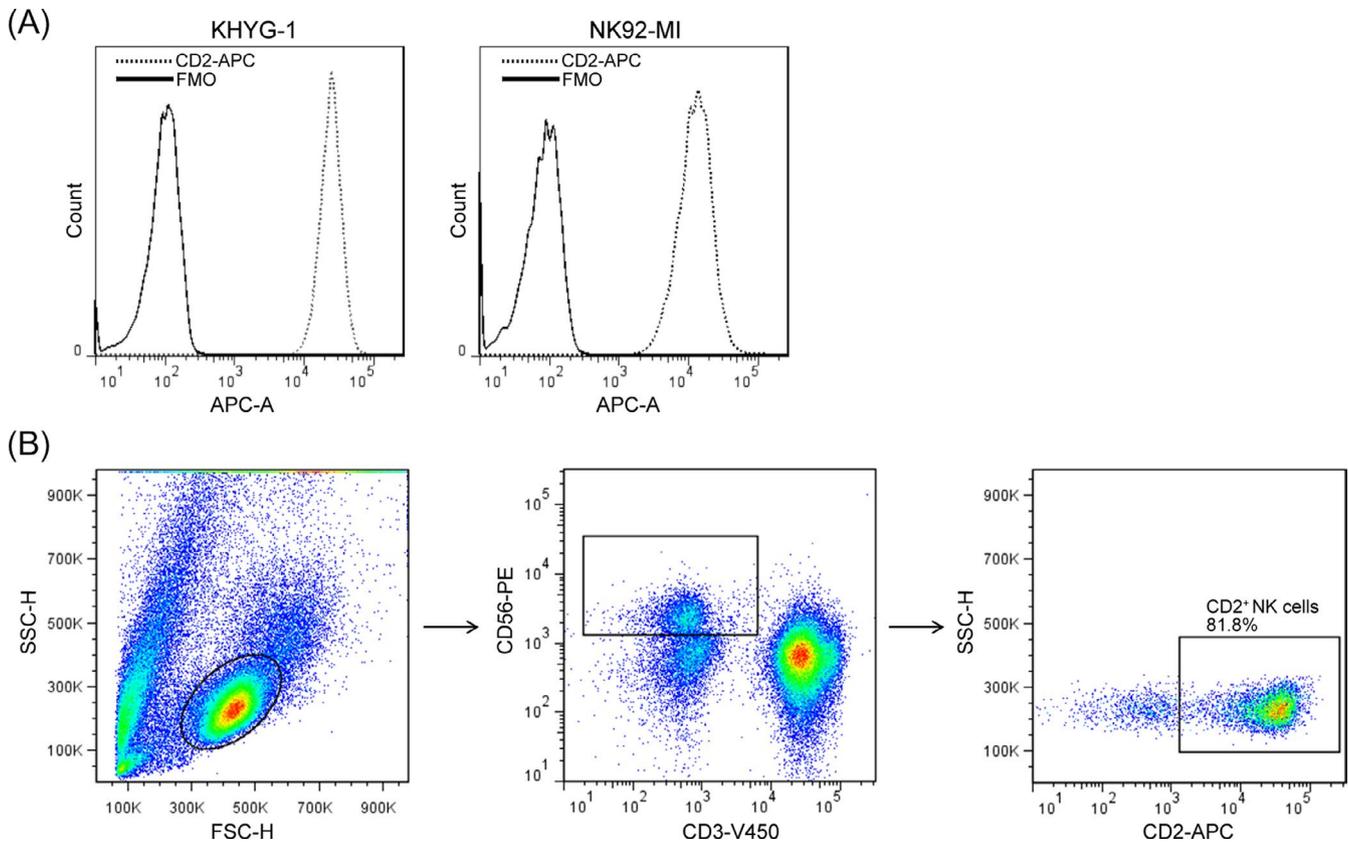
FIGURE 1 Flow cytometric analysis of the expression of CD2 on NK cells. A, Histograms show the expression of CD2 on NK cell line KHYG-1 and NK92. CD2 is 100% positive in KHYG-1 and NK92 cells. A Fluorescence Minus One (FMO) was used as negative control to identify gating boundaries. B, A representative experiment of flow cytometric analysis of the expression of CD2 on human peripheral blood NK cells

