Cell mediated cytotoxicity in “untouched” whole blood

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ABSTRACT

We have developed a quantitative flow cytometry method for cell-mediated cytotoxicity studies, preserving cellular function with minimal sample manipulation. Cytotoxic activity is simply and reproducibly measured as the ability of cytotoxic cells to lyse K562 target cells previously loaded with Calcein-AM vital stain. After spiking a known number of fluorescent viable K562 target cells into whole blood, cell mixtures are incubated for 2 hours in a cell incubator and the remaining spiked cells are counted by flow cytometry. In order to discriminate nucleated cells, erythrocytes, and debris, unlysed blood is vitally stained with a cell permeable DNA fluorescent dye. Cell-mediated lysis is measured by comparing target counts for different target-effector ratios. Since the cytotoxicity of these dyes is relatively low, this method can be broadly applied to studies of innate immune response to tumors and infections, especially where target-killing activity might be compromised by small volume samples or low frequency of cytotoxic cells.

METHODS I

Cell lines and media. K562 and EGFP-K562 cells were grown with 10% heat-inactivated FBS (Biowest) together with RPMI (Biowest), 2 mM l-glutamine, 1 mM sodium pyruvate, 15U/ml penicillin and 15ng/ml streptomycin (PAA). Cytotoxic assays were all performed in the absence of antibiotics and serum, with AIM V medium (Thermo Fisher Scientific). AIM V medium does not require human serum for optimal growth and activation of differentiated lymphoid cells and contains no added cytokines and lymphokines.

Collection of blood samples. Heparinised blood samples were obtained from patients with their written informed consent according to the protocol approved by the Ethical Committee of the Germans Trias i Pujol Hospital. Blood was collected into heparinised tubes and analysed immediately after collection. Blood samples were processed immediately or within 1 hr after collection.

Preparation of green fluorescent target cells: Before each experiment, either EGFP-K562 or WT-K562 cells stained with Calcein AM were counted using flow cytometry. Dead cells were excluded with Propidium Iodide (PI) (Thermo Fisher Scientific) at a concentration of 5µg/ml. EGFP expression was also checked in EGFP-K562 cells before each experiment, to prepare 1 ml of cell suspension containing 1,2x10^5 cells/ml in AIM-V medium (Thermo Fisher Scientific) with 100% EGFP positive K562 cells. We also used wild type K562 cells stained with Cell Calcein Green AM. 2µl of Calcein Green AM (Thermo Fisher Scientific) were added to 1ml cell suspension with a concentration of 1x10^6 cells/ml in PBS (Biowest). Cells were incubated 30 minutes at room temperature and protected from light. Optimal concentration of this fluorochrome was determined empirically. After incubation, cells were washed three times by resuspending the pellet in PBS. WT-K562 stained cells were counted by measuring Calcein AM accumulation by flow cytometry, excluding dead cells as above. WT-K562 cells stained with Calcein AM were then prepared to obtain a 1ml cell suspension with a concentration of 1,2x10^5 cells/ml in AIM-V medium. Calcein AM labeling was optimized to obtain 100% green fluorescent wt-K562 cells before each experiment.

EGFP-K562 cells provide a “ready-to-use” target

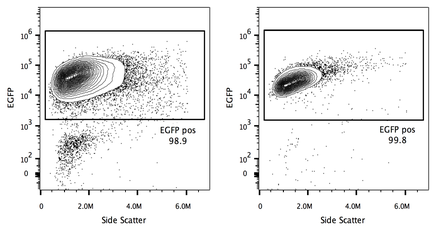

Figure 1. Monitoring stability of EGFP transduction in green fluorescent K562 cells. (**a**) Contour plots showing EGFP-K562 cells after excluding propidium iodide positive cells, before **(a)** and after the selection procedure using a limiting dilution assay **(b)**.

