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Cytotoxicity of Vγ9Vδ2 T cells towards Colon Cancer Cells

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Abstract

Immunotherapies for cancer are widely studied at present. We are currently studying a specific form of "Vγ9Vδ2 T cells" found in the peripheral blood of healthy donors that can be used for the killing of HT-29 colon cancer cells. In order to determine the cytotoxicity of effectors, Vγ9Vδ2 T cells towards target cells, HT-29, it is important to first evaluate the absolute number of Vγ9Vδ2 T cells in a mixed cell population, and next to determine the phenotypic characterization, their activity and cytotoxicity in the presence of target cells. A flow cytometry and bead based assay was developed to evaluate the absolute number of Vγ9Vδ2 T cells in a mixed cell population. Peripheral Blood Mononuclear Cells (PBMCs) were surface stained with monoclonal antibodies (MoAbs) conjugated to fluorochromes that are cross reactive to cell surface markers such as CD3 (T Lymphocytes), γδ2 and were mixed with fluorophore beads. In these assays, no washes and centrifugation steps were performed after the cell surface staining and bead addition. The absolute cell counts were evaluated based on referencing a known concentration of beads. In addition, quantification assays were also performed to measure the cell and bead loss on surface staining that included washes and centrifugation steps and thus found a higher percentage loss of cells than beads. Immunophenotyping assays with four color staining were performed to monitor the phenotypic differentiation of effector cells based on cell surface markers CD27 and CD45RA. Only the naïve ($CD27^+CDRA^+$) and terminally differentiated effector memory ($CD27^-CD45RA^+$) were identified on the assays performed using Vγ9Vδ2 T cells of different donors. A flow cytometry based cytotoxicity (FCC) assay was completed to monitor the effector cell activity ($CD69^+$) in the presence and absence of target cells and also the cytotoxicity was measured based on % specific lysis of target cells at four different effector to target (*E:T)* cell ratios. Only preliminary data were obtained for the FCC assay and the development is still in progress.

Introduction

Colorectal cancer is the second largest leading cause of death in the Western hemisphere. The most common treatment methods for this disease are surgery to remove the tumor area, chemotherapy and radiation. However, there are modern newly developed techniques such as vaccinations and viral therapies to selectively inhibit and kill tumors, as new forms of treatment for this deadly disease. Immunotherapy methods for the treatment of cancer are widely studied at present. Kim et al., 2007 discussed a Natural Killer (NK) cell or T cell mediated cytotoxicity towards leukemia (K562 Cell line) as a form of immunotherapy [1]. Todaro et al., 2013 in addition discussed combinations of immunotherapies together with chemotherapy and other anti-tumor agents are essential for the treatment of colon cancer [2]. γδ T cells are found to be of particular interest for such combined therapies due to their potential for anti-tumor cytotoxicity and the ease of use *in vitro* [3]. These T cell subsets are preferentially considered to bridge innate and adaptive immunity, as they share with cells belonging to the adaptive immune system on the expression of antigen receptors, as well as the cells with innate immunity the expression of Natural Killer Group 2 Member D (NKG2D), and pattern recognition receptors [4, 5]. Nevertheless, γδ T cells recognize antigens independently of Major Histocompatibility Complex (MHC) presentation [6]. The T cell receptor (TCR) for γδ T cells contains γδ chains compared to the traditional TCRs that consists of the αβ chains of MHC dependent CD4+ and CD8+ αβ T cells.

Human γδ T cells are divided into two main populations based on the δ chain expression [7]. They are the Vδ2 and Vδ1 subsets of human γδ T cells and recognize different types of ligands, and thus both exert potential anti-tumor effects [6]. The TCR of Vδ2 recognizes cell derived pyrophosphates, whereas Vδ1 γδ T cells recognize stress associated surface antigens [6]. The Vδ2 T cells account for the majority (50%-95%) of circulating γδ T cells (constituting only 5% of T cells in peripheral blood in healthy humans), whereas Vδ1 are very rare in peripheral blood [6]. However, Vδ1 γδ T cells appear at increased frequencies in mucosal tissues and in the skin [6]. γδ T cells that express the Vδ2 chain paired to the Vγ9 chain are called Vγ9Vδ2 T cells [5]. For the ease and purpose of this report "Vγ9Vδ2 T cells" are collectively referred to as γδ2 T cells and combination of both Vδ1 and Vδ2 are referred to as γδ T cells in this report. γδ2 T cells can also be classified into four subpopulations based on their expression of cell surface markers CD27 and CD45RA: Naïve ($CD27^+CD45RA^+$), Effector Memory ($CD27^-CD45RA^-$), Central Memory ($CD27^+CD45RA^-$) and Terminally Differentiated Effector Memory ($CD27^-CD45RA^+$) [8]. $CD27^+CD45RA^+$ $T_{Naïve}$ cells account for 15-20%, $CD27^-CD45RA^-$ T_{EM} for ~30%, $CD27^+CD45RA^-$ T_{CM} for 40-60% and $CD27^-CD45RA^+$ T_{EMRA} for ~7% of the Vγ9Vδ2 T cell population, with significant inter-individual variability [9, 10].