2.5 | ^{51}Cr-release assay

The ^{51}Cr-release assay was performed as described previously.[10] Briefly, 5×10^6 target cells were loaded for 2 hours with 100 μCi of $Na_2{}^{51}CrO_4$ (Atom-Hitech) at 37°C in the atmosphere of 5% CO_2 in the air. After washing twice with complete medium, the labeled cells were then mixed with NK cells at different E:T ratios in U-bottom 96-well plates (Costar, Corning) for 4 hours at 37°C. Controls included target cells incubated in culture medium alone for spontaneously release and targets in 5% (v/v) Triton X-100 in PBS for maximum release. ^{51}Cr released into supernatants was measured with a gamma detector (USTC Zonkia Scientific Instruments). The PSL was calculated as (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100.

2.6 | Flow cytometry analysis of the CD2 expression on peripheral blood NK cells

Peripheral blood from healthy donors was collected in heparin tubes and processed within 4 hours. Peripheral blood mononuclear cells (PBMCs) were separated by density-gradient centrifugation using a lymphocyte separation medium Ficoll-Paque PLUS. In brief, 5 mL of peripheral blood was layered on 5 mL of LSM and centrifuged at 400 g for 30 minutes at room temperature. PBMCs were collected

and then washed twice with PBS. Next, PBMCs were stained with fluorescence-labeled anti-human antibodies against CD3-V450, CD56-PE, and CD2-APC. The expression of CD2 on the CD3$^-$CD56$^+$ NK cell population was analyzed using a flow cytometer CytoFlex.

2.7 | Statistical analyses

Statistical analyses were performed using the Prism software package 5.0 (GraphPad Software). Data are expressed as the mean ± SEM of at least three independent experiments. The between-group differences of cytotoxicity results were compared by Student's t test. A $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | CD2 is highly expressed on NK cell lines and human peripheral blood NK population

In NK cells, the expression of CD2 has been demonstrated to play an important role in NK cell activation and cytolysis.[11-13] Concerning this specific expression pattern in NK cells, we further evaluate the possibility that CD2 could be used as a marker to discriminate NK cells from target

cells. Firstly, we detected the expression of CD2 on two widely used NK cell lines. As shown in Figure 1A, both KHYG-1 and NK92 NK cell lines show a 100% positive membrane staining of CD2. Secondly, we evaluated the expression of CD2 on primary NK cells from human peripheral blood. As shown in Figure 1B, we found that CD2 is strongly expressed in human peripheral blood NK cells. The percentage of CD2 positive in CD3⁻CD56⁺ NK cell population ranges from 78.3 to 98.5 (n = 4).

3.2 | CD2 is negatively expressed on most tested tumor cell lines

We further investigated the expression of CD2 in some widely used hematological cancer cell lines. As shown in Figure 2A, CD2 is negative in leukemia cell lines K562, HL60, and MOLM13, B-cell lymphoma cell line Raji, as well as multiple myeloma (MM) cell lines NCI-H929, RPMI8226, MM.1S, and KMS11. However, we found that T-cell leukemia cell line Jurkat is CD2-positive (Figure 2B).

