Development and Application of a Multiplexable Flow Cytometry-Based Assay to Quantify Cell-Mediated Cytolysis

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Abstract

Although target cell cytolysis has been widely employed to describe effector function of cells, cytolysis assays as commonly employed do not generate quantitative data. In this report we describe the development and application of a statistically supported flow cytometry-based assay to quantify cell-mediated cytolysis. The assay depends on the use of the fluorescent dye CFSE to distinguish target from effector cells, the DNA intercalating dye 7AAD to distinguish dead from live cell events, and on the establishment of a cytolysis curve that allows for the derivation of statistically robust data. We demonstrate that the cytolysis curve is well described by a four parameter logistic regression model provided that (i) the range of effector to target (E:T) ratios studied allows for full description of the logistic curve, and (ii) an adequate number of data points are collected to estimate the model parameters. We show that the assay is highly reproducible and accurate, and comparable in sensitivity with the standard 51Cr assay. We report on the potential for this assay to generate quantitative data on the cytolytic activity of both CD8 T and NK cells; describe a relationship between the efficiency of effector cell degranulation and target cell cytolysis throughout a range of E:T ratios, and demonstrate the potential to multiplex with other platforms to obtain broader datasets for the effector phenotype of cells. Appropriate use of this assay will enhance the ability to derive quantitative and integrated correlative datasets from basic, translational, and clinical studies.

Key terms
cytolysis; flow cytometry; logistic model; quantification; multiplexing

A large and diverse number of clinical trials have been conducted to evaluate the potential of novel candidate therapeutic regimens to enhance effector function of immune cells. Target cell lysis is one of the hallmark activities associated with immune effector cells including cytotoxic T lymphocytes, natural killer (NK) cells, and in the context of redirected lysis, monocyctic cells.

The most commonly employed assay to measure target cell lysis is the 51Cr release assay (1). Although the 51Cr release assay has enjoyed wide use and has provided useful data in a large number of clinical and basic research studies, the assay suffers from certain intrinsic limitations. Specifically, (i) the use of a radioactive element (51Cr) with attendant issues of licensing, isotope half-life, storage, disposal, and compliance, (ii) the requirement for metabolic labeling, since metabolically slow cells can label very poorly, and cells at different metabolic states may be differentially resistant to lysis, and (iii) despite the fact that cytolysis is reported in the context of effector:target cell ratios (E:T), the read-out from 51Cr-based assays is not based on a per-cell measurement but rather on bulk release of radioactivity into the culture medium.
Over the past few years a number of assays have been proposed as alternatives to the $^{51}$Cr release assay (2–9). In many cases these assays have involved the use of flow-cytometry-based approaches and fluorescent dyes to distinguish target from effector cells and dead from live target cells. Primarily using NK model systems, such assays have been shown to be specific, reproducible, and to generate datasets that compare favorably with the $^{51}$Cr release assay (3,5,6,8–14). Importantly, in both principle and practice flow-based cytolysis platforms allow for the potential to simultaneously evaluate, on both target and effector cells, other phenotypic and effector parameters such as activation status and cytokine production by effector cells, as well as phenotype and apoptotic status of targets (15,16).

One issue that has not been adequately addressed to-date is the need to establish statistical underpinning for cytolysis assays. Since cytolysis curves are nonlinear in nature and individual cytolysis curves most often have different kinetics, quantitative comparisons of the specific cytolysis values for different E:T combinations within and between experiments are difficult to perform. Datasets across experiments are additionally compromised by the variability inherent in biological assays as well as the lack of standardization for assay set-up. Thus, the absence of statistical underpinnings for cytolysis assays has resulted in datasets that are at best, semi-quantitative. These limitations are particularly problematic for the evaluation of clinical trial-related specimens where quantitative data on modulation of effector function may be critical to be able to guide further product development.

In this report we describe the development of a statistically supported flow-cytometry-based assay to quantify target cell cytolysis. The assay is based on the previously described Guava cytolysis assay (Guava Technologies, Hayward, CA), which we adapted for use on standard flow cytometers. This assay uses the fluorescent dye CFSE (carboxy fluorescein diacetate, succinimidyl ester) to distinguish target cells from effector cells and the DNA binding dye 7AAD (7 amino-actinomycin D) to discriminate live from dead cells. We show that the cytolysis curve is well described by a four parameter logistic regression model which requires a minimum number of data point to generate statistically robust model parameter estimates. Additionally, we show that the assay is reproducible, accurate, comparable to the $^{51}$Cr-based assay with regard to sensitivity, and applicable to a variety of E:T combinations. Importantly, we show that application of the statistical supported cytolysis assay allows for the generation of numerical data that allow for the quantitative assessment of cytotoxicity in biological samples. Finally, we provide examples that highlight an important advantage of this assay by demonstrating the potential to multiplex with other assays and simultaneously obtain information about the phenotype and function of the effector cells.

**MATERIALS AND METHODS**

The Clinical Immunobiology Correlative Studies Laboratory operates under principles of Good Laboratory Practice (GLP). All instrumentation is maintained and operated under established Standard Operating Protocol (SOP) by qualified personnel. All laboratory procedures were performed by trained and qualified personnel under established SOP and using qualified assays.

