TITLE:

A novel flow cytometric method to study cytotoxic activity in whole blood samples

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Cell-mediated cytotoxicity in “untouched” whole blood.

We have developed a quantitative 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 whole blood is stained with a cell permeable DNA vital fluorescent dye. Cell-mediated lysis is measured by comparing target counts for different effector-to-target 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.

For several decades, cell-mediated cytotoxicity has been measured using the ^{51}Cr release assay ¹. This radiometric assay has several drawbacks however ², and since degranulation has been shown to be associated with the cytotoxic activity of Natural Killer (NK) cells, cytotoxicity measurement is often performed by flow cytometry in combination with specific antibodies and/or cytokines that indicate degranulation. The flow cytometry assay uses quantification of bound fluorescent CD107a antibody to indicate the level of activation of the effector population ³, whereas the ^{51}Cr release assay quantifies the lysis of target cells. For both methods, the continuous cell line K562, established by Lozzio and Lozzio ⁴ from the pleural effusion of a 53-year-old female with chronic myelogenous leukemia in terminal blast crises, is widely used as a highly sensitive *in vitro* target for natural killer cells.

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 (e.g. pediatric samples). Red cell lysing procedures can similarly cause depletion and dysfunction and should be avoided when possible to circumvent their potential effect on cell cytotoxicity. We have observed depletion of up to nearly 40% of leukocytes by comparing lysed and unlysed specimens [Supplementary Figure I].

We previously reported no-lyse, no-wash protocols to allow “untouched” processing of whole blood and bone marrow by simply adding reagent, including a nucleic acid stain for identifying nucleated cells, and diluting and running analysis at a higher sample rate than is practical for a conventional cytometer ⁵.

Given the importance of cytotoxic assays and the deleterious effects of current sample preparation methods, we have adapted this untouched flow cytometry method to study cytotoxic activity using unlysed whole blood samples and fluorescent-K562 cells. This approach is simple and rapid when compared with other previously described methods and it takes advantage of classic methods that are widely used. It is fast and highly sensitive and avoids red cell lysis, washing and centrifugation steps.

Key to the non-radioactive quantification of K562 target cells by flow cytometry is the fluorescent labeling of the cells prior to incubation with the cytotoxic cell population. Although previous studies have not avoided density gradients and/or red cell lysing procedures, they have explored the use of various labeling strategies. Fluorescent dyes used for flow cytometry of K562 target cells include MitoTracker™ Green ⁶, PKH67 ⁷, DiOC₍₁₈₎3⁸, and BCECF, AM ⁹. Green fluorescent protein (GFP) and the enhanced GFP (EGFP) variant have been also used to obtain stable transfectant or transduced target cells ¹⁰.

We have used two different labeling approaches. In the first approach, EGFP-K562 cells provide a “ready-to-use” target without time needed for labeling or staining, as shown in Figure 1. However, this cell line shows a progressive silencing of the target gene (Figure 1a) which requires repeated isolation of high-purity single cell clones by limiting dilution or by cell sorting (Figure 1b and Figure 1c) [Supplementary Table I]. For the second approach, we performed parallel cytotoxicity assays in the presence of wild type K562 (WT-K562) cells previously stained with a fluorescent dye with spectral properties similar to EGFP. This parallel staining procedure is aimed at avoiding the issues associated with EGFP silencing.

WT-K562 cells were independently stained with three different fluorescent dyes. CellTrace™ Calcein-AM, Vybrant™ DyeCycle™ Green and CellTrace™ CFSE have emission wavelengths comparable to EGFP fluorescence. Figure 2 shows the fluorescent profiles of WT-K562 as a function of the concentration of Calcein-AM (Figure 2a),

DyeCycle Green (*Figure 2b*) and CFSE (*Figure 2c*). Fluorescent intensity values are shown in *Supplementary Table II*. We calculated the optimal concentration of each dye that gave a similar EGFP intensity. The concentrations were as follows: 20nM of Calcein-AM, 170nM of DyeCycle Green, and 10nM of CFSE.