γδ2 T cells recognize phosphoantigens (small organic compounds with phosphate groups) that are produced through the isoprenoid biosynthesis pathways [11-13]. This T cell subset can also be activated, through an indirect mechanism, by aminobisphosphonates (a class of drugs used to treat bone diseases), that inhibit farnesyl pyrophosphate synthase, and cause the accumulation of metabolites such as isopentenyl pyrophosphate (IPP) [14]. γδ2 T cells also recognize, *(E)*- 4-hydroxyl-3-methyl-but-2-enyl pyrophosphate (HMB-PP), which is a natural intermediate of the non-mevalonate pathway of IPP biosynthesis. It is also an essential metabolite in most pathogenic bacteria and malaria parasites, but is not produced in the human host [15]. HMB-PP is structurally closely similar to IPP, and is the most potent human γδ2 T cell activator currently identified [9]. *In vitro* stimulation of γδ2 T cells can be performed by synthetic phosphoantigens (IPP/HMB-PP) in the presence of a cytokine and human interleukin-2 (IL-2). Hence, stimulation with these phosphoantigens and cytokine signaling molecules triggers a rapid, and selective expansion of γδ2 T cells [6]. The $T_{Naïve}$ and T_{CM} subsets of γδ2 T cells proliferate in response to phosphoantigen stimulation and T_{EM} and T_{EMRA} subsets expand in response to cytokines [10].

In order to determine the expression of the γδ2 T cells and study the phenotypic differentiation of expanded γδ2 T cells in Peripheral Blood Mononuclear Cells (PBMCs) by Flow Cytometry (FC) analysis, the cells need to be surface stained with monoclonal antibodies (MoAbs) conjugated to fluorochromes that are cross reactive to cell surface markers such as CD3 (T Lymphocytes), γδ2 CD27, CD45RA and CD69. CD69 is a marker of T-cell responsiveness to antigen stimulus, and can be used as a measure of T-cell activation [16].Flow cytometry measures the fluorescence and other optical properties of individual cells. The principle behind flow cytometry is that, the cell sample is carried in a stream of liquid through a laser beam (2 lasers in BD FACS Calibur), and three primary measurements are made: forward light scatter (cell size), side light scatter (cell refractivity/ granularity) and excited fluorescent dyes emission [17]. These fluorescense components can be correlated with the size, structure and other physical properties of cells [17]. The advantage of flow cytometry is the combination of both multiparameter measurements and high speed analysis [17]. At present, flow cytometric assays are widely used for enumeration and

phenotypic characterization of lymphocytes, measurement of cytokines and other signaling molecules, intracellular signaling, cell function and proliferation [18-20]. Several attempts have also been made to develop flow cytometry based cytotoxicity assays [17]. Flow cytometric cytotoxicity assays provide some advantages, including: the detection of cytotoxicity at the single-cell level; evaluation of all stages of the cytotoxic process; and the possibility of characterizing the phenotypic differentiation of cytotoxic cells [17].

γδ T cells may have a unique role in the immune surveillance against malignancies and also an advantage over αβ T cells because they can directly recognize molecules expressed on cancer cells, independently of antigen processing and presentation [21, 22]. In addition, γδ T cells can migrate as infiltrating lymphocytes into solid tumors [23] and can recognize and eliminate different types of cultured malignant cells [24]. Also, the activation of γδ TCRs promote γδ T cell cytotoxicity through increased secretion of perforin/granzymes, IFN-γ and TNF-α (effector molecules in the immune response against cancer) and up-regulates expression of Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL) [25, 26]. Highest IFN-γ secretion is confined to the $CD45RA^{-}CD27^{-}$ T_{EM} subset, while strong cytotoxic activity exists in the $CD45RA^{+}CD27^{-}$ T_{EMRA} population (produce perforin and granulysin), whereas $CD45RA^{+}CD27^{+}$ $T_{Naïve}$ display low functional activity [9].

Research shows that there is a positive correlation between the γδ2 T cell number and tumor cell death making γδ2 T cells excellent candidates for immunotherapy and we aim to optimize their expansion conditions both in respect to cell number and cytotoxic activity. In order to determine the composition of PBMCs and cytotoxic activity of γδ2 T cells towards adherent cells (HT-29 Cell line), we are developing bead and Flow Cytometry (FC) based methods. By knowing the absolute number of beads (referencing bead counts) and once these beads are combined together with PBMCs or expanded γδ2 T cells, we can estimate the actual number of PBMCs and γδ2 T cells by FACS analysis. Kim et al., developed a bead and FC based method to determine the absolute PBMC/PBMC subset (T/NK and NKT cells) counts by adding fluorospheres (Beckman Coulter) to assay tubes prior to FC acquisition and by estimating the absolute cell counts by referencing the bead counts [1]. Additionally, Kim et al., also developed a new four color flow-cytometry based cytotoxicity (FCC) assay to simultaneously measure NK cell cytotoxicity and NK cell phenotype [1]. A highly sensitive and reproducible multiparametric flow cytometry-based cytotoxicity assay utilizing low numbers of antigen-specific T cells also has been described by Devevre et al. [27]. Furthermore, flow cytometric assays that simultaneously monitor cell-mediated target cell death and effector-cell frequency and activity in clinical trials have also been developed [28]. Oberg et al., on the other hand, optimized a method for calculating the absolute γδ T cell numbers by the use of BD TrueCount™ beads and a cytotoxicity assay against pancreatic cells using a Real Time Cell Analyzer [29].

Therefore, we hypothesize that based on using beads as a reference for counting γδ2 T cells, we can obtain the absolute number of γδ2 T cells in a mixed cell population. In order to measure the cytotoxic effect of γδ2 T cells towards HT-29 target cells, and determine effector cell activity ($CD69^{+}$) in the presence of target cells and monitor the differentiation of their phenotypes (CD45RA and CD27) we have undertaken to develop a new multi-color flow-cytometry based cytotoxicity (FCC) assay.