**Statistical Methods**

The full dose response curve for antigen-specific killing by an effector cell population of antigen-positive and -negative target cells follows an S shape which is well described by a four parameter logistic regression model (17), (Fig. 1). Two elements are critical for appropriate implementation of the four parameter logistic model: (i) the range of E:T ratios used must allow for full description of the logistic curve, and (ii) sufficient data points must be collected across this range to estimate the model parameters.

The parameters of relevance for this model are the upper asymptote of the percent specific lysis which represents the maximum percent specific lysis, the lower asymptote of percent specific lysis which represents the minimum percent specific lysis, the ED50 which represents the E:T ratio at the midpoint or inflection point between the upper and lower asymptotes for percent specific lysis, and the Hill slope, which represents the change in percent specific lysis for a unit change in dilution of effector cells at the inflection point. Although the ED50 value is by definition the point in the curve with lowest variance (i.e., the most sensitive part of the curve), in principle, depending on the specific assay limitations and the availability of appropriate software, lower and higher ED values can be calculated and used to quantify cytolytic activity in cultures. For most experimental situations the four parame-
ter logistic regression model is the preferred choice; however, in experimental situations where the min and max values are known to be fixed, a lower parameter logistic model could be applied if necessary. To allow for assumption of equal variances throughout the curves we first transformed the ratio value for the dilution of effector cells relative to a target cell dilution factor of 1 \( E(x)/T(y) \) to a log scale and then implemented a model which estimates the loge ED50 values to allow for comparisons of this parameter for two samples on the transformed scale.

To estimate the numeric values for the parameters we exploited nonlinear least squares implemented through the software package GraphPad Prism (version 5.0.1 for Windows, GraphPad Software, San Diego, CA, www.graphpad.com). The estimated numeric values for these four parameters serve to describe the specific lytic activity of an effector cell population. Comparison of the lytic activity across cell populations can be made by assessing if the 95% confidence intervals based on a Student’s \( t \) distribution for comparable parameter estimates (i.e., the Hill slope for two cell populations) overlap; non-overlapping confidence intervals indicate a statistically significant difference.

**Cell Lines and Cell Culture**

UPN035 is an oligoclonal population of primary CD3+ CD8+ T cells transfected to express a chimeric ScFv immunoreceptor that directs specific cytolysis of CD19+ target cells. UPN035 is a clinical product and was generated by successive in-vitro expansions using anti-CD3 mAb (OKT3) and rhIL-2, essentially as per (18). UPN035 is \([95\% \text{ CD}3+/\text{CD}8+\text{, essentially 100}\% \text{ of cells express the chimeric immunoreceptor on the surface, and the chimeric immunoreceptor directs specific cytolysis of CD19-positive target. For these studies UPN035 was used under an Institutional Review Board (IRB) approved protocol (COH IRB#08027), was additionally expanded for one cycle as described above with the addition of 10 ng ml\(^{-1}\) rhIL-15, and evaluated either fresh or after freezing and subsequent thaw according to established laboratory SOP.

PBMC from patients and aphaeresis discard samples were obtained according to institutional guidelines and under IRB approved protocols (COH IRB#08152 and COH IRB#05096, respectively). For patient samples, whole blood was collected in lavender top tubes containing potassium EDTA (Becton Dickinson #365974). PBMC samples were processed using Accuspin columns (Sigma), and frozen according to established laboratory SOP.

NS0, a mouse B cell myeloma cell line (ATCC# CRL-2695) and NS0/CD19 (NS0 transfected to stably express a membrane-truncated CD19 gene product in >95\% of cells), were generous gifts from the Jensen laboratory (COH), and were grown using standard laboratory methodologies in high glucose DMEM medium supplemented with 10\% fetal calf serum.

Daudi (ATCC# CCL-213), Jurkat (ATCC# CRL1990), and K562 (ATCC# CCL-243) were obtained from the ATCC and cultured using standard laboratory methodologies in RPMI medium supplemented with 10\% fetal calf serum.

**Antibody Reagents**

PE-conjugated antihuman monoclonal antibody to CD107a was obtained from BD Bioscence (San Diego, CA). PC5-conjugated antihuman CD8\(\beta\) monoclonal antibody was obtained from Beckman Coulter (Fullerton, CA). PC-7-conjugated antihuman CD3 and PC-5-conjugated antihuman CD56 monoclonal antibody were purchased from BD Bioscience (San Diego, CA).

**Cytotoxicity Assays**

**Cell preparation.** Effector cells were used either fresh from culture or thawed from frozen stock and washed; in the case of PBMC, cultures were used immediately after thaw or rested overnight in rhIL-2 as indicated. Target cells were obtained from cultures maintained at a minimum of 85\% viability. Cell counts and viability were assessed using a GuavaPcA96 system (Guava Technologies).

51Cr release assay. Standard 4-h 51Cr release assays were performed and specific cytolysis determined as per (2).