DyeCycle Green is a cell membrane permeant DNA-selective dye that is fluorescent upon binding to double-stranded DNA. Calcein-AM and CFSE are cell-permeant dyes that are colorless and non-fluorescent until acetoxymethyl or acetate groups respectively are cleaved by intracellular esterases in the cytosol. DyeCycle Green was eliminated because it had cytotoxic properties even at lower concentrations. Although CFSE required lower dye concentrations to obtain the same fluorescence intensity levels, Calcein-AM was chosen because it showed less day-to-day fluorescent signal variability. Calcein-AM pre-stained WT-K562 cells provide a cell model for specifically studying the lysing of target cells with 100% positive fluorescent cells, however cells must be prepared prior to use. Optimal dye concentration should be determined empirically and extra-time is needed for cell labeling prior to performing the cytotoxic assay. Generally, loading times of between 15 minutes and 1 hour are sufficient, although periods of about 30 min are generally optimal. Despite these minor disadvantages, EGFP-K562 cells or Calcein-AM pre-stained WT-K562 cells provide safer alternatives to the standard ^{51}Cr release assay.

Cell counting linearity and reproducibility was assessed by performing serial blood dilutions with a fixed number of EGFP-K562 cells. Blood cell counts were also performed in the absence of EGFP-K562 cells. All measurements were done in triplicate immediately after EGFP-K562 spiking. *Figure 3* and *Supplementary Table III* show the lymphocyte count vs. blood dilutions. Values are expressed as Mean $\pm$ SD, and cell counting was performed using acoustic flow cytometry.

Flow cytometry sample acquisition can be time-consuming, especially for highly diluted blood samples. In addition, low sample rates can result in non-homogeneous cell suspensions, giving non-representative counting results. In order to optimize the acquisition time, we evaluated the accuracy of cell counting by using different sample rates. We aimed to set the fastest sample rate while maintaining the resolution and the sensitivity of our measurements. *Figure 4* shows a representative experiment with blood and mixed EGFP-K562 cells. Samples were acquired using the Attune™ Acoustic Focusing Flow Cytometer (Thermo Fisher) and acquisition was stopped when 1000 lymphocytes were counted using a region in the Forward Scatter vs. Side Scatter plot. Immunology protocols for blood immunophenotyping widely accept the use of a region to encircle lymphocytes on a Side Scatter vs. Forward Scatter plot. Cell mixtures were acquired using increasing sample rate (100 $\mu\text{l}/\text{min}$, 200 $\mu\text{l}/\text{min}$, and 500 $\mu\text{l}/\text{min}$). Although mean fluorescence intensity of EGFP-K562 cells was comparable for all acquisition rates, the 500 $\mu\text{l}/\text{min}$ sample rate gave lower lymphocyte scatter resolution. We chose 200 $\mu\text{l}/\text{min}$ as the optimal sample acquisition rate for this protocol (*Figure 4*).

Cytotoxic activity is measured as the ability of cytotoxic cells from unlysed whole blood to effectively lyse K562 cells. EGFP-K562 and Calcein-K562 target cells were co-incubated for 2h with the blood at four different effector-to-target (E/T) cell ratios (1:3, 1:1, 5:3 and 10:3) (*Table 1*). Controls were prepared without target cell co-incubation using the described E/T ratio but with fluorescent target cells being spiked into blood cells following the 2h incubation. EGFP-K562 and Calcein-K562 cells co-incubated (test) and without co-incubation (control) were counted as shown in *Figure 5*. K562 cell-lysing was calculated as a percentage with the following formula: $[(\text{number of control cells} - \text{number of test cells}) / \text{number of control cells}] \times 100$. *Figure 6* shows a representative experiment of the cytotoxic activity at different effector-to-target ratio and *Figure 7* shows a comparison between EGFP-K562 cells and WT-K562 cells stained with Calcein-AM in a cytotoxicity assay (See also *Supplementary Figure II*).