Materials and Methods

Blood samples and PBMC isolation

Blood samples were obtained from healthy male and female donors of ages ranging from 21-46 (n=20) and were collected into EDTA coated vacutainer tubes. The samples were collected in accordance with the ethical standards and all donors signed a consent form prior to phlebotomy. Donor blood tubes were processed within 1 hour of phlebotomy and was diluted (1:1) with Sterile Phosphate Buffered Saline (PBS), pH 7.4 in separate 50mL conical tubes. PBMCs were isolated by Histopaque®-1077 (Sigma, USA) density gradient centrifugation. They were centrifuged at 400 x g for 30 min. The white mononuclear ring was collected into separate tubes and was washed with 1X PBS twice, and the pellet was re-suspended in pre-warmed complete growth media (RPMI-1640 with 25 mM HEPES (Thermo Scientific, USA) +5% human serum + 1% Penicillin and Streptomycin (PenStrep) + 1% L-Glutamine). The recovered PBMCs were counted and assessed for viability in Trypan blue dye 0.4 % (v/v) (Sigma, USA), and the concentration of the cell suspension was set to 1 x 10^6 cells/mL.

Determination of absolute counts of PBMCs by using fluorophore beads

CaliBrite Phycoerythrin (PE) bead stock (BD Biosciences, USA) was gently vortexed and diluted (1:10, 1:50, 1:100) with 1X PBS prior to their addition to the FACS tubes. These PE beads are collectively referred to as "beads" throughout this report, unless otherwise stated as different types of fluorophore beads. The number of beads were counted manually by adding 10uL of the diluted beads (optimal dilution factor = 10), into a hemocytometer with a cover slip and observed under the microscope. The beads were also counted on the Countess™ (Invitrogen, USA). The actual counts of the PE beads from microscopy and Countess counting ranged from 1.80 x 10^6 - 2.50 x 10^6 beads/mL. The number of cells were also counted manually using the microscope and Countess to compare and contrast the ratio of PBMCs: beads to ratio of cell events: bead events based on FACS analysis. The diluted beads were then vortexed again and different volumes of 25uL, 50uL and 100uL was added into three separate FACS tubes that contained equal volumes of PBMC cell suspension (500uL = 500,000 cells). An additional tube with a control (beads only) was added to this assay. These samples were then incubated on ice for 30mins and samples were tested on flow cytometry. In parallel, three additional tubes with 500uL of cell suspension and different bead volumes (same as previous samples) were washed twice with 1X PBS and centrifuged at 1400 rpm for 7mins and cell pellet re-suspended in 400uL of 1X PBS, were also tested by flow cytometry. The FACS ratio of PBMCs: beads was determined using formula: cell events/bead events.

Cell surface staining for PBMC subsets and absolute counts

Isolated PBMCs were surface stained by two methods that included no wash and wash steps with centrifugation. For the surface stain with no wash, 500,000 harvested cells (500uL) were added into three separate FACS tubes. To each tube PE anti-human CD3 (UCHT1) and FITC (Fluorescein isothiocynate) anti-human TCR γδ2 (B6) monoclonal antibodies (Biolegend, USA) were added at dilutions recommended by the manufacturer. In addition, 25uL, 50uL and 100uL of diluted beads were added into the three tubes and all samples together with negative control

(PBMC only) and single color staining controls were incubated in the dark for 30 min at 4^{o}C. Flow cytometry was performed on the samples right after the 30 min incubation. For the antibody stains with wash steps, PBMCs containing 25uL/50uL/100uL diluted beads were washed with 1mL of ice cold 1X PBS and the cell pellet was re-suspended in 100uL of ice cold 1X PBS and same volumes of antibodies were added into three tubes with different dilutions of beads. The samples were then incubated on ice in the dark for 30mins and washed with 500uL of 1X PBS with centrifugation speed of 1400 rpm for 7mins and the cell pellets were re-suspended in 400uL of PBS. The assay was repeated with the same conditions that is with the same antibody volumes and bead volumes, but by only changing the centrifugation speed to 1900 rpm during the wash steps and then flow cytometry was performed on these samples.

The absolute number of PBMC and PBMC subsets were determined using the formula: (Cell events/ Bead events) x actual bead counts. For absolute cell count and reproducibility assays isolated PBMCs were re-suspended in RPMI 1640 free of Phenol Red (*Life technologies*, USA) without any human serum, L-Glutamine and antibiotics (incomplete growth media).

Determination of loss of PBMCs and beads after antibody staining and washes

The same PBMC and Bead based assay was repeated with the same conditions as above and after antibody staining and washes, 50uL of diluted (1:10) CaliBrite FITC beads (BD Biosciences, USA) was added to the final suspension of PBMC and PE beads prior to FACS acquisition. The purpose of the addition of FITC beads (with known actual number) was to use it as a reference to evaluate the percentage loss of PE beads and PBMCs after antibody staining with wash steps. A detailed step by step analysis of the formulas for calculating the actual % loss of PBMCs and PE beads are shown below.