3.3 | Gating strategy for the CD2 staining–based FCC

Flow cytometric analysis in this assay involves the detection of two parameters: NK cell staining, by detecting CD2 in the channel of APC; and dead target cell staining, by detecting Annexin V-FITC and SYTOX Green in the same channel of FITC in CD2-negative cell population. After data acquisition, the gating strategy in Figure 3 was used to analyze data. First, debris was excluded in an FSC-H/SSC-H plot. NK cells are then identified by gating on CD2-positive cells. Thereafter, dead target cells are gated in the APC-A/SSC-H within the CD2-negative cell population and quantitatively
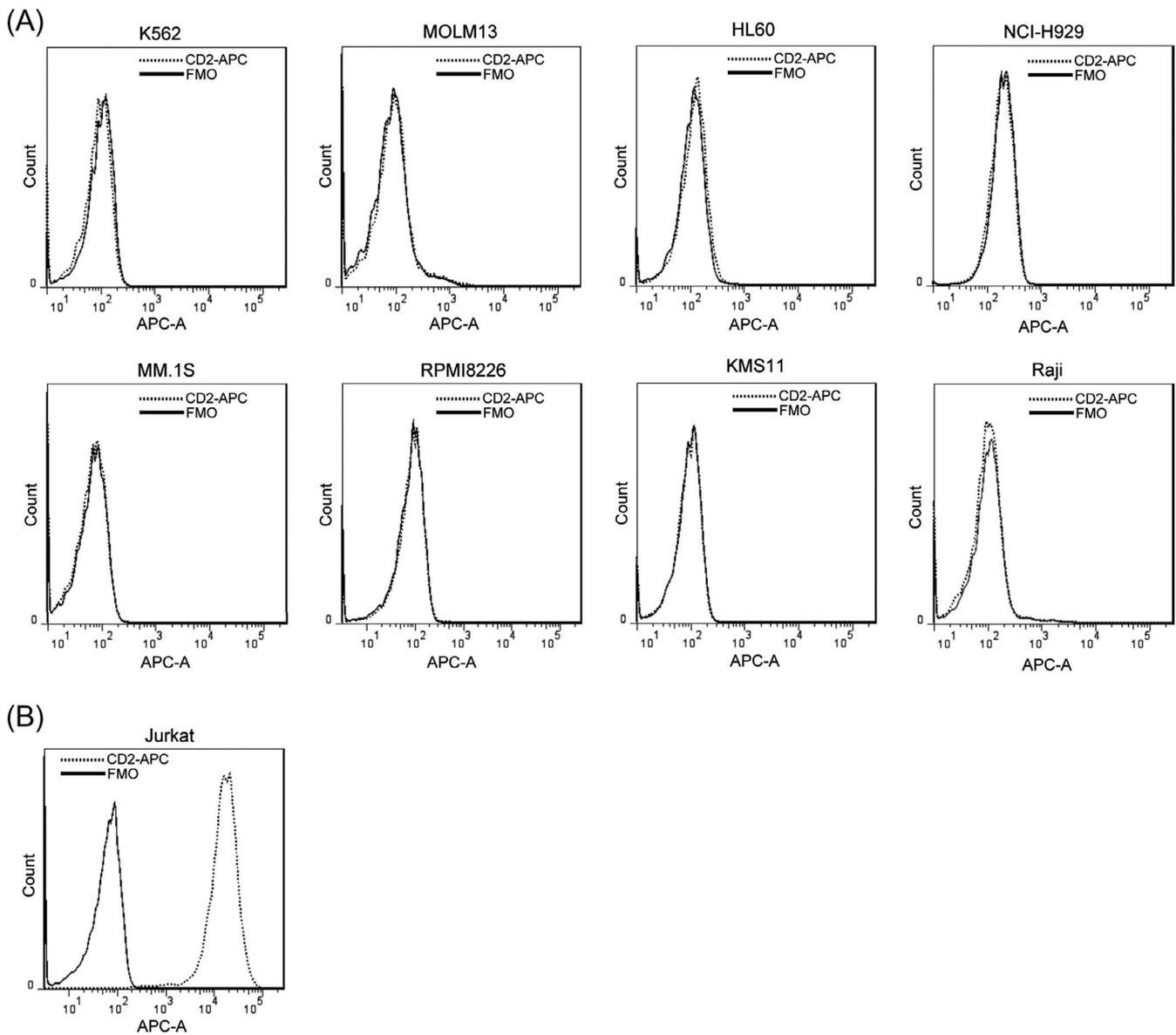

