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A Novel Approach to Measuring Cell-Mediated Lympholysis Using Quantitative Flow and Imaging Cytometry

G.M. La Muraglia II^a, M.J. O'Neil^a, M.L. Madariaga^a, S.G. Michel^a, K.S. Mordecai^b, J.S. Allan^{a,c}, J.C. Madsen^{a,d}, I.M. Hanekamp^{a,#,*}, and F.I. Preffer^{b,#,*}

^aCenter for Transplantation Sciences, Department of Surgery, Massachusetts General Hospital, Boston, MA, USA

^bDepartment of Pathology, Massachusetts General Hospital, Boston, MA, USA

^cDivision of Thoracic Surgery, Department of Surgery, Massachusetts General Hospital, Boston, MA, USA

^dDivision of Cardiac Surgery, Department of Surgery, Massachusetts General Hospital, Boston, MA, USA

1. Introduction

The chief function of CTLs resides in their ability to carry out directed cell-mediated cytotoxicity on specific target cells presenting a unique extracellular antigen. The recognition of the antigen is mediated by the T cell receptor (TCR) and MHC class I antigen interactions, followed by the induction of target cell lysis by means of granzymes, perforin, serglycin, TNF- α or FAS-L pathways (Shresta et al., 1998). The target specificity of these cytolytic mechanisms, along with their regulation, is commonly measured by functional *in vitro* cytotoxicity assays. Using these methods, it is possible to detect the presence of CTLs *in vitro* and thereby assess potential *in vivo* immune function.

CML has traditionally been enumerated by loading target cell populations with the radioactive isotope ⁵¹Cr that diffuses into live cells and is released upon the induction of apoptosis and subsequent lysis as mediated by CTL effector cells (Brunner et al., 1968). Specific lysis is then quantified by the amount of ⁵¹Cr that is released into the supernatant following the reaction. The requirement of radioisotope use for this assay limits its suitability to those labs able to work with radioactive materials since these are inherently dangerous and waste disposal is expensive. Furthermore, although the ⁵¹Cr release assay is widely accepted as the 'gold standard', cytotoxic activity against the target cells cannot be quantified at the single cell level and the assay does not allow for the evaluation of lysis within distinct immunophenotypically defined cellular subsets of either the target or effector

#Corresponding author: PREFFER@HELIX.MGH.HARVARD.EDU.

*Co-senior author

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cell populations. In order to derive more information on the specificity of cell-mediated lympholysis, we developed an improved flow-based assay capable of better elucidating these effector-target cell interactions.

The goal of this study was to develop an isotope-free approach to the quantification of CML in the context of transplantation immune monitoring, by moving from ^{51}Cr release as the metric of target cell lysis to determining the total loss of healthy target cells as measured by flow cytometry. While the most current isotope-free methods for the detection of CML have been established using immortalized cell lines (Zaritskaya et al., 2010), we needed a reliable assay that would work with MHC defined primary peripheral blood mononuclear cells (PBMC) (Gianello and Sachs, 1996). In addition, due to high levels of spontaneous effector lysis in the CML, the ability to specifically detect target cell death was of paramount importance. Previous studies in our lab have utilized the release of intracellular dyes or enzymes (LDH, Calcein AM, proteases) to track lysis of PBMC in a CML (I. Hanekamp, unpublished data). However, while many of these methods worked well with tumor cell lines, these assays lacked sensitivity when PBMC served as the targets, possibly due the smaller cell volume of the primary cells. To be more clinically applicable and to evaluate donor-specific MHC alloimmunity as defined by in vivo transplantation tolerance, primary lymphocytes were preferable as targets. Although the assay described in this study has been tailored to transplantation and immune monitoring, it is applicable to any model involving CTL responses to specific target cells.

In order to adapt the traditional ^{51}Cr release assay with a flow cytometry platform, target labeling was performed with eFluor670 membrane dye. eFluor670 is a fluorescent dye that binds to any intra-cellular protein containing primary amines, thereby providing a method for cellular tracking of the target cell population (Johnson et al., 2013). An additional benefit of eFluor670 is that it allows the use of a wider range of primary cells as targets without the increased spontaneous leakage of the dye (Quah and Parish, 2012) that can occur with longer incubation periods using ^{51}Cr (Zaritskaya et al., 2010) thus facilitating the possibility of future studies investigating slower apoptotic mechanisms (Jedema et al., 2004), such as Fas-Ligand and TNF- α (Waring and Mullbacher, 1999).