Ratio microscopy (manual) = PBMC counts/ Beads (PE+ FITC) counts

Ratio FACS = Number of Cell events/ Number of Bead (PE+FITC) events

Number of PE Beads after wash = [PE Bead Events/ FITC Bead Events] x [FITC Bead Counts x FITC Bead Volume]

Loss of PE Beads = PE Bead counts before wash – PE bead counts after wash

Number of PBMCs after wash = [Cell Events/ FITC Bead Events] x [FITC Bead Counts x FITC Bead Volume]

Loss of PBMCs = PBMC counts before wash – Number of PBMCs after wash

% loss of PBMCs = [PBMC Counts- Loss of PBMCs]/ PBMC Counts x 100

% loss of PE Beads = [PE Bead Counts – Loss of PE Beads]/ PE Bead Counts x 100

In vitro expansion and purification of γδ2 T cells

8mM HMBPP stock was diluted in 0.15M NH_3/EtOH and 8pM HMBPP was transferred per well in a 6-well plate and was let dry. Five milliliters of cell suspension (1 x 10^6 cells/mL), with complete growth media (RPMI-1640 free of phenol red (*Life technologies*, USA) + 25 mM HEPES+ 5% human serum+ 1% PenStrep+ 1% L-Glutamin) was transferred per well. The cells were incubated at 37 ^{O}C in the atmosphere of 5% CO_2 in air for 3 days and 20U/mL IL-2 (Sigma, USA) was added to cells and the same was repeated after day 5 and day 7. The cells were fed with 1mL of fresh growth media every 2 days until day 10. After 10 days, cells were pooled together into a 50mL conical tube (under sterile conditions) and were counted using a hemocytometer and 5.0 x 10^5 cells/mL were stained with PE anti-human CD3 (UCHT1) and FITC anti-human TCR γδ2 (B6) for FACS analysis to determine the % total of $CD3^+/γδ2^+$ in sample compared to % total of $CD3^+/γδ2^+$ in sample on day 0. γδ2 T cells were purified from the expanded PBMCs by the use of a magnetic bead cell separation kit (Miltenyi Biotec, Germany). The separation procedure was followed according to manufacturer's instructions. The purity of isolated γδ2 T cells was >90% determined by FACS analysis. Finally, purified γδ2 T cells were used for immunophenotyping and cytotoxicity assays.

Immunophenotyping assay

A four-color antibody cocktail with MoAbs APC (Allophycocyanin) anti-human CD3 (UCHT1), FITC anti-human TCR Vδ2 (B6), PerCP-Cy 5.5 (Peridinin chlorophyll- Cyanine) anti-human CD45RA (HI100), PE anti-human CD27 (O323) (all from BioLegend, USA) was prepared at dilutions recommended by the manufacturer and ice cold 1X PBS was added to make up a total volume of 100uL. In order to make a 1:1 mixture of MoAbs and PBMCs, 100uL of PBMCs were incubated in FACS tubes containing 100uL of Ab cocktail in the dark at 4 ^{O}C for 30 min. The Ab cocktail stained cells were washed twice with 500uL of 1X PBS with centrifugation speed of 1400 rpm for 5 min and cell pellets were re-suspended in 400uL of ice cold 1X PBS.

Single colors with the same isotype control MoAbs were used in parallel to the samples. Fluorescence Minus One (FMO) staining was also performed on N-$CD3^+$ APC ($CD45RA^+$ PerCP-Cy5.5/$CD27^+$ PE/$γδ2^+$ FITC), N-$CD45RA^+$ PerCP-Cy5.5 ($CD27^+$ PE/$γδ2^+$ FITC/$CD3^+$ APC) N-$CD27^+$ PE ($γδ2^+$ FITC/$CD3^+$ APC/$CD45RA^+$ PerCP-Cy5.5) N- $γδ2^+$ FITC ($CD3^+$ APC/$CD45RA^+$ PerCP-Cy5.5/$CD27^+$ PE). These controls and samples were then run on FACS to determine the phenotypic differentiation of CD27 and CD45RA populations of γδ2 T cells. These assays were run on separate tubes and independently of cytotoxicity assays.

Culturing of adherent cells (HT-29 cell line)

HT-29, human colorectal adenocarcinoma cells (ATCC # 30-2007) were cultured in complete growth media (DMEM/HIGH Glucose (Thermo Scientific, USA) + 10% Fetal Bovine Serum (FBS) + 1% PenStrep + 1% L-Glutamine) in a T75 flask. These cells were incubated at 37 ^{O}C in the atmosphere of CO_2 in air and subcultured based on 70%-80% confluency for 2 weeks by rinsing cell layer with sterile 1X PBS, pH 7.4 and 0.25% (v/v) Trypsin-0.53mM EDTA was added to detach the adherent cells and cell layer dispersal was observed under an inverted microscope. After the cell layer dispersal the cells were then filled with complete growth media

at a subcultivation ratio of 1:8. Adherent cells were harvested and counted (cell concentration set to 1.0 x 10^6 cells/mL in 1X PBS) for CFSE staining in the cytotoxicity assay.

In this report, the adherent cells (HT-29) are referred to as "target cells" and γδ2 T cells are referred to as "effector cells".