**Flow-based cytolysis assay (FBCA).** A detailed SOP that describes this assay is included in the Supporting Information for this manuscript. Briefly, effector cells were harvested, counted, washed, and resuspended to \(1 \times 10^6\) cells ml\(^{-1}\) in T-cell media. Effectors and CFSE-labeled target cells were mixed at a range of E:T, either in sterile 96-well round bottom plates (Corning, Lowell, MA) plating \(1 \times 10^4\) targets/well and variable numbers of effector cells, with triplicate wells/condition, or in sterile FACS tubes (BD Falcon) which contained \(1.5 \times 10^5\) targets/tube and variable numbers of effector cells. Cultures were incubated for 4 h at 37°C under 5\% CO2. The 7-AAD was then added to samples and 1% Saponin to the max samples, and cultures were incubated for 30 min in the dark, washed, and resuspended in 900 \(\mu\)l FACS buffer (PBS, 0.5\% BSA, 0.006\% Sodium Azide); 100 \(\mu\)l of beads \((1 \times 10^6\) ml\(^{-1}\), (Bangs Laboratories, Fishers, IN) were added to each sample.

**Data acquisition and analysis.** After staining, samples were placed on ice and data collected immediately on an FC500 flow cytometer (Beckman Coulter), with each collection acquiring 20,000 bead events (∼20% of each sample). Data were analyzed using FCS Express V3.0 software. The gating strategy is described in the SOP included in the Supporting Information, and a graphic description of the gating strategies is presented in Supporting Information Figure 1. Because cytolysis can destroy cell architecture cytolysis, % live cells were recorded for each E:T and % cytolysis was calculated as 1 – % live cells. Since detergent lysis destroys cell architecture, the max % cytolysis value was established as the maximum possible cytolysis, determined by: 1 – [(live cell events\(_{max}\)/total number cells\(_{max}\)] × 100.

\% specific lysis (% SL) for each sample was calculated as:

\[
\% SL = \left[ \frac{\% cytolysis_{sample} - \% cytolysis_{min}}{\% cytolysis_{max} - \% cytolysis_{min}} \right] \times 100
\]
CD107 Degranulation Assay

The CD107 degranulation/mobilization assay was performed as previously described using only the CD107a antibody (9), either using aliquots from the cytolytic assays or using frozen PBMC rested overnight. For surface marker staining following degranulation, after the 4-h incubation period for the cytotoxicity assay cells were washed twice with 2 ml staining buffer and stained with mAbs (CD3 and CD8β or CD56) for 30 min at room temperature in the dark, and washed again before flow analysis. Samples were acquired on a FC500 (Beckman Coulter). A total 100,000 cell events were acquired and samples were analyzed using FCS Express v3.0 software. The analysis was performed on gated cells that fell within the lymphocyte population with subsequent gating on the CD3+ or CD8β+ populations.

Luminex Bead Array Cytokine Analysis

Following the 4-h incubation period for cytotoxicity assays, 50 μl supernatant were collected from each condition for Luminex-based array cytokine analysis. A Bio-Plex Luminex 100 XYP instrument was used for data acquisition and Bio-Plex Manager 5.0 software (Bio-Rad Laboratories, Hercules, CA) was used for analysis. Cytokine multiplex analysis was performed using a human cytokine 30-Plex antibody bead kits (Invitrogen, Camarillo, CA) as per the manufacturer’s protocol. A standard curve cut-off value at 70% observed/expected values was employed. With the exception of IL1RA (48 pg ml⁻¹), MCP-1 (52 pg ml⁻¹), and IL-7 (18 pg ml⁻¹), the lower limit of quantification for all cytokines was less than 11 pg ml⁻¹. Each sample was evaluated in duplicate and the average value determined; % CV in all cases was less than 16.7%.

RESULTS

Evaluation of the FBCA Curve

Cytolysis curves have been shown to conform to a four parameter logistic regression model (17). Two elements are critical for appropriate implementation of the four parameter logistic model, (i) the range of E:T ratios used must allow for

Table 1. Relative efficiencies for quantifying model parameters based on the number of data points used to generate the cytolytic curve

| ROW | DATA POINTS (EFFCTOR CELL RATIOS) USED | RELATIVE VARIANCE INFLATION |
|-----|--------------------------------------|-----------------------------|
|     |                                      | N  | LOG_{e} ED50 | HILL SLOPE | LOWER | UPPER |
| 1   | 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10, 0.05 | 10 | 1.0 | 1.0 | 1.0 | 1.0 |
| 2   | 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, **0.10** | 8  | 1.0 | 1.0 | 1.4 | 1.3 |
| 3   | 6.25, 3.13, 1.56, 0.78, 0.39, **0.20** | 6  | 1.0 | 1.2 | 2.1 | 1.8 |
| 4   | 3.13 1.56, 0.78 0.39 | 4  | 1.1 | 1.8 | 4.0 | 2.8 |
| 5   | 25, 12.5, 6.25, **3.13**, 0.39, 0.20, 0.10, 0.05 | 8  | 2.5 | 1.2 | 1.1 | 1.2 |
| 6   | 25, 12.5, **6.25**, 0.20, 0.10, 0.05 | 6  | 10.1 | 2.3 | 1.3 | 1.6 |
| 7   | 25, 12.5, 0.10, 0.05 | 4  | 58.8 | 7.1 | 1.9 | 2.3 |
| 8   | 26.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.05 | 8  | 1.0 | 1.1 | 1.3 | 1.3 |
| 9   | 12.5, 6.25, 3.13, 1.56, 0.78, 0.20, 0.10 | 6  | 1.4 | 1.7 | 1.8 | 1.7 |

Relative efficiency was determined by comparing the Variance Inflation Factor (VIF) described by Gabrielsson and Weiner (19). The formula for relative variance inflation = VIF reduced model/VIF full model where (VIF) = (X’X)⁻¹ and X is the gradient or 1st derivative matrix. For Rows 2–4, 5–6, and 8–9, data points removed from the subsequent row are highlighted in bold.