METHODS

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). The labs of J.B. and R.P. provided K562 and EGFP-K562 cells respectively. Cells were maintained in a humidified incubator at 37°C and 5% CO₂ for 35 days, subculturing 1/12 every 2-3 days. Cytotoxic assays were all performed in the absence of antibiotics and serum, with AIM V™ Medium (Thermo Fisher). 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 1h 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) at a concentration of 5µg/ml. EGFP expression was also checked in EGFP-K562 cells before each experiment, to prepare 1ml of cell suspension containing 1,2x10⁵ cells/ml in AIM-V Medium (Thermo Fisher) with 100% EGFP positive K562 cells. We also used wild type K562 cells stained with CellTrace™ Calcein Green AM. 2µl of CellTrace™ Calcein Green AM (Thermo Fisher) were added to 1ml cell suspension with a concentration of 1x10⁶ 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 1,2x10⁵ cells/ml in AIM V Medium. Calcein AM labeling was optimized to obtain 100% of green fluorescent wt-K562 cells before each experiment.

Preparation of effector cells. Lymphocyte counting was immediately performed prior to cytotoxicity experiments. Unlysed whole blood was prepared for absolute counting of lymphocytes using acoustic 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) for 10 minutes at 37°C and light-protected. After incubation, the sample was diluted 1/8 with Focusing Fluid 1x (Life Technologies) and acquired on a flow cytometer to provide absolute lymphocyte counts. Lymphocyte dilutions were prepared with AIM V Medium (Thermo Fisher) as follows: a) 8x10⁵ lymphocytes/ml, b) 4x10⁵ lymphocytes/ml, c) 2.4x10⁵ lymphocytes/ml, and d) 4x10⁴ lymphocytes/ml. Dead cells were excluded with Propidium Iodide (PI) (Life Technologies) at a concentration of 5µg/ml.

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 a four different effector-to-target (E/T) cell ratios (1:3, 1:1, 5:3 and 10:3). Cell were mixed to a final volume of 1ml 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) at a concentration of 10µM, for 5 minutes at 37°C and protected from light. Propidium Iodide (PI) (Thermo Fisher) 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 flow cytometry tube will be needed for each condition tested. Samples were diluted 1/8 in acoustic focusing fluid and filtered using a nylon mesh of 52µm.

Flow cytometry. Data was collected on an Attune™ Acoustic Focusing Cytometer (Thermo Fisher) 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.).

Strategic planning. For the assay of the cytotoxic activity in unlysed whole blood, a minimum of 2h incubation and an additional 2h for the preparation of effector and target cells are required, including flow cytometry measurement of cell-mediated lysing of target cells. Furthermore, heparinised blood samples are processed immediately or within 1h after collection. Although it has been described that heparinised collection tubes can be stored for 24h at room temperature without a significant variation in cytotoxic activity, we strongly recommend storing sample collection tubes no more than 2h. Moreover, this method has not been compared with any of the established methods, given that for the first time, either peripheral blood mononuclear cell density separation and erythrocyte lysing procedures have been completely avoided.

Careful preparation of target and effector cells is crucial to avoid different sources of error. Improperly prepared cells, inaccurate calibration of pipettes, and poor pipetting technique can cause improper dilutions, with consequences in the application of insufficient or excessive amount of cells that may affect target and effector ratios. In addition, cell suspensions should be as homogeneous as possible. K562 are large cells in comparison with normal blood cells, and will sediment comparatively faster than the lighter normal blood cells.

Importantly, 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. A dedicated cell incubator will help to avoid deviations from the expected conditions, as repeated opening and closing of the incubator causes temperature and gas variations.

Almost all commercially available flow cytometers can be used to perform this assay, but many of them cannot directly provide the cell concentration or absolute count of cells in a sample. Absolute cell counts are generally obtained either by combining a separate cell concentration determination from a hematology analyzer with flow cytometric population data (dual-platform testing) or by adding an internal microsphere counting standard to the flow cytometric sample (single-platform testing). The main requirement for the experimental setup is a violet laser, or alternatively ultraviolet (UV) or near-UV lasers. Keep in mind that violet laser powers below 15mW may be inadequate to resolve DCV blue emitting cells in unlysed whole blood, and that each laboratory must establish its own expected ranges based on its instrument. DCV stain is fluorescent upon binding to double-stranded DNA and can also be used with UV excitation, having emission at ~440 nm. This live cell stain can be used for simultaneously staining with antibodies and fluorescence compensation may be required for FITC and PE channels.