As the extent of effector and target cell death varies between samples, flow cytometric collection based upon cell number does not provide an accurate assessment of cell viability. In order to accurately quantitate the number of viable target cells remaining, and thereby the extent of target cell death, fluorescent counting beads were added to each tube and a standard number of beads was collected. In this way, the same reaction volume was collected and analyzed for each sample (Jedema et al., 2004). Our new method establishes a functional replacement for and improvement over the conventional ^{51}Cr CML assay by providing greater insight into specific cellular interactions. In addition to eliminating the need for radioactive labelling of the target cells, it is also possible to obtain phenotypic information regarding both the effector and target cell populations in this assay system. However, traditional flow cytometry cannot definitively identify the cells responsible for causing lysis, or evaluate the extent of accessory cell involvement.

Recent advancements in flow cytometry have incorporated the analytical properties of cellular imaging derived from the ImageStream^X technology, which captures multiple images in different emission spectra as each event passes through the flow cell core (Basiji et al., 2007). This technology enabled us to further characterize cellular interactions at the single cell level, specifically confirming that CD8⁺ T-cell effector lymphocytes interacted with eFluor670⁺ target cells at different stages of lysis. In these studies generalized nuclear morphology, or nucleus presence *versus* loss, was used as the metric for cell lysis, serving as a “snapshot” rather than a cumulative measurement. Detailed changes in nuclear morphology such as fragmentation and blebbing can potentially be quantitated statistically, but require extensive sample-dependent refinement of gating strategies within the specific context of primary cells used in the assay. Measures of changes in nuclear morphology are therefore less generalizable across different laboratory conditions and are more robust in cultured cells and relatively large tumor cells due to the size of their nucleus. (Helguera et al., 2011)

Imaging flow cytometry (Amnis ImageStream^X MkII, EMD-Millipore) is an established platform for the accurate quantification of single cells, doublets, aggregates, particles and other cellular debris in suspended cell preparations. The technology couples high acquisition rates of hydrodynamically focused cells in suspension and accurate object segmentation with bright field and fluorescence microscopy. The emitted and transmitted photons are spatially registered on two 6-channel 12-bit cameras. The capacity to acquire large numbers of events permits statistical discrimination of objects based on the combination of differences in appearance and spatial distribution of signal. This unique form of statistical microscopy was instrumental during the assay development phase as it enabled the greater understanding of cellular interactions in specific CD8⁺ T-cell effector lymphocyte populations with eFluor670⁺ target cells at different stages of lysis. Interestingly, some of these observations included prolonged association of antigenic target cell fragments with effector cells, suggesting a sustained increase in CTL stimulation and activation (Wiedemann et al., 2006).

In this report, we have developed an isotope-free assay system utilizing primary PBMCs as targets. The use of primary cells rather than immortalized cells or cell lines (Jedema et al., 2004) is of particular importance for the experimental transplant studies ongoing in our laboratory. While similar experimental protocols to detect early apoptosis by assessing markers of membrane integrity in immortalized cells (Lecoeur et al., 2001) or caspase expression in activated PBMC (Liu et al., 2004) have been reported, the use of naive primary lymphocytes is an absolute requirement for our studies. Although these methods can identify cells earlier in the process of apoptosis, this level of sensitivity is not necessary for routine immune monitoring in our experimental transplantation models.

Currently, within the field of clinical transplantation, the use of *in vitro* assays to assess the presence of anti-donor CTL activity is of great importance. Previous work in our lab (Leight et al., 1977) has determined that detection of anti-donor CTLp *in vitro* is predictive of immune-mediated graft rejection. These assays are currently being utilized for both living donor transplant pairs (Rakha et al., 2014) and cadaver donors in which PBMC are frozen at the time of organ harvest (Kraus et al., 2003). While these methods relied upon ⁵¹Cr labeled target cells, recently the ⁵¹Cr technique has been successfully applied to studies with

naive human PBMC in our laboratory (G. La Muraglia II, manuscript in preparation). Through the introduction of single cell analysis in clinical immune monitoring, we hope to provide a platform able to facilitate the development of tailored cellular therapies based on the presence of anti-donor CTL within certain recipient PBMC cell subsets.