Flow cytometry based cytotoxicity (FCC) assay

Target cells were stained with Carboxyfluorescein succinimidyl ester (CFSE) for 1h at 37 ^{O}C in the atmosphere of CO_2 in air. The stained cells were washed twice in 1X PBS, re-suspended in complete growth media (RPMI-1640 + 10% Fetal Calf Serum (FCS) + 1% L-Glutamine + 1% PenStrep) and counted using a hemocytometer. One hundred microliters of each target cells at a concentration of 1.0 x 10^5 cells/ mL was transferred into 6 different FACS tubes (4 *E:T* ratios and 2 controls). The effector cells at a desired concentration of 5.0 x 10^6 cells/ mL was added to FACS tubes containing target cells, resulting in four different *E:T* ratios of 50:1, 25:1, 12.5:1 and 6.25:1. Additional complete growth media was added in order to make the total volume up to 200uL in these four tubes. The two control tubes used were target cells only with complete growth media and target cells with 5% Triton to measure specific lysis. All 6 tubes were incubated at 37 ^{O}C in the atmosphere of CO_2 in air for 4 hours. After incubation termination all tubes were placed on ice and 7-amino-actinomycin D (7-AAD) (BioLegend, USA) was added to every tube at a dilution recommended by the manufacturer and incubated for 5min at 4 ^{O}C. For absolute count measurements 25uL of well vortexed diluted (1:5) beads were added to tubes and FACS acquisition was performed immediately. Next, specific lysis was measured for all four *E:T* ratios. The percentage of specific lysis was calculated using the following formula:

$$\% \text{ Specific Lysis} = \frac{\text{Live target cells in media control} - \text{Live target cells remaining in sample}}{\text{Live target cells in media control}}$$

In parallel to tubes set up for measurements of cytotoxicity, 4 tubes with *E:T* ratios of 50:0, 25:0, 12.5:0 and 6.25:0 (controls) and 4 tubes with *E:T* ratios of 50:1, 25:1, 12.5:1 and 6.25:1 were also incubated at 37 ^{O}C in the atmosphere of CO_2 in air for 4 hours to monitor the effector cells activated in the presence of target cells. The target cells were stained with CFSE, but the effector and target cells were not stained with 7-AAD after incubation. In order to determine the effector cells activated in the presence of the target cells, PerCP-Cy-5.5 anti-human CD69 (FN50) (Biolegend, USA) antibody was added at a dilution recommended by the manufacturer to the assay tubes immediately after termination of incubation as described in Kim et al., 2007 [1]. Single color isotype control MoAb was also placed in a control tube. FACS acquisition was performed to detect the Mean Fluorescence Intensity (MFI) of effector cells in the presence and absence of targets.

Gating strategies used for flow cytometry

For PBMC and bead assays with no stain and wash, wash and no stain, the gating used for the control (beads) was standard Forward Scatter (FSC) vs Side Scatter (SSC) and SSC vs FL2 channels on two different dot plots. Gating used for PBMC and bead samples without antibody

stain included only a dot plot of FSC vs SSC and a polygon gate was applied to PBMCs and a square gate was applied to beads (Figure 1).

In PBMC and bead assays with antibody staining and washes and antibody stain-no wash, the gating were similar as above, however, in order to determine the PBMC subsets (CD3+ lymphocytes and γδ2+ T cells) additional dot plots were analyzed with FL2 vs FL1 channels (FL2 = PE channel, FL1 = FITC channel). Spectral overlap was compensated in FL1 and FL2 channels using single color isotype control monoclonal antibodies (MoAbs). A quadrant was placed on the dot plot of FL2 vs FL1 to capture the number of CD3+ events and γδ2+ events (Figure 1). When applying quadrants, the upper right quadrant is double positive (++), lower right quadrant is single positive (+-), lower left is double negative (--) and upper left is single positive (-+). A dot plot of FSC vs SSC is usually plotted on a linear scale, whereas any corresponding dot plots with FL1-FL4 channels are typically plotted on a logarithmic scale. For absolute count calculations and reproducibility assays the gate was only set on lymphocytes in a dot plot of FSC vs SSC.

In immunophenotyping assays additional dot plots were analyzed using FL-3 and FL-4 channels (FL-3 = PerCP-Cy 5.5 channel, FL-4 = APC channel). Isotype control MoAbs were run to compensate spectral overlap between FL2-FL1, FL2-FL3 and FL3-FL4 channels. FMO staining was performed to validate compensation settings by analyzing whether fluorescent is present in the fluorescence minus one of interest (e.g. N-$CD3^+$ PE). The gates were set on lymphocytes in a dot plot of FSC vs SSC and the lymphocyte gating was applied to dot plots of FL1 vs SSC and FL1 vs FL4. A fourth dot plot of FL3 vs FL2 was analyzed based on CD3+/γδ2+ cells to determine the percentages of phenotypes in γδ2+ T cells (Figure 9).

For flow cytometry based cytotoxicity assay, target cells with media only control was analyzed and live target cells were gated on a dot plot of SSC vs FSC and was applied to all samples. Next, control tube with target cells, 7-AAD and 5% Triton was analyzed on a dot plot of FL1 vs FL3 and a quadrant was placed to capture the 7-AAD positive events (FL1 = CFSE channel, FL3 = 7-AAD channel). In addition, other control tubes used were target cells (no CFSE stain) only to determine gating size, CFSE stained target control to observe compensation for FL1 channel, lysis control (CFSE stained targets+7-AAD) to detect 100% lysis and effector cells only control for gating cells (Figure 11).

FACS acquisition was performed on the FACS Calibur (BD Biosciences, USA) and the data were analyzed using Cell Quest Pro (BD Biosciences, USA) and FLOWJO (Treestar, USA) software.

Results and Discussion

Ratios of PBMCs and beads based on microscopy and FACS counting

The initial step was to compare the ratios between manual microscopy counts and FACS counts. Based on the calculated ratios for different bead volumes (25uL, 50uL, 100uL) with the same volume of PBMCs (500uL), there were major differences between manual microscopy counts and

FACS counts. These PBMCs were just mixed with PE beads without any antibody staining and washes and were run on FACS directly. The calculated ratios are shown in Table 1.

