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Research paper

A novel approach to measuring cell-mediated lympholysis using quantitative flow and imaging cytometry

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ABSTRACT

In this study, we established a novel isotope-free approach for the detection of cell-mediated lympholysis (CML) in MHC defined peripheral blood mononuclear cells (PBMCs) using multiparameter flow and imaging cytometry. CML is an established in vitro assay to detect the presence of cytotoxic effector T-lymphocytes precursors (CTLp). Current methods employed in the identification of CTLp in the context of transplantation are based upon the quantification of chromium (^{51}Cr) released from target cells. In order to adapt the assay to flow cytometry, primary porcine PBMC targets were labeled with eFluor670 and incubated with major histocompatibility complex (MHC) mismatched effector cytotoxic lymphocytes (CTLs). With this method, we were able to detect target-specific lysis that was comparable to that observed with the ^{51}Cr-based assay. In addition, the use of quantitative cell imaging demonstrates the presence of accessory cells involved in the cytotoxic pathway. This innovative technique improves upon the standard ^{51}Cr release assay by eliminating the need for radioisotopes and provides enhanced characterization of the interactions between effector and target cells. This technique has wide applicability to numerous experimental and clinical models involved with effector–cell interactions.

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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 ^{51}Cr 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 ^{51}Cr 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 ^{51}Cr 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 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

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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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 labeling 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$^{\times}$ 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$^{\times}$ 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 ^{51}Cr labeled target cells, recently the $_{fc}$CML technique has been successfully applied to studies with naive human PBMC in our laboratory (La Muraglia, 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 transplants are

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