FIGURE 2 Flow cytometric analysis of the expression of CD2 on hematologic cancer cell lines. A, CD2 is negative in K562, MOLM13, KMS11, HL60, NCI-H929, MM.1S, RPMI8226, and Raji cells. B, CD2 is positive in Jurkat cells
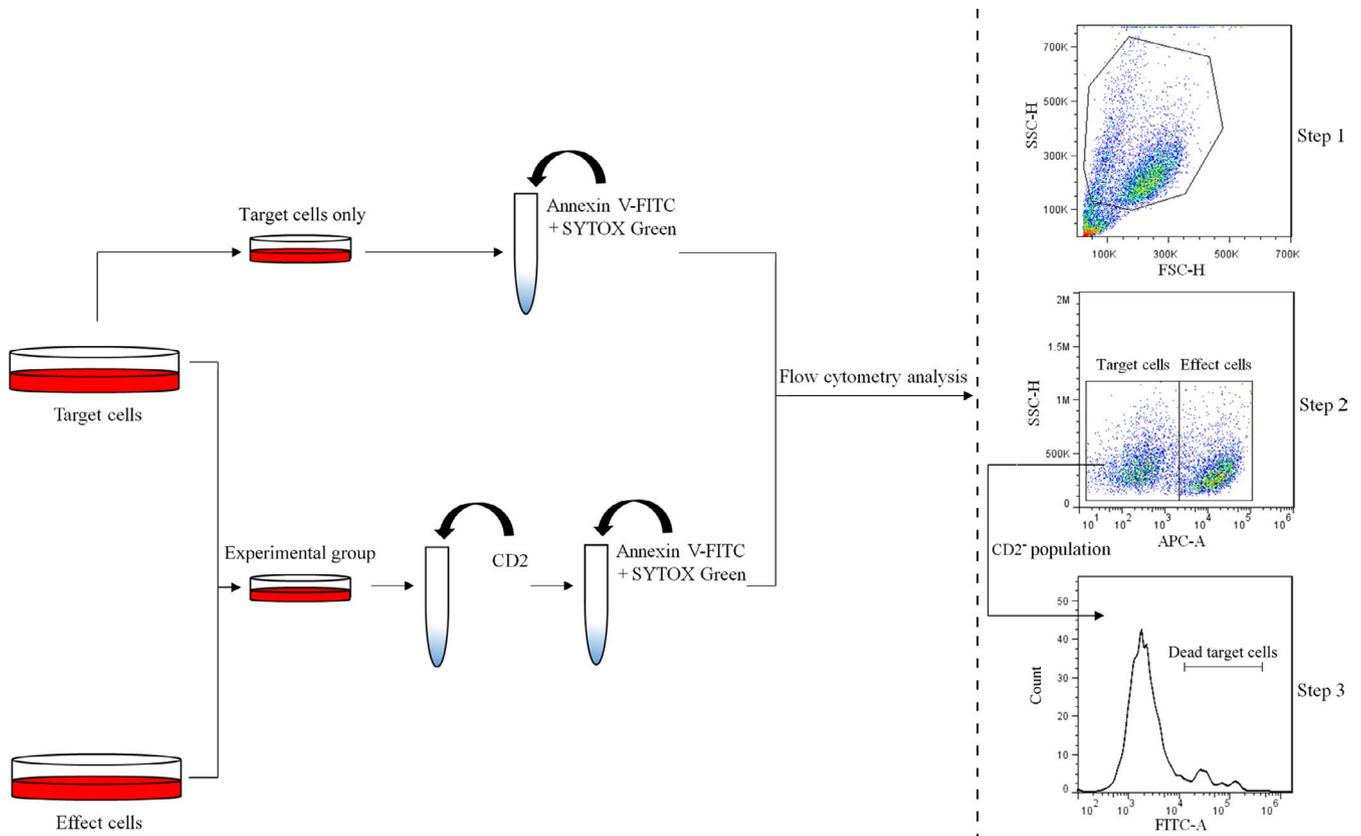
FIGURE 3 The gating strategy used to distinguish NK cells from target cells. NK cells and target cells mixed at different E:T ratios were incubated for 4 h, and target cells only were used as the negative control. The cocultured cells were then harvested and stained with CD2 antibody and SYTOX Green/Annexin V-FITC. Thereafter, flow cytometric analysis was performed to detect the target cell death. In step 1, debris is excluded in an FSC-H/SSC-H plot. In step 2, NK and target populations are identified by gating CD2+ cells and CD2- cells, respectively. In step 3, within the plot of CD2-, SYTOX Green+ and Annexin V-FITC+ are identified as the dead target cells

analyzed by counting Annexin V-FITC and SYTOX Green-positive events.

3.4 | Quantitative measurement of NK cell cytotoxicity against K562 cells using the CD2 staining–based FCC

Representative results of NK cell–mediated killing of K562 or MM.1S cells following the above gating strategy are illustrated in Figure 4. NK cells (KHYG1) against K562 (or MM.1S) cells at two E:T ratios (1:1 and 5:1) were used to assess the consistency of the assay. A good correlation was observed between PSL and E:T ratios. Moreover, a real-time E:T ratio monitored by CD2-positive or CD2-negative populations could also be shown simultaneously.