As an extension to these studies, we also describe an imaging flow cytometric assay capable of characterizing MHC defined effector- target cell interactions at a level that has not been previously explored. The combined use of imaging flow cytometry and standard analytical flow cytometry enabled us to evaluate and verify key steps during development, then transfer the assay to a standard flow cytometry platform for more wide-spread access and simplicity of result readout. This technology will enable us to identify which cells are responsible for lysis, as well as any other cells that are acting as accessories, through the technology's unique ability to analyze multi-cellular aggregates and thereby the intra-cellular interactions occurring during CML. Thus in the course of these studies, we have developed a flow cytometric assay that will enable us to evaluate the differential susceptibilities of subsets of target cells to cell-mediated lysis and to characterize the cells which are directly and indirectly responsible for lysis.

2. Materials and Methods

2.1 Animal Model

Responder and stimulator cells were isolated from the blood of our inbred MGH miniature swine. The immunogenetic characteristics of this herd have been described previously (Sachs et al., 1976). Third party stimulation controls were isolated from outbred Yorkshire swine (MHC^{York}). Transplant donors and recipients were selected from our herd of partially inbred miniature swine (age, 3–6 months; weight, 15–30 kg). MHC^{dd} (Class I^d/II^d) donor organs were transplanted into MHC^{cc} (Class I^c/II^c) recipients to achieve a 2-haplotype full MHC Class I and Class II mismatch. All recipients demonstrated significant *in vitro* anti-donor cytotoxic activity (>20% specific lysis) before organ transplantation. Institutional review board approval was obtained for this study. The surgical procedure (Madsen et al., 1998), immunosuppressive protocol and clinical outcomes of these transplanted is summarized in our publication in the American Journal of Transplantation (Madariaga et al., 2013). All animal care and procedures were in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 2011.

2.2 Preparation of Porcine PBMC

Freshly heparinized whole porcine blood was diluted 1:2 with HBSS (Invitrogen), and the mononuclear cells were obtained by means of gradient centrifugation with Histopaque 1077 (Sigma Aldrich). The mononuclear cells were washed once with HBSS to remove any platelets and the remaining contaminating red cells were lysed with ammonium chloride potassium lysing buffer (ACK lysis buffer, Bio Whittaker). Cells were then washed with HBSS and re-suspended in tissue culture medium.

2.3 Effector Cell Cultures

The same bulk effector cell cultures were used for both the ^{51}Cr CML and for the f_c CML. Responder lymphocyte cultures containing 1ml of responder and 1ml of irradiated stimulator PBMC, both at 4×10^6 cells/ml, were set up in 6 well plates; three wells provided sufficient effectors for most assays. The cultures were incubated for 6 days at 5% CO_2 , 37°C in CML medium (RPMI-1640 (Invitrogen), 6% fetal bovine serum (FBS; Atlanta Biologicals), 100 U/mL penicillin, 135 mg/mL streptomycin (Invitrogen), 50 mg/mL gentamicin (Invitrogen), 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; Fisher Scientific), 2 mM L-glutamine (Invitrogen), 1mM sodium pyruvate (Bio Whittaker), nonessential amino acids (Bio Whittaker), and $5 \times 10^{-5}\text{M}$ 2-mercaptoethanol (Sigma Aldrich). Bulk effector cell cultures were harvested, counted and resuspended in Basal Medium Eagles (BME; Invitrogen) supplemented with 6% bovine growth serum (BGS; Hyclone), just prior to addition to the CML cultures.

2.4 ^{51}Cr Labeling of Targets

PBMC were isolated from whole blood as previously described and stimulated with phytohemagglutinin (M-form; Invitrogen) for 18–24 hours. After washing, 3×10^7 cells were labeled with 0.3mCi of ^{51}Cr (Perkin Elmer) for 2 hours. The ^{51}Cr labeled target cells were washed, counted and resuspended in BME + BGS just prior to addition to the CML cultures.