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tion of Rows 5, 6, and 7, respectively. In each of these cases, the ability to estimate log ED50 values is dramatically impacted, with relative variance inflation values starting at 150%). The ability to estimate Hill slope values is also significantly impacted, particularly when more than two central points are removed.

Rows 8 and 9 show the consequences of removing intermittent points from the curve on the relative variance inflation for the calculated log ED50 and Hill slope values, with 2 and 4 points removed for Rows 8 and 9, respectively. When two intermittent points are removed from this analysis, both loge ED50 and Hill slope values are calculated with efficiencies near 1 (Row 8). When four intermittent points are removed both loge ED50 and Hill slope values are significantly impacted (variance values increasing 40% (1.4 vs. 1) and 70% (1.7 vs. 1), respectively (Row 9). Relative variance inflation values for the lower and upper curve parameters were significantly impacted in most cases when more than two data points were removed (Rows 3, 4, 6, 7, and 9).

Specificity of FBCA
To evaluate the ability to use the FBCA to measure antigen-specific cytotoxicity we performed a series of experiments using UPN035 as effector cells and a series of CD19-expressing and nonexpressing target cells. Figure 2A presents the cytotoxic activity of UPN035 cells against Daudi (CD19-positive) (closed squares) and Jurkat (CD19-negative) (open squares). Cytolysis of CFSE-painted NS0 transfected to express the CD19 gene product: -[●]-- and CFSE-painted NS0 parental --[○]--. Data are representative of greater than 12 independent experiments.

Reproducibility of FBCA
To evaluate the reproducibility of the FBCA we performed a series of experiments using as effectors UPN035 cells expanded by OKT3 stimulation in the presence of IL-2 and IL-15, and frozen 23-days poststimulation. Effector cells were independently tested in three assays over a course of 6 weeks against NS0/CD19 and NS0 targets. Figure 3 presents the average percent specific cytolysis for the three experiments. For cytolysis of the NS0/CD19 targets, the maximal Standard Deviation (SD) was 12.3% (for ratio 0.78:1 E:T), and for most data point was less than 8%. Background cytolysis of NS0 targets was minimal and highly reproducible at all E:T ratios. These results demonstrate that when utilized appropriately this assay shows high reproducibility.

Accuracy of FBCA
To evaluate how accurately the FBCA could quantify antigen-specific cytolytic activity UPN035 effector cells were titrated into allogeneic PBMC at two different ratios and cytolysis of NS0-CD19 and NS0 parental targets was measured. For these experiments, UPN035 effector cells were titrated to comprise either 50 or 10% of the effector cell population. Representative results from these experiments are presented in Figure 4. A difference in CD19-specific cytolysis curves could be observed qualitatively for the 50 and 10% UPN035 curves, with the curve generated using the 50% UPN035 effector cell population showing a higher specific cytolysis essentially throughout the titration range; very low cytolysis could be detected against the NS0 parental targets by the 50% UPN035 effectors (closed triangles) or the 10% UPN035 effectors (not shown). Use of the four parameter logistic regression model allowed for a quantitative measurement of the cytolysis for
both the effector cell populations. Although an additional
dilution point would have been optimal to define the lower
parts of these cytolyis curves, as presented in Table 2 the
obtained data points were adequate to sufficiently estimate the
logED50 and Hill slope values. As is also shown in Table 2,
although the Hill slope values for the two curves (a measure of
effector cell potency) were statistically indistinguishable,
comparison of the log, ED50 values for the two specific cytolyis
curves demonstrated a 4.3-fold higher activity for the 50%
UPN035 effector cell population compared to the 10%
UPN035 effector cell population. This 4.3-fold difference is
within 14% of the actual five-fold difference in the specific
effector cell composition of the two populations and demon-
strate that the data from FBCA analyzed using nonlinear least
squares can be used to accurately quantify specific effector cell
activity in cell populations.

Comparison of FBCA and 51Cr-Based Cytolysis Assays

To directly compare the results obtained using the FBCA
with a standard 51Cr-based assay we used the same popula-
tions of effector cells (UPN035 frozen at Day 23 postexpansion
and freshly thawed), and the same culture of target cells (NS0/
CD19 and NS0 parental) either labeled overnight with 51Cr or
labeled with CFSE. As shown in Figure 5, comparable specific
cytolysis curves were obtained for the two methodologies
using both antigen-positive (CFSE assay closed squares, 51Cr
assay closed triangles) and negative target cells (CFSE assay
open squares, 51Cr assay open triangles). As shown in Table 3,
for all four parameter estimates the 95% confidence intervals
overlap, indicating the models for the two methods are not
statistically different. The standard errors for the parameter
estimates from four parameter logistic regression model were
notably higher for the 51Cr assay, a phenomenon we consis-
tently observed over multiple experiments using different
effector and target combinations. These results demonstrate
that compared to the standard 51Cr assay, the FBCA demon-
strates similar sensitivity and lower variability across the titra-
tion curve.