Table 1: PBMCs to bead ratios for manual microscopy counts and FACS counts without any staining and washes. The manual microscopy counts for PBMCs were 8.3 X 10^5 cell/mL and Beads were 2.45 X 10^6 beads/mL. Ratio for manual microscopy counting was calculated by using formula: (PBMC counts/ Bead counts) and for FACS counts: PBMC events/ Bead events.

Bead Volume	Manual Ratio	FACS Ratio No wash and No Ab Stain
25uL	6.78	4.26
50uL	3.39	4.75
100uL	1.69	1.97

There is a large difference (2.52) between the manual ratio and FACS ratio for the assay tube that contains 25uL of beads and thus, the FACS ratio is lower than the manual ratio. However, for assay tubes with both 50uL and 100uL bead volumes the FACS ratio has been higher than the manual ratio with a difference of 1.36 for 50uL and 0.28 for 100uL. The differences in the ratios of no wash FACS counts and manual counts is that in FACS analysis all of the cells are not gated and counted in comparison to the manual counts in microscopy. In manual counting, when the cells are observed under the microscope all macrophages, monocytes, minor fractions of dendritic cells and some contaminated erythrocytes are counted and taken into consideration for the total counts. However, when gating on FACS, cells are gated from the lymphocytes to the right including some parts of the monocytes eliminating the contaminated erythrocytes, macrophages and minor fractions of dendritic cells.

The PBMC and bead assay without any washes or staining was eliminated in the next assay and an assay with PBMC and beads with washes (no antibody stain), and antibody stain and washes was performed. The results of ratio calculations for this assay are shown below in Table 2.

Table 2: PBMC to bead ratios for manual microscopy counts and FACS counts with wash and no antibody stain or antibody stain and washes. The manual microscopy counts for PBMCs were 9.1 X 10^5 cell/mL and beads were 2.32 X 10^6 beads/mL.

Bead Volume	Manual Ratio	FACS Ratio	
		Wash and No Ab Stain	Ab Stain and Wash
25uL	7.84	4.80	5.84
50uL	**3.92**	**3.04**	**3.03**
100uL	1.96	2.10	1.30

There were differences for manual ratios and FACS ratios for assay tubes with 25uL and 100uL of beads with no antibody staining and wash steps and antibody staining and wash steps. However, the assay tube with 50uL bead volume had comparable results to both manual ratios

and FACS ratios. However, there is a decrease in ratio for both assays with washes and antibody staining. The decreased ratio was 0.88 for wash steps without antibody staining and 0.89 for antibody staining and washes for assay tubes that contain 50uL of diluted bead suspensions (the actual ratios are highlighted in bold, Table 2). Therefore, the most optimal bead volume to add for isolated PBMC assay tubes to calculate the PBMC and bead ratios was finalized to 50uL of diluted beads. The next assays were performed using a set volume of 50uL of diluted (1:10) beads and 500uL of PBMCs.

The PBMC and bead assays performed with increasing centrifugation speeds during wash steps had not much of an impact in avoiding the decrease in ratios of PBMCs: beads when the ratio was compared to manual counts as the ratios slightly decreased in centrifugation speed of 1900rpm in comparison to the speed of 1400rpm. However, increase in centrifugation speeds should in fact be easier to pellet out cells and thus increase the number of cells. Nevertheless, cells are larger in size and less dense in comparison to beads that are smaller in size and denser. Therefore, even when centrifugation speeds are increased in wash steps and are analyzed on FACS, the number of cells and beads are decreased.

Flow cytometry for PBMC and bead assays

The dot plots for the PBMC and bead assay with antibody staining and washes performed with cells from a single donor is shown in Figure 1.