2.5 eFluor Labeling of Targets

As previously described, PBMC were isolated from fresh whole blood. Thirty million PBMC were then incubated in HBSS containing 6 μM eFluor 670 (eBioscience) for 10 minutes at 37°C . The target cells were then quenched with 1 volume of FBS and 3 volumes of media containing serum, washed with HBSS twice and re-suspended in 10 mL of CML media. Targets were stored at 4°C until use in the CML (12–18 hours). Cells were washed, counted and resuspended in BME + BGS just prior to addition to the CML cultures.

2.6 ^{51}Cr CML Cultures

Chromium based CML assays with porcine cells have been described previously (Leight et al., 1977). Effector cells were incubated for 5 hours with ^{51}Cr labeled target cells in triplicate at effector/target ratios of 100:1, 50:1, 25:1, and 12.5:1 in 96-well round-bottom plates (Corning). For the 100:1 ratio, 5×10^5 effectors and 5×10^3 targets were added to each reaction well; the same number of targets were used in each well for the other ratios, while the effector cells were proportionally decreased. Supernatants were harvested by using the Skatron collection system (Skatron, Sterling, VA), and ^{51}Cr release was determined on a gamma counter (Micromedics, Huntsville, AL). The results were expressed as a percentage of specific lysis and calculated as follows:

$$\text{Percentage of specific lysis} = \frac{(\text{Experimental}[\text{cpm}] - \text{Spontaneous}[\text{cpm}])}{(\text{Maximum}[\text{cpm}] - \text{Spontaneous}[\text{cpm}])} \times 100$$

2.7 Flow Cytometric Assay for Cell-mediated Lymphocytotoxicity (fcCML)

Effector and eFluor 670 labeled target cells were co-cultured at 5% CO₂, 37°C for 5 hours with target cells in triplicate at ratios of 100:1, 50:1, 25:1, and 12.5:1 in 96-well round-bottom tissue culture plates (Corning). Following incubation, cell suspensions were transferred to 5 mL polystyrene round bottom tubes (Corning). The cells were washed once in HBSS containing 0.1% Bovine Serum Albumin (BSA Fraction V, Fisher Scientific). Following the wash, cells were re-suspended in 300uL of HBSS containing 0.1% BSA and 0.05% NaN₃. Prior to acquisition of the samples, 25ul of CountBright absolute counting beads (25,000 beads; Invitrogen) were added to each tube. Tubes were mixed well just prior to collection on a Becton Dickinson FACSCalibur™ running CellQuest™ software Version 3.3 (San Jose, CA); all events within 3200 beads were collected per tube to ensure that the same volume was being analyzed in each sample. Data analysis was performed using FlowJo V_10 (Treestar) software. The results were expressed as a percentage of overall cytotoxic lysis and calculated as follows:

$$\% \text{Targets Lysed} = 100 - [(\# \text{experimental targets} / \# \text{spontaneous ctrl targets}) \times 100]$$

2.8 Imaging Flow Cytometry

Samples were prepared in parallel for standard flow and imaging flow cytometry. In addition, cells were stained with αCD8-PE (BD Biosciences) for 30 minutes at 4°C in the dark in order to observe CD8+ T-cell effector cell interactions. Cells were then washed once with HBSS containing 0.1% BSA and 0.05% NaN₃ and re-suspended in fixation buffer (BioLegend) containing 4% paraformaldehyde for 20 minutes at room temperature. Following fixation, the cells were washed twice and resuspended in 50 ul of HBSS containing 0.1% BSA, 0.05% NaN₃ and 3 μM 4'6'diamino-2-phenylindole2HCl (DAPI; Invitrogen). For each experimental sample, acquisition of 100,000 objects >3 microns in bright-field (BF) area was performed on the AMNIS ImageStream^X MK-II running INSPIRE v200.755.0 acquisition software. 2000 event single color controls were collected for the spectral overlap. Post-acquisition compensation and data analysis was performed using the IDEAS software (Version 6.0; AMNIS Millipore).