Quantification of NK Cytolytic Activity Directly
in Patient PBMC

We next sought to evaluate the potential to apply the
FBCA to quantify NK cytolytic activity directly ex vivo in
PBMC samples by measuring cytolyis of the NK—sensitive
target cell line K562. For these analyses we obtained under
IRB approval whole blood samples from a renal cell carcinoma

| PARAMETER     | 10%   | 95% CI  | 50%   | 95% CI  |
|---------------|-------|---------|-------|---------|
| Hill Slope    | 1.31  | 0.090   | 1.06–1.55 | 1.47  | 0.10   | 1.18–1.75 |
| Loge ED50     | 0.99  | 0.051   | 0.85–1.13 | 0.23  | 0.068  | 0.045–0.42 |
| Upper limit   | 84.2  | 1.24    | 80.8–87.6 | 82.0  | 0.63   | 80.2–83.8 |
| Lower limit   | −1.46 | 2.19    | −7.55–4.63 | 9.87  | 2.89   | 1.84–17.89 |

Estimated values, the standard error (S.E.) and the 95% confidence interval (95% C.I.) are based on a t-distribution test for each pa-
parameter and cell population.
were either labeled overnight with 51Cr or cultured overnight and express the CD19 gene product (NSO/CD19) and NS0 parental.  

Multiplexable Flow Cytometry-Based Assay

According the percentage of CD3$^+$ cells in the samples was determined by flow cytometry by recytolysis assay, samples were thawed and the percentage of NK cells was essentially identical. Although limitations in the available volume of clinical samples obtained for this study precluded the ability to generate an upper asymptote for specific cytolytic activity of the two samples across the cytolysis curves, it was necessary for us to perform these analyses in replicate tubes; to this end, a portion of the cytolysis cultures was removed at the start of the cytolysis assay and subjected to an FBCA as a representative example, the lytic activity in the PBMC cultures is a result of specific degranulation of the NK cell population in response to targets NS0/CD19 and NS0. As shown in Figure 7, top panel, cytolytic activity in this assay increased with increasing E:T ratio. Because of detection limitations with the instrumentation used for these analyses, it was necessary for us to perform these analyses in replicate tubes; to this end, a portion of the cytolysis cultures was removed at the start of the cytolysis assay and subjected to a CD107 degranulation assay, followed by surface staining for CD3$^+$ and CD56$^+$ cells. As shown in Figure 6A, higher specific cytolytic activity was observed in both the pre and post-IL-2 treatment samples (Fig. 6). As shown in Figure 6B, when normalizing for the percentage of NK cells in each sample (6.5% pre- and 17% post-IL-2 administration) the cytolytic activity of the two samples across the cytolysis curves was essentially identical. Although limitations in the available volume of clinical samples obtained for this study precluded the ability to generate an upper asymptote for specific cytolytic activity and therefore a full S-shaped cytolysis curve to generate logED50 values in this analysis, the essentially absolute overlap of the two curves throughout the range which would in all probability include the logED50 value demonstrates that the NK-specific cytolytic activity in the pre- and post-IL-2 treatment samples are indistinguishable. Similar results were obtained in vitro using healthy donor PBMC incubated overnight with "low" (10 IU) or "high" (100 IU) rhIL-2 (data not shown).

Tracking CD107 Degranulation and Cytolytic Kinetics

One of the advantages of the FBCA is the potential to multiplex this assay with other platforms and assays, thus providing a more comprehensive clinical correlative dataset. Cytolysis of target cells is associated with the release of cytotoxic granules which contain perforin and granzyme B by effector cells. The release of the cytotoxic granules can be tracked by accumulation of LAMP-1 and -2 proteins (CD107a and b) on the surface of effector cells (9). The flow-cytometry-based nature of this assay allowed us to quantify both target cell cytolysis and the efficiency of the degranulation by effector cells. For these experiments we examined the degranulation properties of effector subsets in PBMC incubated overnight in 100 U ml$^{-1}$ IL-2 and then cocultured with NK-sensitive K562 cells; as shown in Figure 7, top panel, cytolytic activity in this assay increased with increasing E:T ratio. Because of detection limitations with the instrumentation used for these analyses, it was necessary for us to perform these analyses in replicate tubes; to this end, a portion of the cytolysis cultures was removed at the start of the cytolysis assay and subjected to a CD107 degranulation assay, followed by surface staining for CD3$^+$ and CD56$^+$ to identify NK cells (CD3$^-$/CD56$^+$), NK T cells and activated T cells (CD3$^+$/CD56$^+$), and CD3 lymphocytes (CD56$^-$/CD3$^+$), Figure 7, middle panel. As shown in Figure 7, bottom panels (A–C) using the 6.25:1 E:T as a representative example, the lytic activity in the PBMC cultures is a result of specific degranulation of the NK cell population (CD56$^+$/CD3$^-$), while the NK T cell and CD3 lymphocyte subsets do not demonstrate specific degranulation in response to K562 targets.