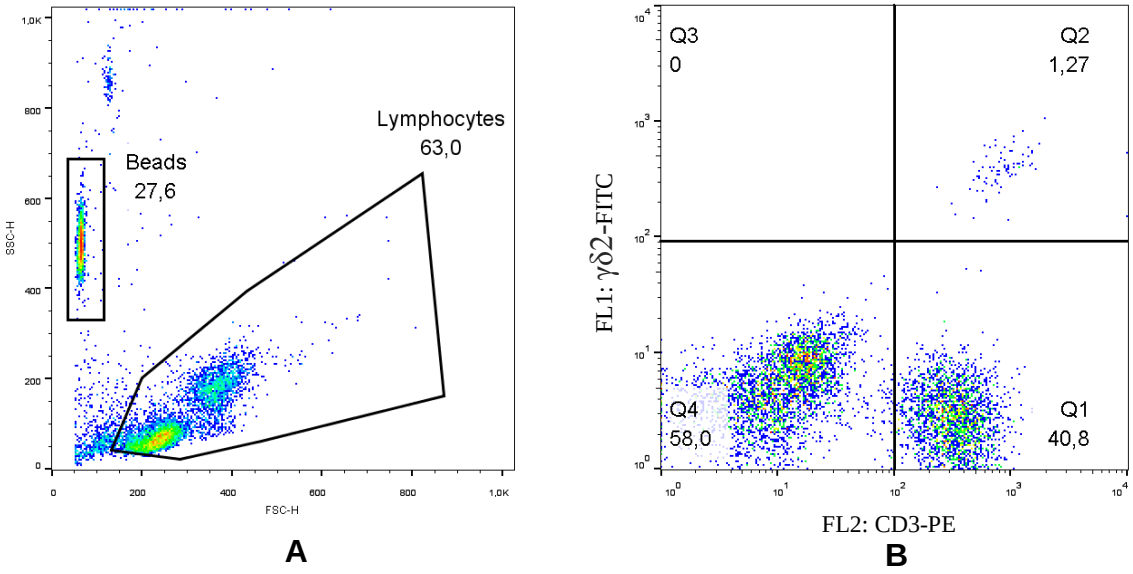


Figure 1: Gating strategies used to estimate the number of PBMCs in the presence of beads and to calculate the ratios of cells/beads. Bead events were gated by a square and cell events gated by a polygon in a dot plot of SSC Vs FSC (A). B. A quadrant is set on a dot plot of FL1 vs FL2, to discriminate between the various populations. CD3+ events are displayed in the right quadrants, and in the upper right quadrant γδ2+CD3+ events are displayed. The CD3- γδ2- events are displayed in the lower left quadrant.

The number of events and percentages for beads, PBMCs and PBMC subsets are shown in table 3. Based on the excel data exported from FLOWJO software, the ratio of PBMC: bead events

after antibody staining and washes were calculated and compared with manually calculated ratios of PBMCs: beads.

Table 3: The number of events and % total of gated events for beads, PBMCs, population of CD3+ lymphocytes and populations of γδ2+ T cells for one single donor. Number of manually calculated, 500uL cell suspension of PBMCs were 3.9 x 10^5 cells/mL and 50uL of diluted beads were 1.3 x 10^5 beads/mL.

Name	# Events	Statistic (%)
Beads	2763	27.6
PBMCs	6299	63
CD3+ lymphocytes	2567	40.8
γδ2+ T cells	84	1.27
CD3- γδ2-	3614	58.0

The manually calculated ratio for PBMCs: beads was 3.0 and in comparison, the FACS ratio after antibody staining with washes was 2.30. In parallel, an assay tube with antibody stain and no wash was performed and the ratio calculated from FACS analysis was 2.90 a slight difference (~0.1) from the manually calculated ratio. Since, there is a significant decrease in the ratios of PBMCs and beads after antibody staining and washes a quantification assay was performed in order to determine the loss of PBMCs and beads using Calibrite FITC beads.

Quantification assay to determine PBMC and bead loss in antibody staining and washes

Flow cytometry was performed after addition of diluted FITC beads to the PBMC and bead assay tubes that were antibody stained and washed. The dot plots for the data analysis are shown in Figure 2.

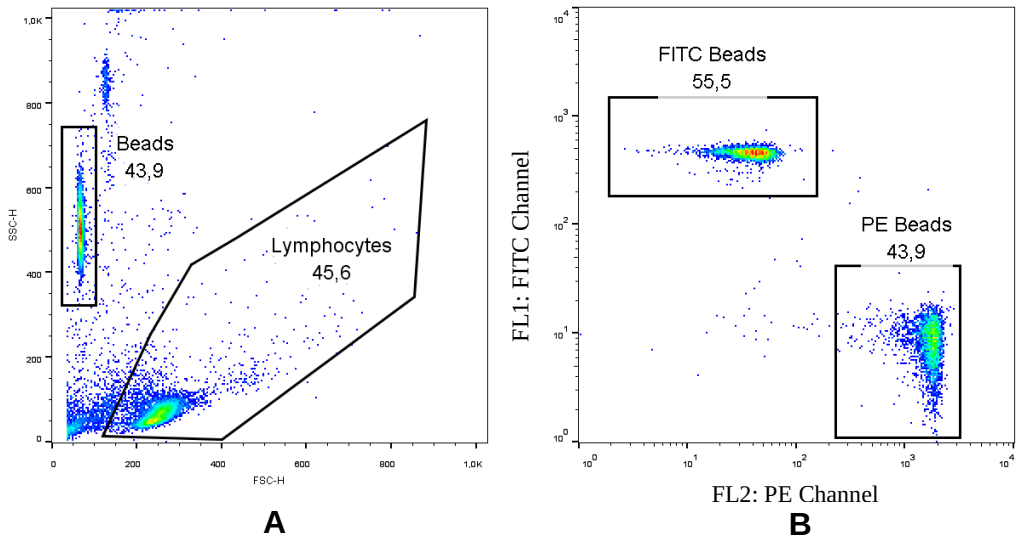


Figure 2: Gating strategies used for calculating the loss of PBMCs and PE beads in the presence of FITC beads. A. Cell events gated by a polygon and total bead events (PE+FITC beads) gated by a square on a dot plot of FSC Vs SSC. B. Gate is set on total beads on a dot plot of FL1 vs FL2 to discriminate FITC beads from PE beads. The total number of events acquired in FACS acquisition was 15,000 for data analysis.

The calculated % losses for both PBMCs and PE beads are shown in table 4 below.

Table 4: The PBMCs: beads manual ratio and FACS ratio after antibody staining and washes with PE beads and FITC beads performed on PBMCs isolated from one donor. Manual PBMC count for 500uL cell suspension of PBMCs was 3.85 x 10^5/ mL. The manual counts for 50uL diluted bead suspensions of PE and FITC beads were 1.05 x 10^5 beads/mL and 8.05 x 10^4 beads/mL respectively.