Gating-Strategy-and-Rationale—Compensated image files were first gated on DAPI positive events obtained from a single color histogram (Figure 3a, left column). Next, 'Intensity' vs. 'Raw Max Pixel' bivariate plots were generated for eFluor 670 (R2) and CD8 PE (R3). From eFluor 670+ and CD8+ events, a new population was created using the Boolean operation R2 OR R3. Using the R2 OR R3 population and using the DAPI+ events (R1), we plotted DAPI Area vs. DAPI Aspect Ratio (intensity weighted). 'Raw Max Pixel' is the single brightest pixel within a masked region of an image and when plotted against total intensity for the same fluorochrome can be used to indicate punctate or particulate staining patterns within positive events. DAPI Area vs. DAPI Aspect Ratio was used to assess morphological features as an indication of size and roundness. These values were used to display and differentiate singlets, doublets, aggregates, and debris (3a bottom). By back-gating populations derived from fluorescence intensity plots onto morphology plots [*e.g.*, DAPI area vs. DAPI aspect ratio], direct observation of the contribution of each

fluorochrome used in each morphologically derived population can be determined with a high degree of accuracy (see Figure 4b–d for representative images and back-gating). Note cells marked with amber and white crosses (A) second from top and C) are almost identical with respect to total intensity (and therefore differences are indiscernible in standard flow cytometry. Raw max pixel vs versus total intensity plots clearly distinguish differences in signal distribution and size, etc. See also B) back-gated on the same plot (A0 second from top) to reveal doublets (blue) and cell fragments (magenta).

3. Results

3.1 Controls and experimental setup

A spontaneous target cell lysis control was developed in order to determine background levels of target cell death. For this control, effectors were generated by co-culturing with irradiated “self” cells. The control effectors were then run in the same set of dilutions as all of the other experimental effectors. A secondary internal control accounting for non-specific target cell lysis was also created by using misprimed effectors; for example, MHC^{cc} responders were stimulated with 3rd party MHC^{York} cells and then challenged with MHC^{dd} targets. The total number of events collected for each sample was determined by a set number of micro beads which were discriminated by their high intensity in 585/42 and 670LP emission spectra when measured on our FACSCaliburTM. The micro beads provide a normalizing control so that the same volume is collected with each sample and thereby mitigate any error introduced by effector cell death. Through the use of this control, absolute counts of remaining healthy target cells can be applied as the sole metric for percent specific lysis.

3.2 Correlation of the flow cytometry-based cell-mediated lympholysis assay and the conventional ⁵¹Cr release assay

To determine if the results of the traditional ⁵¹Cr release assay were comparable to the flow cytometry-based cell-mediated lympholysis, the assays were run in parallel by independent investigators. The parallel assays shared the same bulk effectors to ensure the same level of cytotoxic capacity within each assay. Briefly, MHC^{cc} anti-MHC^{dd} effector cells were generated and incubated with eFluor670 or ⁵¹Cr labeled MHC^{dd} target cells at ratios of 100:1, 50:1, 25:1 and 12.5:1 for 5.5 hours, harvested and analyzed as described in the Materials and Methods. Figure 1 Illustrates the gating design used in FlowJo V_10 to analyze the flow cytometric data collected on the FACSCaliburTM. Events were first discriminated for healthy lymphocytes by means of scatter (Wlodkowic et al., 2011)(Fig. 1A), followed by (+) target cell events for eFluor670 (Fig. 1 C,D). From the events collected within the (G4) gate, the absolute number of events could be calculated relative to the spontaneous control (G3) to determine percent specific survival. The inverse of the survival was taken to provide results of percent specific lysis and present the results in a manner analogous to that of the ⁵¹Cr release. An identical gating strategy was used for both the full and class I MHC mismatch data.

Results of the ⁵¹Cr method were compared directly to that of the flow based _{fc}CML. To confirm correlation between the two methods (Figure 2), we assessed the results of the ⁵¹Cr