In the course of evaluating the degranulation properties of effector subsets, we observed that the percentage of degranulating effector cells decreased as the E:T ratios of cultures increased. To examine this issue in more detail we tracked the degranulation kinetics of effector cells in response to targets over a broad range of E:T ratios using as effector cells UPN035 and as targets NS0/CD19 and NS0 cells. As can be seen in fig-

**Table 3. Statistical comparison of the FBCA and 51Cr assays**

| PARAMETER | FBCA | 51Cr |
|-----------|------|------|
|           | VALUE | S.E.  | 95% CI    | VALUE | S.E.  | 95% CI |
| Hill Slope | 1.45 | 0.194 | 0.97−1.92 | 1.56 | 0.91 | −0.66−3.77 |
| Log ED50   | −0.25 | 0.097 | −0.49−0.017 | −0.33 | 0.40 | −1.31−0.65 |
| Upper limit | 61.6 | 1.51 | 57.9−65.3 | 74.4 | 6.71 | 57.9−90.8 |
| Lower limit | 5.7 | 1.99 | 0.77−10.6 | 13.6 | 9.24 | −9.0−36.2 |

Estimated values, the standard error (S.E.), and the 95% confidence interval (95% C.I.) are based on a t-distribution test for each parameter and cell population.
Figure 6. Quantification of NK cytolysis from patient derived PBMC using FBCA. (A). PBMC collected from a patient undergoing high dose IL-2 therapy were collected pre- and post- high dose IL-2 therapy and frozen. PBMC samples were thawed, counted, and incubated with CFSE-painted K562 target cells at the indicated E:T for 4 h at 37°C. ■—post- IL2 therapy, — ——: pre- IL2 therapy (B). Cytolysis data normalized for % NK cells in each PBMC culture. ——: post- IL2 therapy, — ——: pre- IL2 therapy. The 125:1 data point for the pre-IL-2 therapy was excluded as an outlier based on subjective visual assessment (> 3 fold higher than flanking data points).

Multiplatforms for Cytotoxic Activity Assessment

To evaluate the ability to multiplex the FBCA with other platforms we performed the potential to detect and quantify cytokines specifically secreted during the time course of the assay. For this analysis we utilized the Luminex bead array platform. Fifty-microliter aliquots of cytolytic assay supernatants were collected just prior to harvesting the cytotoxicity cultures and evaluated for the presence of the following 30 pro- and anti-inflammatory cytokines and other factors: IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ, GM-CSF, MIP-1α, MIP-1β, IP-10, MIG, Eotaxin, RANTES, MCP-1, VEGF, G-CSF, EGF, FGF-basic, and HGF.

We performed this analysis in two assay systems. First we collected and evaluated supernatants from the CD8 T cell cytolytic assay presented in Figure 5 (UPN035 vs. NS0/CD19 and NS0 parental targets). As summarized in Table 4, IL-2p40/p70, TNF-α, MIP-1α, and MIP-1β were specifically detected upon coculture of UPN015 with NS0/CD19 but not NS0 parental targets. Additionally, high levels of RANTES (112–125 pg ml⁻¹) were detected in both cultures. None of the remainder of factors (IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-α, IFN-γ, GM-CSF, IP-10, MIG, Eotaxin, MCP-1, VEGF, G-CSF, EGF, FGF-basic, and HGF) were detected under the conditions of this assay. Because NS0 cells are murine in origin and the antibodies specific for IL-12 p40/p70 and TNF-α do not recognize the murine homologues (Invitrogen 30-plex package insert) it is clear that these factors were produced by the effector and not the target cells; since it is not known whether the MIP1α and MIP1β antibody reagents distinguish between the human and murine species, it is theoretically possible that the detected factors are produced by target cells. Second, we collected and evaluated supernatants from an NK cytolytic assay with healthy PBMC cultured overnight with 100 IU human IL-2, and K562 targets. As can be seen in Table 5, IL-1b, IL-12, IL-10, and HGF could be specifically detected in the coculture conditions but not in cultures comprised of PBMC or K562 alone. Additionally, levels of RANTES (42–84 pg ml⁻¹), MIP1b (11–18 pg ml⁻¹), IL-8 (36–170 pg ml⁻¹), IL1Rz (42–150 pg ml⁻¹), and IL-2 (15–22 pg ml⁻¹) were detected in the effector + target or effector alone cultures but not in the target alone cultures; the remainder of cytokines were not detected under the conditions of this assay. Qualitatively similar results were obtained using PBMC cultured overnight in lower concentrations of IL-2. Since both effector and target cells are human in origin in this instance, it is theoretically possible that the measured cytokines are produced by the target and not the effector cells; intracellular cytokine staining in conjunction with surface staining for effector and target-specific markers would allow for this discrimination to be made conclusively. It should be emphasized that this dataset is presented as illustrative for the potential to multiplex the flow-based cytolytic assay with cytokine detection; clearly, kinetics