Table 1. Layout for the 12-Well Plate for the Cytotoxicity Assay

	1	2	3	4
A	1ml of AIM V medium Effector: 50µl Blood Dilution I (40.000 lymphocytes) Target: 100µl test cells (12.000 cells) E/T = 10:3	1ml of AIM V medium Effector: 50µl Blood Dilution II (20.000 lymphocytes) Target: 100µl test cells (12.000 cells) E/T = 5:3	1ml of AIM V medium Effector: 50µl Blood Dilution III (12.000 lymphocytes) Target: 100µl test cells (12.000 cells) E/T = 1:1	1ml of AIM V medium Effector: 50µl Blood Dilution IV (4.000 lymphocytes) Target: 100µl test cells (12.000 cells) E/T = 1:3
B	1ml of AIM V medium Effector: 50µl Blood Dilution I (40.000 lymphocytes) Target: 100µl control cells (12.000 cells) E/T = 10:3	1ml of AIM V medium Effector: 50µl Blood Dilution II (20.000 lymphocytes) Target: 100µl control cells (12.000 cells) E/T = 5:3	1ml of AIM V medium Effector: 50µl Blood Dilution III (12.000 lymphocytes) Target: 100µl control cells (12.000 cells) E/T = 1:1	1ml of AIM V medium Effector: 50µl Blood Dilution IV (4.000 lymphocytes) Target: 100µl control cells (12.000 cells) E/T = 1:3
C	Blank	Blank	Blank	Blank

Table 1. Layout for the 12-Well Plate Used in Cytotoxicity Assay. Different number of effector cells were added (4×10^4 , 2×10^4 , 1.2×10^4 and 4×10^3) while keeping the volume constant (50 µL) to meet the performance requirements. Absolute lymphocyte counts were obtained by flow cytometry. Blood dilutions were all made with AIM V™ Medium. The first dilution was prepared at 8×10^5 lymphocytes/ml (**Dilution I**). The second dilution was a 1:2 dilution of the first solution. The concentration of lymphocytes in the second dilution was 4×10^5 lymphocytes/ml (**Dilution II**). The third solution was a 5/3 dilution of the first solution to obtain 2.4×10^5 lymphocytes/ml (**Dilution III**). The fourth solution was made by a 1/10 dilution of the first solution to obtain 4×10^4 lymphocytes/ml (**Dilution IV**). Next, 100µl of EGFP-K562 cells or Calcein-AM stained K562 target (T) cells with 1.2×10^5 cells/ml concentration were added to each well. Target cells were added in row **A** at the beginning of incubation (t=0h; *Test cells*) and in row **B** at the end of incubation (t=2h; *Control cells*). Wells in row **C** were left intentionally empty.

Figure 1

Figure 1. Monitoring green fluorescent K562 cells stably transduced with EGFP. 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). (c) Percent of fluorescent cells observed over time after thawing, and characterization of the presence of necrotic cells. The decrease in over the first 10 days cells is due to recovery of the culture from freeze/thaw.

Figure 2

Figure 2 a

Figure 2 b

Figure 2 c

Figure 2. Fluorescent intensity of WT-K562 cells as a function of the concentration of fluorescent dyes. (a) Analysis of the fluorescent intensity at different concentrations of Calcein-AM. (b) Analysis of the fluorescent intensity at different concentrations of DyeCycle Green (DCG). (c) Analysis of the fluorescent intensity at different concentrations of CFSE.

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 (lymphocytes) or blood and a constant volume of EGFP-K562 cells (lymphocytes mixture). All measurements were done in triplicate and values are expressed as Mean $\pm$ SD.