technique (2A) and flow cytometry based assay (2B) across a full MHC mismatch. Data from a single experiment that is representative of a total of 10 independent assays is shown in Figure 2 (A,B), for two effector sets: MHC^{cc} anti-MHC^{dd} (blue) and MHC^{cc} anti-MHC^{York} (black) co-cultured with MHC^{dd} target cells. Natural day to day variation of both the donor and recipient PBMC combined with minor histocompatibility antigen discrepancies prevents the normalization of individual experiments (Roopenian et al., 2002). The matched stimulator to target effector set (blue) at the ratio of 100:1 (effector: target) showed 51% specific lysis in the ⁵¹Cr-based assays, and 50% in the flow cytometry based approach, while the mismatched stimulator to target control (black) resulted in 4% specific lysis in the ⁵¹Cr and 1% in the flow cytometry-based assay. The titration of lytic activity with decreasing effector to target cells ratios was similar in both methods. In order to test the sensitivity of the assay, we also performed both the ⁵¹Cr and flow cytometry-based CML methods with cells that only had a class I MHC disparity (Figure 2. D,E); representative data is shown in Figure 2D and 2E (n=6). In these experiments, two effector sets were generated, MHC^{dd} anti-MHC^{cd} (blue) and MHC^{dd} anti-MHC^{York} that were then co-cultured with MHC^{cd} targets. Consistency between the two methods can also be seen here with 100:1 ratio specific lysis of 47% by ⁵¹Cr and 49% by flow cytometry. Again, as with the full mismatch, the reduction in lysis that was observed with decreasing effector: target cell ratios correlated well in both the ⁵¹Cr and flow cytometric methods. To show assay correlation, percent specific lysis in the ⁵¹Cr and _{fc}CML method for each experiment are plotted against each other with a perfect fit line to show agreement between the two methods, in the full MHC mismatch (2C) and the class I MHC mismatch (2F).

In order to apply the _{fc}CML to investigate transplantation tolerance, we performed the assays in parallel with our conventional ⁵¹Cr CML that is routinely used for confirmation of *in vivo* tolerance induction. The anti-donor tolerance experiments were performed in the same manner as the full and class I MHC mismatched CML assays (Fig. 2) using responder PBMC [MHC^{cc}] from a full MHC mismatch heart and kidney allograft [MHC^{dd}] recipient 100 days post-transplant (Fig. 3). Serial CML assays were performed to assess the immune competence in recipients of combined heart/kidney allografts. All heart/kidney recipients demonstrated loss of donor-specific responsiveness (Fig. 3 A,B) in CML assays by POD 30 (data not shown) but maintenance of third-party reactivity (Fig. 3 C,D). Donor-specific hyporesponsiveness was maintained for the duration of the experiment (>200 days) (Madariaga et al., 2013). Data between the two methods correlated well in both the anti-donor MHC^{dd} CML (Fig. 3 A,B) and the anti-3rd party MHC^{YO} CML (Fig. 3 C,D). The titration of lytic activity with decreasing effector to target cells ratios was in general agreement between the two methods.

3.3 Further investigation of effector - target cell interactions by imaging flow cytometry

In order to refine our understanding of the intercellular events occurring in this process, we decided to investigate the interaction of the effector and target cells using imaging flow cytometry. One of the advantages of this technology is that cell-cell interactions are more likely to be preserved during sample acquisition due to the decreased pressure of the sheath fluid and increased size of the flow core. The assay was set up in the same manner as the FACS CML with the addition of anti-CD8 PE antibody in order to identify the CD8+

effector cells. These qualitative assays were performed in parallel with the conventional ^{51}Cr in order to provide confirmation of the results. Effector cells primed against one MHC (MHC^{cc} anti MHC^{York}) and incubated with mismatched targets (MHC^{dd}) served as the negative controls. The gating methods used for this portion of the experiment are illustrated (Figure 4). In order to assess the morphological changes within the target cell population of interest, events of single cells (R4) and events of multicellular aggregates (R5) were analyzed separately. Collected events were first discriminated to only include those with at least one nucleus (R1) and then further refined to include at least one eFluor670⁺ event (R2) and/ or at least one CD8⁺ event (R3). Aspect ratio (y-axis) segregates DAPI positive events (i.e. nuclei) based on roundness. The (R5) gate identifies nuclear doublets (aspect ratio of ~0.5 and larger area in the x-axis than the singlet gate (R4)).

Analysis of the collected target lymphocyte events was then performed on a basis of visual confirmation using the IDEAS software. By labeling the PBMCs after the CML reaction with DAPI nuclear stain, it can be observed in the ImageStream^x images that over a time titration, the target cell nuclei condense, fragment and finally disappear altogether (data not shown). Figure 5 (left) illustrates representative images of eFluor670⁺ nucleated target lymphocytes after 2 hours of CML reaction, likely to be in the process lympholysis while conjugated to active CD8⁺ effector cells. The right side of Figure 5 presents eFluor670⁺ target cells after 4 hours of CML reaction, that have already undergone lympholysis, leaving only membrane fragments associated with the CD8⁺ effectors.