Figure 8A. The efficiency of effector cell degranulation is strongly dependent on the E:T ratio. Maximum efficiency of degranulation was observed at E:T ratios below 0.16:1. The efficiency of degranulation was very sensitive to E:T ratios from 0.3:1 up through 2.5:1 (reflecting the linear nature of the cytolytic curve at those ratios), and at higher E:T the efficiency of degranulation was substantially reduced. A subset of the degranulation of effector cells is additionally presented in the form of flow cytometry dot plot data in Figure 8B. These results suggest that optimal effector function is likely to occur under conditions where target cells are in significant excess; this observation, not possible using ⁵¹Cr assays, should be taken into account when using cytolytic values from high E:T to make quantitative assessments of the specific cytotoxic activity of effector cell populations. Supporting Information Figure 2 further supports this point by presenting an overlay of cytolytic and degranulation curves from cytolytic and degranulation assays performed in parallel using UPN035 effector and NS0/CD19 target cells.
Figure 7. Assessment of effector cell degranulation in FBCA cultures. Aliquots from PBMC:K562 FBCA assay cultures were incubated for 4 h at 37°C in the presence of monensin, anti-CD28, -CD49a, and -CD107 antibodies as described in Materials and Methods. In following, cultures were washed, stained with antibodies specific for CD56 and CD3 and analyzed by flow cytometry. CFSE-positive target cells were excluded from the analysis following compensation. Data presented are from the 6.25:1 E:T condition and are representative of what was observed at all E:T. Top panel: Cytolysis curve for assay. Middle panel: Gating strategy to identify lymphocyte subsets. A: FSC/SSC gate to identify lymphocytes; B: CFSE- gate to eliminate residual target cells; C: CD56-PECy5 vs. CD3-PECy7 Bottom panels: Degranulation phenotype of individual lymphocyte subsets. D: NK cells (CD56+/CD3-), Subset a in panel C; E: NK T cells and activated T cells (CD56+/CD3+), Subset b in panel C; F: CD3 lymphocytes (CD56-/CD3+), Subset c in panel C. E only: PBMC alone; E:T 6.25:1: PBMC + K562 at 6.25:1 E:T. Values inside quadrant indicate the percentage of de-granulating cells as a percentage of each subset. At least 100,000 total events were collected for each analysis. These data are representative of three independent experiments.
of cytokine production and secretion will not in most cases optimally conform with the time-course of a 4-h cytolysis assay. Furthermore, these types of data have the potential to be complemented by ICS analyses of effector and target cell populations over a range of E:T ratios to provide insights into the numbers and absolute amounts of cytokines produced by individual cell subsets in the cytolysis assay.

DISCUSSION

In this report we describe the development, evaluation, and application of a statistically supported, flow-based assay to quantify antigen-specific cytolysis. The methodology as described is shown to be comparable to the $^{51}$Cr release assay in terms of sensitivity, and to generate reproducible and accurate data that can be used to quantify the cytolytic activity of both CD8 T and NK effector cells. We have demonstrated the utility to use this methodology to evaluate and quantify cell-mediated cytolysis of suspension cells; in principle the same methodology can be applied to evaluate cytolysis of adherent cell types, providing cell integrity is maintained upon detachment. Importantly, use of this assay has the potential to significantly enhance the quality of data obtained from analysis of clinical samples, by allowing for the generation of quantitative data.

Table 4. Cytokines detected in 4 h CD8 T cell cytolysis assay

| E:T COMBINATION          | IL-12 | TNF-α | MIP1α | MIP1β |
|--------------------------|-------|-------|-------|-------|
| UPN035 + NS0/CD19        | 27.4  | 14.9  | 33.1  | 160.3 |
| UPN035 + NS0             | ND    | ND    | ND    | 15.1  |

Values are from the average of duplicate measurements and are expressed as picograms detected/ml serum. %CV in all cases was less than 16.7%. ND = below the limit of detection. Lower limits of detection were: IL-12: 4.7 pg ml$^{-1}$; TNF-α: 2.5 pg ml$^{-1}$; MIP1α: 3.1 pg ml$^{-1}$; MIP1β: 5.4 pg ml$^{-1}$. Data is shown from the 6.25:1 E:T condition.

Table 5. Cytokines detected in 4 h NK cytolysis assay

| E:T COMBINATION          | IL-1B | IL-12 | IP10 | HGF  |
|--------------------------|-------|-------|------|------|
| PBMC + K562, 100:1 E:T   | 37.76 | 7.52  | 13.87| 32.95|
| PBMC + K562, 25:1 E:T    | 10.15 | ND    | ND   | ND   |
| PBMC only                | ND    | ND    | ND   | ND   |
| K562 only                | ND    | ND    | ND   | ND   |

Values are from the average of duplicate measurements and are expressed as picograms detected/ml serum. %CV in all cases was less than 12.4%. ND = below the limit of detection. Lower limits of detection were: IL-1B: 6.6 pg ml$^{-1}$; IL-12: 4.7 pg ml$^{-1}$; IP10: 2.8 pg ml$^{-1}$; HGF: 8.2 pg ml$^{-1}$.
values for cytolytic activity and simultaneously the evaluation of other functional and effector functions in samples. The present work expands on previously published flow-cytometry-based cytotoxicity assays (3,5,6,8–14) in two significant ways by demonstrating the methodological approach and potential to quantify cytolytic activity and the significant potential and advantage for multiplexing the FBCA assay with other platforms to obtain more comprehensive and integrated datasets.

The integration of statistical underpinnings and the FBCA results in the critical advantage of generating quantifiable data for each of the parameters (log, ED50, Hill slope, maximum and minimum cytolysis values) that can be measured in a cytolysis assay. However this integration also has a number of important consequences with regard to assay setup. Specifically, (i) assays need to be set-up so that a minimum of eight data points are obtained per E:T combination, and (ii) data need to be generated so that they span the entire lysis curve. By selectively eliminating data points from a 10-point full cytolysis curve, we demonstrate that the ability to accurately calculate estimates for log, ED50 and Hill slope values is significantly impacted by removing as few as two internal data points, while the removal of external data points has similar significant impact on estimating maximum and minimum cytolysis values.