3.5 | Combinational staining using SYTOX Green and Annexin V significantly increases the sensitivity to detect dead target cells

Another major difference from the traditional FCCs is that we use both DNA dye and the early apoptosis marker Annexin V to detect

the dead target cells. As shown in Figure 5, compared with the samples stained with SYTOX Green or Annexin V-FITC alone, the combinational staining shows a significantly higher detectable rate of death in the target cell population. Compared to the double staining, DNA dye SYTOX Green staining alone can only detect a lower number of necrotic and late apoptotic target cells; similarly, Annexin V staining alone only detects a lower number of early and late apoptotic target cells.

3.6 | Comparison of the CD2 staining–based FCC with the CRA and the conventional 7-AAD/CFSE-based FCC

To further validate that the CD2-based FCC is reliable, we compared it with the CRA and a conventional 7-AAD/CFSE FCC using KHYG-1 cells as effectors and K562 cells as targets. After 4 hours co-incubation at different E:T ratios ranging from 10:1 to 1:1, we quantitatively analyzed the target cell lysis using these three different assays in parallel. As shown in Figure 6, although these three assays show that strong lysis of target cells was induced in an E:T ratio-dependent manner, leading to cell death of the majority target cells (>60% for the E:T ratio of 10:1). As expected, the results of the three arrays