Figure 4. Scatter analysis of EGFP-K562 cells and blood at different sample acquisition rate. Counting by flow cytometry of the number of lymphocytes and EGFP-K562 cells of a sample analyzed at different sample acquisition rate: 100μl/min, 200μl/min and 500μl/min. K562 cells are characterized by greater size (high forward scatter and intermediate side scatter), relative to lymphocytes and granulocytes.

Figure 5. Representative gating strategy to study the numbers of target cells in unlysed whole blood. (a) A Side Scatter versus Vybrant™ DyeCycle™ Violet stain (DCV) contour plot was used to discriminate nucleated cells from erythrocytes and debris. (b) Nucleated cells were further selected in a forward scatter versus side scatter contour plot. (c) Necrotic cells were then excluded using propidium iodide staining. (d) A Calcein-AM vs. DCV contour plot was used to count green fluorescent target cells. (e) Histogram showing green fluorescent staining.

Figure 6. Representative cytotoxicity experiment using unlysed whole blood. Lysis of Calcein-K562 cells at different effector-to-target (E/T) 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.

A)

B)

Figure 7. Comparison between EGFP-K562 cells and WT-K562 cells stained with Calcein-AM in a cytotoxicity assay. WT-K562 cells stained with Calcein-AM (a) and EGFP-K562 cells (b) were discriminated from blood by their green fluorescence. DCV was used to discriminate erythrocytes and debris from nucleated cells. For contour plots displayed in (a) and (b) respectively, the first row represents control cells and the second row represents the cell-mediated lysis analyses.

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Supplementary Table I

Time (days)	% Propidium Iodide (-)	% EGFP (+)
1	78.57	99.95
2	79.50	99.90
3	81.95	99.89
6	91.86	99.92
10	97.97	99.88
35	96.72	99.74

Supplementary Table I. Analysis of the fluorescence decay observed over time after thawing, and characterization of the presence of necrotic cells.

Supplementary Table II

	Concentration (nM)	Viable cells (%)	Green cells (%)	Fluorescent intensity (a.u)
EGFP-K652	0	95	99	144653
Control	0	84	0	543
DMSO	0	83	0	593
Calcein10	10	89	100	78911
Calcein25	25	94	100	228461
Calcein40	40	94	100	365863
Calcein100	100	94	100	706058
EGFP-K562	0	95	100	142898
Control	0	88	0	549
DMSO	0	88	1	607
DyeCycle10	10	88	91	3961
DyeCycle100	100	93	100	97227
DyeCycle1000	1000	96	100	1375340
DyeCycle10000	10000	94	100	7732890
EGFP-K562	0	96	99	143656
Control	0	61	1	643
DMSO	0	64	1	706
CFSE10	10	63	100	70677
CFSE50	50	63	100	392511
CFSE100	100	80	100	1086809
CFSE1000	1000	74	100	7776340

Supplementary Table II. Fluorescence intensity of WT-K562 cells as a function of the concentration of fluorescent dyes. (a) Analysis of the fluorescence intensity at different concentrations of Calcein-AM. **(b)** Analysis of the fluorescent intensity at different concentrations of DyeCycle Green. **(c)** Analysis of the fluorescent intensity at different concentrations of CFSE.

Supplementary Table III

Blood Volume (μl)						
		Replicate	5	15	25	50
Lymphocytes	Blood	1	127	341	445	905
		2	136	262	487	971
		3	131	292	526	864
		Mean	131	298	486	913
		SD	5	40	41	54
	Blood + Cells	1	169	329	514	954
		2	139	327	488	973
		3	133	343	560	910
		Mean	147	333	521	946
		SD	19	9	36	32
K562	Blood + Cells	1	241	403	387	325
		2	306	340	360	314
		3	369	371	355	300
		Mean	305	371	367	313
		SD	64	32	17	13

Supplementary Table III. Number of lymphocytes and K562 in different dilutions of blood.

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

Supplementary Figure I

Supplementary Figure I. Red cell lysing effect on human bone marrow cells. The Attune™ Acoustic Focusing 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.

Supplementary Figure II

Supplementary Figure II. 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.