METHODS II

Preparation of effector cells. Lymphocyte counting was immediately performed prior to cytotoxicity experiments. Unlysed whole blood was prepared for absolute counting of lymphocytes using flow cytometry. In order to discriminate dead cells, erythrocytes, and debris, 50µl of whole blood were incubated in the presence of 10µM Vybrant™ DyeCycle™ Violet Stain (DCV) (Thermo Fisher Scientific) for 10 minutes at 37ºC and light-protected. After incubation, the sample was diluted 1/8 with Attune™ Focusing Fluid 1x (Thermo Fisher Scientific) and acquired on a flow cytometer to provide absolute lymphocyte counts. Lymphocyte dilutions were prepared with AIM-V medium (Thermo Fisher Scientific) as follows: a) 8x10^5 lymphocytes/ml, b) 4x10^5 lymphocytes/ml, c) 2.4x10^5 lymphocytes/ml, and d) 4x10^4 lymphocytes/ml. Dead cells were excluded with Propidium Iodide (PI) (Thermo Fisher Scientific) at a concentration of 5µg/ml.

WT-K562 staining as an alternative to EGFP

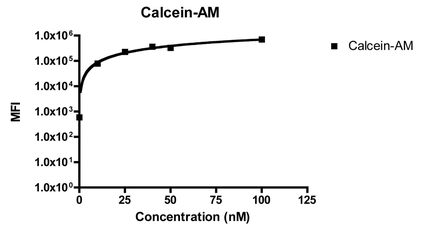

Figure 2. Fluorescent intensity of WT-K562 cells as a function of the concentration of dye. Analysis of the fluorescent intensity at different concentrations of Calcein-AM.

METHODS III

Cytotoxicity assays. Cytotoxicity assays were performed in a 12 well plate using unlysed whole blood. EGFP-K562 target cells and pre-stained-K562 target cells were used at four different effector-to-target (E/T) cell ratios (1:3, 1:1, 5:3 and 10:3). Cells mixed to a final volume of 1 ml in AIM V medium were incubated for 2h in a cell incubator. Control cells were prepared without cell co-incubation. In order to discriminate nucleated cells from erythrocytes and debris, cells were stained with Vybrant DyeCycle Violet Stain (DCV) (Thermo Fisher Scientific) at a concentration of 10µM, for 5 minutes at 37ºC and protected from light. Propidium Iodide (PI) (Thermo Fisher Scientific) was used at a concentration of 5µg/ml prior to analysis in order to discriminate dead cells. Following incubation, 500 µL of cell mixtures were immediately prepared for flow cytometry analysis to directly detect the presence of green fluorescent target cells. One tube was needed for each condition tested. Samples were diluted 1/8 in acoustic focusing fluid and filtered using a nylon mesh of 52µm.

WT-K562 to lymphocyte ratio

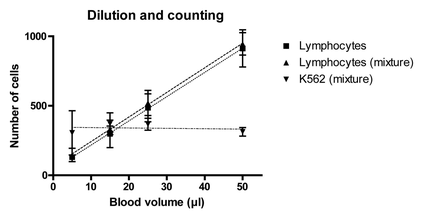

Figure 3. Number of lymphocytes vs. blood volume added. Analysis and counting by flow cytometry of the number of lymphocytes present in several dilutions of blood (blood only) or blood and a constant volume of EGFP-K562 cells (blood + cells). All measurements were done in triplicate and values are expressed as Mean ± SD.

METHODS IV

Flow cytometry. Data was collected on the Attune™ Flow Cytometer (Thermo Fisher Scientific) equipped with a 50-mW violet laser operating at 405nm and a blue laser operating at 488nm. The sample delivery rates used were 100µl/min, 200µl/min and 500µl/min. The filter combination consisted of 450/40nm band pass (blue), 530/30nm band pass (green), 640 long pass (red), 574/24nm band pass (orange) and 500 dichroic long pass filters. Forward Scatter and Side Scatter were collected on linear scale, and a blue fluorescence threshold was set to exclude erythrocytes but not lymphocytes with low forward scatter. Analysis was performed with FlowJo software (Tree Star, Inc.).