4. Conclusions

In these studies, we have developed an assay system capable of evaluating CML in primary PBMC by successfully adapting the conventional chromium release assay to a flow cytometry-based platform capable of measuring cell-mediated cytotoxicity. In doing so, we have developed an assay that not only eliminates the need for radioactive materials but also makes it possible to further define the cell populations involved in cytotoxicity using antibodies to phenotypic markers. Additionally, we have been able to confirm the validity of the fcCML method in models of solid organ allotransplantation tolerance by demonstrating anti-donor hyporesponsiveness with both the fcCML and ^{51}Cr CML methods. Thus, the fcCML assay presents a novel method for immune monitoring in transplant patients (Mehrotra et al., 2015). While current clinical transplant assays may rely on ^{51}Cr release assays (Leventhal et al., 2013), our method provides the ability to obtain additional phenotypic data in both the effector and target cell populations. This assay can be combined with current CFSE based mixed lymphocyte reactions (MLR) to simultaneously provide an assessment of anti-donor CTL functionality and anti-donor lymphocyte proliferation (Ashokkumar et al., 2009; Tanaka et al., 2012). Such comprehensive data from a limited patient sample is crucial for the development of tolerance induction protocols and for rapid diagnosis of graft rejection (Tanaka et al., 2012).

Many current immunosuppressive protocols rely on the use of broad spectrum calcineurin inhibitors for maintenance of donor allografts (Karam and Wali, 2015). These immunosuppressive therapies have many side effects due to their non-specific nature and high potency. Current endeavors in clinical transplantation are focused on the development

of individual immunosuppression protocols tailored directly through immune monitoring data (Sommerer et al., 2014). This is of great importance in that such regimens could dramatically reduce the deleterious side effects of immunosuppression. The methods described within this manuscript can be applied to transplantation studies in order to define the anti-donor CTL mechanisms and characterize allograft tolerance and rejection at their cellular and molecular levels.

Further investigation utilizing imaging flow cytometry enabled us to visualize, troubleshoot and further understand these cytotoxic processes at the single and multiple cell level. Future studies have the potential to assess the role of functional molecules with this method. Image flow cytometric analysis allows us to examine the cellular interactions occurring and provides additional detail through the analysis of apoptotic target cells and their associated effector and accessory cells.

The data for nuclear condensation and fragmentation (not shown) yielded inconsistent results when used as the sole indicator for target cell lympholysis and will require further optimization in order to be used in future studies. We believe this was due to the smaller cellular and nuclear volume of the primary porcine PBMC in contrast to the tumor cell lines utilized by other researchers (Helguera et al., 2011). However, the data for nuclear disappearance provided a reliable metric for relative levels of target cell apoptosis within the context of involuted target cells and membrane fragments. Although these methods did not prove to be an efficient method of quantifying CML on a large scale, their utility will be paramount in future mechanistic experiments.

Interestingly, preliminary data from these studies indicated the presence of helper and/or accessory cells in the process of CML. Furthermore, while the data presented showed that target cell lympholysis was mediated by CD8+ effector cells, we think that other effector cell subsets, including NK cells, may also play a role in the anti-donor MHC CML response. Experiments to characterize the effector and target cell populations are currently underway.

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Abbreviations

CML	cell-mediated lympholysis
CTL	cytotoxic lymphocyte
CTLp	CTL precursors
MHC	major histocompatibility complex
PBMC	peripheral blood mononuclear cell

TCR	T-cell receptor
⁵¹ Cr	chromium-51
TNF-α	tumor necrosis factor-alpha

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Highlights

- The establishment of a flow based CML utilizing primary PBMCs.
- Provides isotope-free approach to *in vitro* allo-response monitoring.
- Ability to phenotypically characterize the effector and target cell populations.
- Application of imaging cytometry to evaluate effector-target cell interactions.
- Allows for future studies investigating the mechanism(s) of cell mediated lysis.