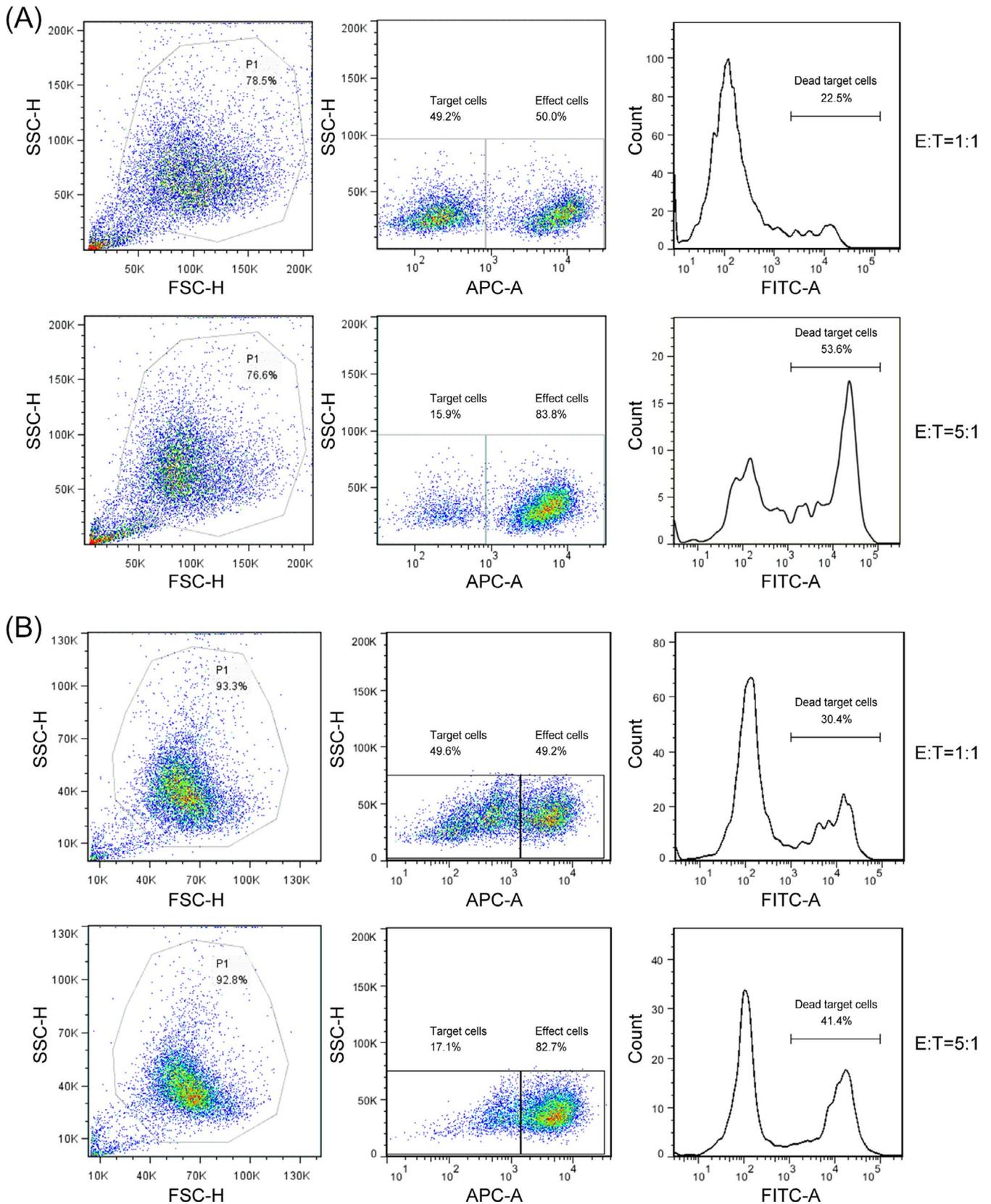
FIGURE 4 Representative results of the flow cytometric method. A, Representative results of KHYG-1 cells against K562 cells at E:T ratio of 1:1 and 5:1, using the gating strategy described in Figure 3. B, Representative results of KHYG-1 cells against MM.1S cells at E:T ratios of 1:1 and 5:1, using the gating strategy described in Figure 3
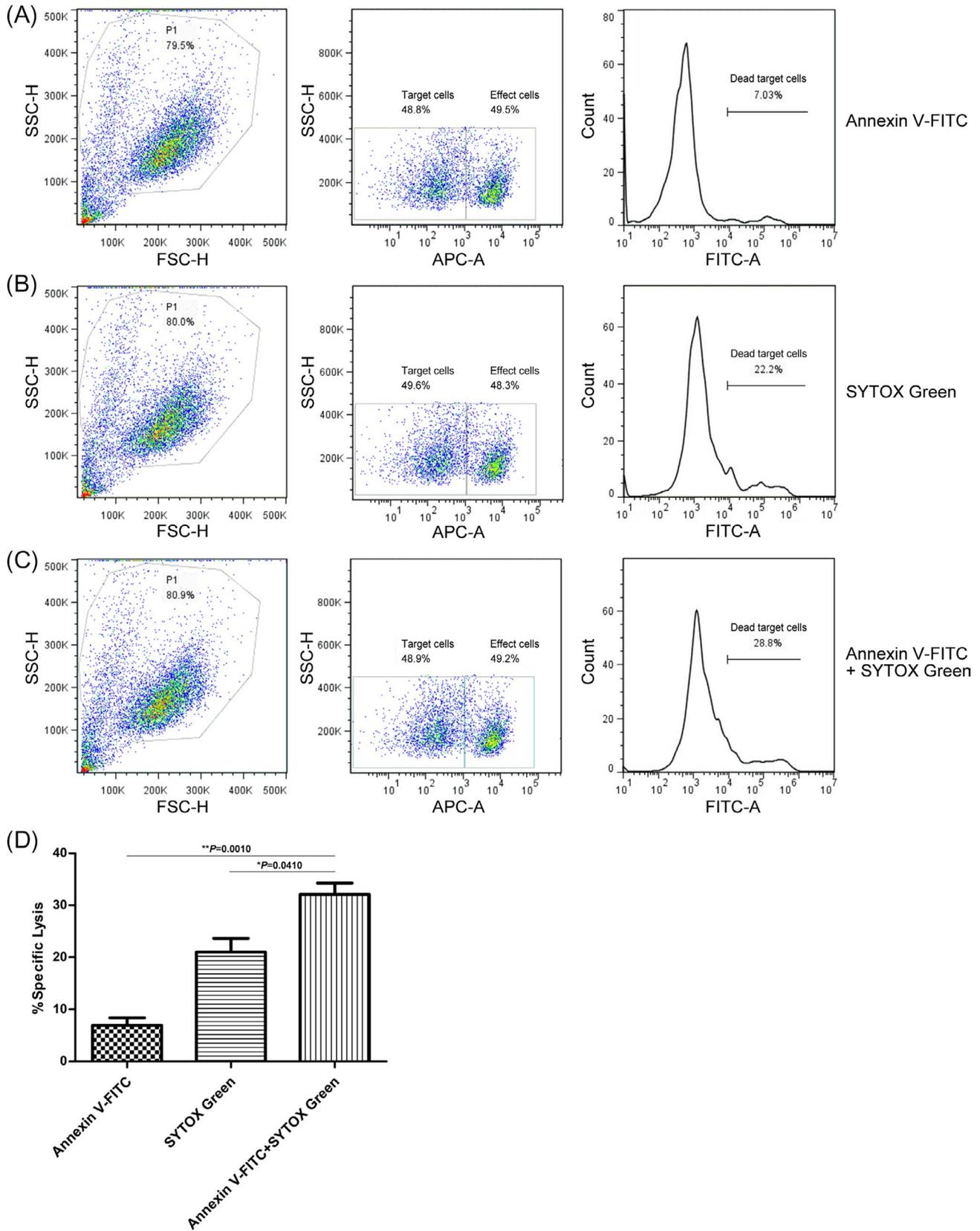
FIGURE 5 The effects of SYTOX Green/Annexin V-FITC single or double staining on the detection of target cell death in the same samples. KHYG-1 cells were incubated with K562 cells for 2 h at an E:T ratio of 1:1, and then, flow cytometric analysis was performed using the gating strategy described in Figure 3. A, A representative result of SYTOX Green staining alone. B, A representative result of Annexin V-FITC staining alone. C, A representative result of the Annexin V-FITC and SYTOX Green double staining. D, Statistical analysis of the effects of SYTOX Green/Annexin V-FITC single or double staining on the detection of target cell death (*$P < .05$, **$P < .01$, n = 3)
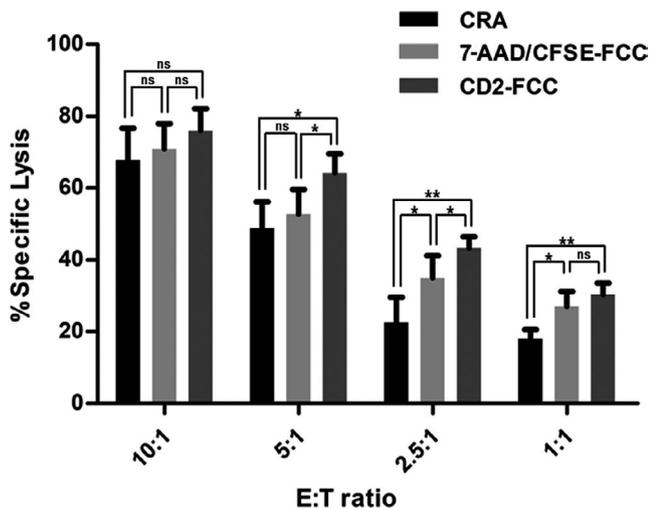
FIGURE 6 Comparison of the three assays for evaluating NK cell cytotoxicity. KHYG-1 cells were incubated with K562 cells at different E:T ratios for 4 h, and then, the PSLs of the three different assays were calculated based on the formula described in materials and methods (*$P < .05$, **$P < .01$, $n = 3$)