Effect of red cell lysing on leukocyte counting

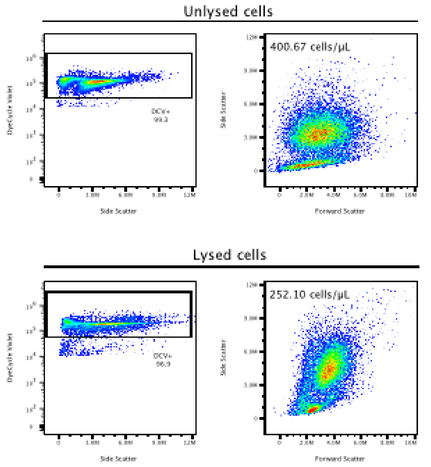

Figure 4. Red cell lysing effect on human bone marrow cells. The Attune Flow Cytometer enables absolute counting, eliminating the need to use counting beads. Human bone marrow cells from a healthy donor were stained in duplicate with Vybrant DyeCycle Violet stain, with and without red cell lysing procedures. By means of volumetric analysis, a cell depletion of up to nearly 40% of leukocytes was observed, when unlysed (top) and lysed specimens (bottom) were compared.

Cytotoxic activity in ulysed whole blood

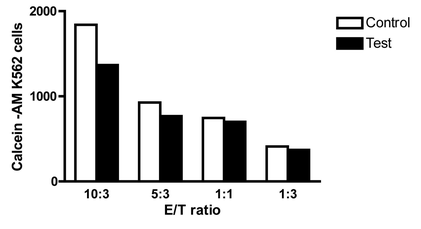

Figure 5. Representative cytotoxicity experiment using unlysed whole blood. Lysis of Calcein-K562 cells at different effector-to-target ratio. Values are expressed as the number of cells detected in figure 5. Cytotoxic activity was measured as the ability of cytotoxic cells from whole blood to effectively lyse K562 cells.

Method for counting Calcein-K562 cells

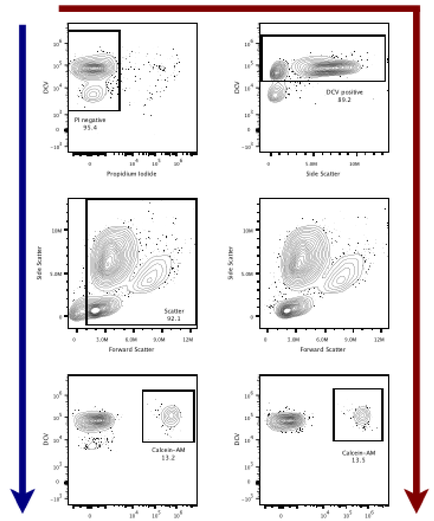

Figure 6. Comparison between different gating strategies. WT-K562 cells stained with Calcein-AM were discriminated from blood by their green fluorescence. DCV was used to discriminate erythrocytes and debris from nucleated cells.

CONCLUSIONS

Among the most important principles of this assay is the use of unlysed whole blood. Current cytotoxicity assays usually isolate peripheral blood mononuclear cells by density gradient centrifugation. This step can cause cellular depletion and cell dysfunction, and may result in erroneous measurements, especially when very few cytotoxic cells are present or when sample size is very small.

Red cell lysing procedures can similarly cause depletion and dysfunction and should be avoided when possible to avoid their potential effect on cell cytotoxicity.

Improperly prepared cells, inaccurate calibration of pipettes, and poor pipetting technique can cause erroneous dilutions, with consequences in the application of insufficient or excessive amount of cells that may affect target and effector ratios.

Cell suspensions should be as homogeneous as possible. K562 are large cells in comparison with normal blood cells, and will sediment faster than the smaller ones.

Cytotoxic assays should be performed with serum-free medium and in a preferably dedicated cell incubator. Advantages of using serum-free media include precise evaluation of cellular function, and elimination of lot-to-lot variation in the assay.

Many commercially available flow cytometers can be used to perform this assay, but many of them cytometers cannot directly provide the cell concentration or absolute count of cells in a sample. The main requirement for the experimental setup is a violet laser, or alternatively ultraviolet (UV) or near-UV lasers.