We demonstrate that the flow-based platform can be used to quantify antigen-specific cytosis mediated by CD8+ T cells as well specific cytosis mediated by NK cells in multiple settings. By quantifying NK cytolytic activity in patient samples we showed that the higher NK activity observed in PBMC samples obtained from a patient post high dose IL-2 therapy could be attributed to a higher percentage of NK cells in the sample post IL-2 treatment, not to increased cytolytic activity by NK cells as previously reported (22–24). These results are at least in part discordant with the proposed function of IL-2 to enhance NK cell functionality in vivo, and highlight the important advantage of being able to quantify NK cytolytic activity on a per cell basis in cytolysis assays.

An important issue related to the use of dyes such as CFSE to label and distinguish target from effector cell populations is the potential for the dyes to be released by labeled target cells either as a consequence of cytolysis or passively and subsequent uptake by effector cells. To address this issue, we evaluated the uptake of CFSE by effector cells at the conclusion of an FBCA, taking advantage of the CD8b surface marker on UPN035 cells. As presented in Supporting Information Figure 3 and summarized in Supporting Information Table 1, cells that stain positively for CD8b can in fact be detected in the CFSE+ quadrant at the conclusion of the cytolysis assay for both target cells. Although the absolute number of CFSE+/CD8+ events does not increase dramatically across the evaluated E:T ratios, the percentage of CFSE+/CD8β cell events increases significantly with increasing E:T for both the NSO–CD19 (2.9% at E:T of 0.2:1 to 21% at E:T of 12:5:1) and NS0 (0.6% at E:T of 0.2:1 to 9.4% at E:T of 12:5:1) targets. These data also indicate that robust target cell lysis is not required for the generation of increased numbers of CD8+/CFSE+ cells.

As presented in Panel B of Supporting Information Figure 2, back-gating of the CFSE+/CD8+ cell populations and evaluation of the forward and side scatter properties reveals that these cells to have size properties distinct from the effector cells and very similar to that of the target cell population. These results clearly demonstrate that the use of dyes such as CFSE to stain target cells has the potential to introduce a degree of error, most apparent at high E:T ratios, and supports the use, if available, of alternative cell identification methodologies such as surface markers to distinguish target from effector cells in FBCA. Notably, although the contamination of the CFSE+ gate by small numbers of effector cells could be responsible for the observed difference in % cytolysis at high E:T ratios in the FBCA vs. 51Cr assays observed in Figure 5, as shown in Table 3 the two assays generated statistically indistinguishable log ED50 and Hill slope values, supporting the validity of the applying the FBCA assay as an alternative to 51Cr release assays to quantify target cell cytolysis. These results highlight the need to optimize CFSE or other dye staining of targets, carefully establish gates that maximally separate stained unstained cells, and maximize viability of both target and effector cells that go into the assay.

One of the emerging themes in correlative science is the increasing appreciation for the need to evaluate biological samples in terms of multiple functionalities, as demonstrated by the example of polyfunctional T cells are associated with positive outcomes (16,25–27). A clear advantage for the flow-based cytolysis assay is the ability to multiplex this assay with other assays and platforms to provide a more comprehensive data set to evaluate effector and other cell functionality. We provide two examples of this multiplexing ability. First we demonstrate the ability to multiplex the FBCA with a flow-based CD107 degranulation assay and thus obtain information on the surface phenotype and effector functionality of effector cells in the cytolysis cultures. An important observation from these analyses is that at E:T ratios that are typically used to evaluate and describe cytolytic activity in T cell cultures (E:T > 5:1), the degranulation of effector cells is substantially less efficient than at lower E:T values, suggesting that at the higher E:T cytolytic activity may be underestimated. This has implications both for the ability to accurately quantify cytolytic activity, but also should be considered when comparing two cultures with significantly different numbers of specific effector cells. Although not illustrated in this report, the combination of CD107 degranulation assay and the cytolysis assay could be further used to analyze the cytotoxic potential of subpopulations of effector cells (for example, central vs. effector memory vs. naïve CD8+ T cells) and to correlate the phenotype of degranulating cells with specific cytolytic activity. Analyses that seek to correlate the phenotype of cells with specific cytolytic activity need to keep in mind and consider the possibility that surface markers may be modulated as a result of target cell recognition and cytolysis.

Finally we demonstrate the ability to multiplex the flow-based cytolysis assay with the Luminex bead array platform to
quantify secreted factors in the cytokine culture. We demonstrate that in a 4-h cytosis assay, both CD8 T cells and NK cells produce low levels of IL-12 in response to specific targets, and beyond that a mostly divergent set of immune factors, with the CD8 T cells studies producing most notably TNF-a, MIP1a and MIP1b, and NK cells IL-1b and IP-10. As discussed above, at least for the case of CD8+ T cells, the production of IL-12 cannot be attributed to the target cells which are murine in origin. The biological significance of this observation is unclear at this point but may be relevant in the post target cell engagement activation, proliferation, and differentiation process for T and NK cells. Although this demonstration is illustrative in nature, we believe it highlights the significant advantage of this assay to allow for the generation of additional data sets to more comprehensively obtain insights into the biology of systems under evaluation.

ACKNOWLEDGMENTS

The authors thank Cherilyn Bautista and Christine Wright in the Jensen laboratory for excellent technical support performing the 51 Cr release assays, and Jeff Longmate for critical reading of the manuscript.

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