are strongly and positively related to each other. Of note, at low E:T ratios of 2.5:1 and 1:1, higher levels of specific lysis were observed with two FCCs, compared to the CRA. In addition, we found that CD2-based FCC is more sensitive than the FCC using 7-AAD/CFSE FCC at lower E:T ratios of 2.5:1 and 1:1. These findings may suggest that CD2-based FCC is more reproducible and sensitive.

4 | DISCUSSION

The CRA has been widely considered the gold standard for measuring the cytolytic activity of effector cells since 1968.[8] While CRA has several positive features, it still has several limitations, including the use of radioactivity, single time point readout, short half-life, time-consuming, specialized radioactive training, the high cost of safe disposal of radioactive waste, and spontaneous release of ^{51}Cr in the medium. Thus, several alternative methods have been developed, among which include FCCs that prevent those limitations related to the use of radioactivity. Compared to CRA, FCCs show several significant advantages, including the detection of cytotoxicity at the single-cell level, measurement at multiple time points, highly reproducible, and the possibility of characterizing the phenotype of the effector cells.[8,9,14,15] For most FCCs, they use a wide range of different fluorescent dyes, such as calcein acetoxymethyl ester (CAM), CFSE, Vybrant DiO (DiO), and MitoTracker Green (MTG) to label target cells, and then use nucleic acid dye like propidium iodide (PI) or 7-Aminoactinomycin D (7-AAD) to determine the lysed cells.[16] However, most of the currently used FCCs have two major disadvantages: One is the high spontaneous release of the labeled fluorescent dyes and the possible uptake by the neighbor unlabeled cells; the other is the potential effects of the labeling dyes on many