Figure 1. Gating on the Target Cell Population

(A) Scatter dot plot of an f_c CML reaction tube representative of all samples; shown is porcine MHC^{cc} responders stimulated with irradiated MHC^{dd} PBMC and then incubated for 5 hours with eFluor670 labeled MHC^{dd} target cells at effector to target ratio of 100:1. Region **G1** is gated on healthy, intact porcine lymphocytes in terms of conventional forward (FSC) versus side scatter (SSC) pattern. (B) Shows the gating method of (**G2**) to discriminate the fluorescent bead stop gate. (C, D) Analysis of the cells in G1 to determine the absolute number of remaining target cells by gating on eFluor670 positive cells, with

(G3) being a non-reactive spontaneous lysis control, while (G4) shows a reactive response on the basis of absolute target cell loss. An identical gating strategy was employed for the class I MHC mismatch data.

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Figure 2.

Comparison of the ^{51}Cr and flow cytometric CML methods in a Fully Disparate MHC Mismatch (A–C) and a Class I MHC Mismatch (D–F) at 100:1, 50:1, 25:1 and 12.5:1 effector:target ratios. Representative percent specific lysis of target cells using ^{51}Cr (A) or eFluor670 (B) by porcine MHC^{cc} responders stimulated with irradiated MHC^{dd} (Blue) or third party control (MHC^{York}, Black). (C) Correlation graph of the two methods with the full MHC mismatch at all effector target ratios (10 independent experiments) with a perfect-fit line. Representative percent specific lysis of MHC^{dd} responders stimulated with irradiated MHC^{cd} (Blue) or third party control (MHC^{York}, Black) in the ^{51}Cr release assay (D) and the ^{51}Cr CML assay (E) in the Class I MHC mismatch. (F) The correlation graph of the two methods with the Class I MHC mismatch at all four effector:target ratios (6 independent experiments) with a perfect fit line.

Figure 3. Monitoring of anti-donor MHC^{dd} (A,B) and the anti-3rd party (C,D) CML responses post-transplant

Percent specific lysis was determined at 100:1, 50:1, 25:1 and 12.5:1 effector:target ratios.

(A) CML assessment of donor type hyporesponsiveness - ^{51}Cr release assay with naïve porcine MHC^{cc} responders (blue) or responders from experimental animals (MHC^{cc}; transplanted with MHC^{DD} heart and kidney (CC HK)) stimulated with donor type MHC^{DD} PBMC (Black). (B) f_c CML performed with same cells. (C) ^{51}Cr assessment of recipient immune competence - naïve (blue) and experimental (black) responders stimulated with irradiated 3rd-party MHC^{YO} PBMC (D) Corresponding f_c CML to assess immune competence.

Figure 4. Gating Strategy for Imaging Flow Cytometry

(A) Shows the gating strategy (left column, top to bottom) as defined in Methods. Briefly, from DAPI+ (R1) events, eFluor 670 (R2) and CD8+ (R3) populations were combined with a Boolean operation “OR” to form a combined population that includes at least one eFluor 670 and/or CD8+ event. The rationale for this strategy is that it includes both single cells (R4), doublets (R5) and aggregates. (B) Depicts representative images containing one effector cell with a non-nuclear target cell fragment (top) and effector cell with a nuclear target cell (bottom). Images are pseudocolor overlays of CD8 PE (green), DAPI (purple) and

eFluor 670 (red). **(C)** Illustrates additional information that distinguishes diffuse from punctate fluorescence signal intensity and distribution. **(D)** Top to bottom, are representative examples of target cells alone (top), and effector cell – nonnucleated targets, effector cell-nucleated targets and aggregated cells.

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Figure 5. Image analysis of eFluor 670⁺ target cells interactions with CD8⁺ effector T-lymphocytes

Representative images of 100,000 collected events were taken on the ImageStream^X at a magnification of 60×, populations were then analyzed using IDEAS software. **(Left)** eFluor 670 labeled target cells potentially undergoing cell mediated lysis by CD8⁺ cytotoxic lymphocyte effector cells after 2 hours of CML reaction. This is classified as early stage cell lysis, with the nuclei of the target cells still intact. **(Right)** eFluor 670 labeled target cell membrane fragments after 4 hours of CML reaction, with absent nuclei, still associated with CD8⁺ effector cells; these were designated late stage lysis.