aspects of cell function. Meanwhile, the use of the nucleic acid dye PI or 7-AAD to detect dead target cells also has significant drawbacks, since it can only determine the necrotic and/or late apoptotic cells that merely account for the part undergoing lysis. For those in the early stages of apoptosis, the intact cell membrane inhibits the entrance and binding of nucleic acid dye with DNA. Given that NK cells can kill target cells by either necrotic or apoptotic mechanisms, as well as mixed forms, it is therefore important to detect both apoptotic and necrotic cells simultaneously.[17-19] Therefore, the traditional FCCs using DNA dye alone are unable to find these early apoptotic target cells. In the current study, combinational staining using Annexin V-FITC and SYTOX Green allows both apoptotic and necrotic cells to be quantified. Correspondingly, it shows higher sensitivity than the conventional 7-AAD/CFSE-based FCC at lower E:T ratios. Our findings strongly indicate that detecting lysed target cells using DNA dye is not enough since it cannot show early apoptotic cells, which represent the major ones occurring from early death but with intact cell membranes.

Discrimination of target and effector cells is critical for such NK cytotoxicity assays. Here, CD2 was used for distinguishing NK cells from target cells. Indeed, we observed that CD2 is strongly and exclusively expressed in NK cell lines KHYG-1 and NK92 and is negative in 8 kinds of widely used hematological cancer cell lines. However, we found that T-cell leukemia cell line Jurkat is CD2 positive. Given that CD2 was found on almost all mature peripheral T cells and thymocytes,[20] it strongly suggests a limitation that this CD2-based FCC could not be used for measuring NK cell-mediated killing of T-cell malignancies and other CD2-positive cancers. Moreover, focusing on the expression of CD2 on primary NK cells, we confirmed a highly positive CD2 expression in peripheral blood NK cell populations. In this regard, previous studies have shown that CD2 is highly and stably expressed on CD56^{bright} than the CD56^{dim} NK cell subset.[21-23] CD56^{dim} population is predominant in peripheral blood, and CD56^{bright} NK cells are primarily from secondary lymphoid tissue and other tissues,[24] and this also limits the use of CD2 to trace NK cell population in cytotoxicity assay, to a certain degree.

Another major advantage of this CD2-based FCC is that there is no interference with the experimental system. The major difference of the current CD2-based FCC from other assays is that all staining procedures are performed after the co-incubation of effectors and targets. Although it has been accepted that labeling cells with fluorescent dyes such as CFSE are non-toxic, it still cannot exclude the other side effects on cell function. For example, the leakage and uptake of fluorescent dye by unlabeled cells would inevitably increase the systemic errors. In this regard, for the CD2-based FCC, there is no prior labeling of the cells with chromium or fluorescent dye. Theoretically, it could not lead to any potential effects on the cell function.

NK cell cytotoxicity is tightly related to the expression of a wide array of surface and intracellular molecules.[22,25] To monitor the phenotypic heterogeneity of NK cells and the molecular interactions between NK and target cells, it is essential for better understanding the mechanisms of NK cell–mediated death of various targets.

This new CD2-based FCC assay offers an opportunity for a multi-parameter definition of the relationship between lytic activity and the dynamic molecular changes during the interaction of NK and target cells. By using additional antibodies against various phenotypes of NK cells such as the activation status, cytokine and signaling molecule expression, this FCC assay can be further adapted to be a tool for investigating the molecular mechanisms of NK cells contributing to target lysis.

5 | CONCLUSIONS

In conclusion, we described a straightforward CD2-based FCC to assess NK cell cytotoxicity. This modified method is not only sensitive and reliable but also offers several advantages over the previously developed FCCs. Firstly, there is no prior labeling necessary, all staining procedures are performed after co-incubation, and therefore, it does not disturb the function of both effector and target cells. Secondly, combinational staining target cells with SYTOX Green and Annexin V-FITC can efficiently detect the major types of target cell death, including necrosis and apoptosis.

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