Donor	Manual Ratio	FACS data after antibody staining and washes		
		Ratio	% loss of PBMCs	% loss of PE Beads
1	2.10	1.05	61	40

FITC beads are not lost in the process as they are added after the antibody staining and washes before FACS acquisition. In addition, the number of FITC beads are known prior to FACS acquisition. The rationale behind the inclusion of FITC bead counts and volume in the formula described above is that FITC beads are calculated based on hemocytometer calculations, and thus to know the exact amount of FITC beads added to tubes, the counts and volume (actual concentrations) are important. There is significantly a higher decrease (50%) in the FACS ratio of PBMCs: beads after antibody staining and washes in comparison to the manual counts. In addition, a higher % loss of PBMCs which is 61% compared to % loss of PE beads which is 40% (Table 4). The ratio after antibody staining and washes is significantly reduced because there is a considerable loss of both PBMCs and beads. PBMCs are larger in size and less dense than the PE beads. Due to the properties of the swinging bucket rotor in centrifugation in the wash steps, and beads with a higher density are compacted, and PBMCs that are lower in density are lost in the process. Hence, higher loss of PBMCs than beads in antibody staining and wash steps.

Based on these data, PBMC and bead assays were repeated with antibody stain without any washes with PBMCs from two different healthy donors. In parallel, assay tubes with antibody stains and washes were assessed for comparisons.

Data analysis of the comparisons of antibody staining with and without washes

The dot plots of the data analysis of PBMC and bead assays with and without washes for two different donors are shown in Figures 3 and 4.

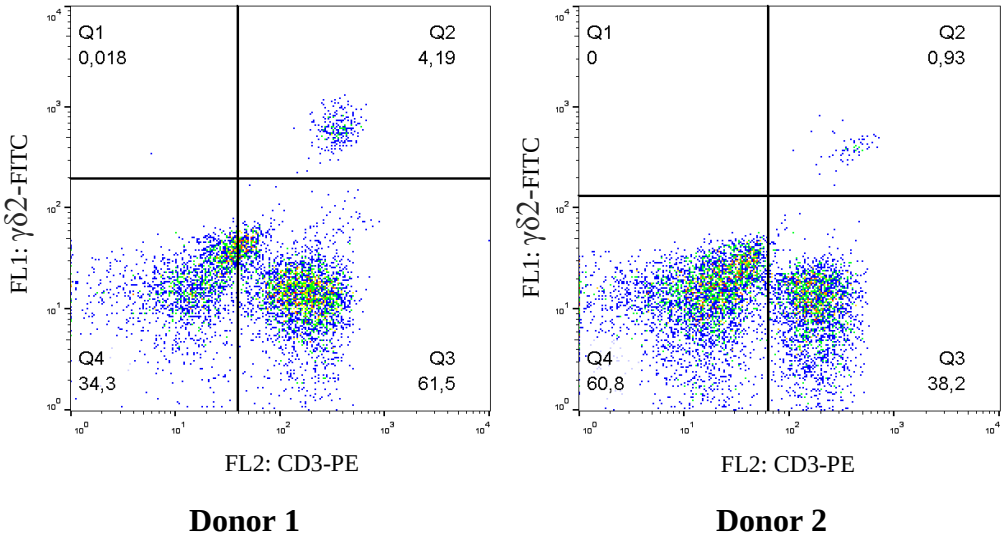


Figure 3: Dot plots of FL1 vs FL2 for two different donors with antibody stain and no wash.

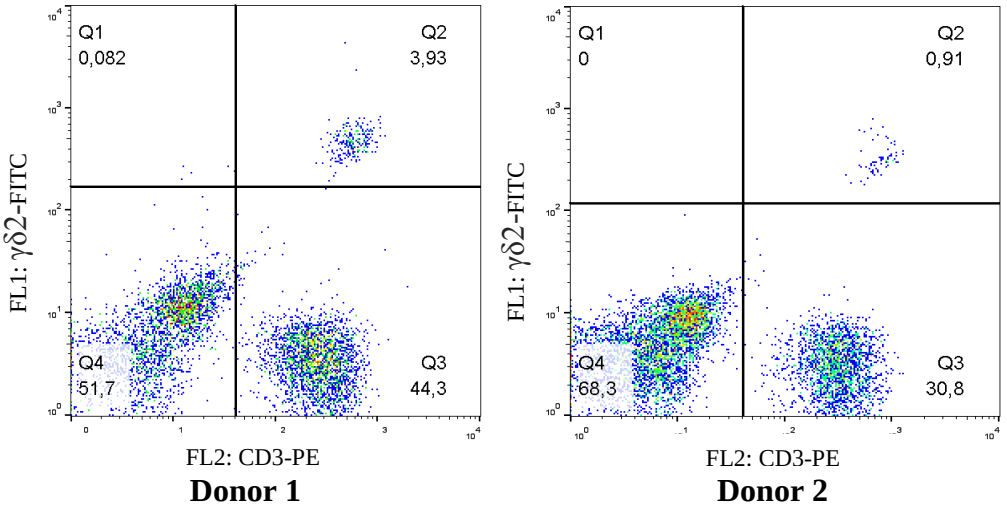


Figure 4: Dot plots of FL1 vs FL2 for two different donors with antibody stain and wash.

No clear separation between the CD3- and CD3+ populations was visible for either donor when no washing step was included (Figure 3), compared to the assay when a washing step was included after antibody staining (Figure 4). There was a decrease in ratios of PBMCs: beads in antibody staining with washes for both donors compared to no washes in antibody staining. This is due to the fact that there is a loss in both beads and PBMCs in the wash steps (previous data, table 4). Hence, no wash steps in antibody staining is feasible to avoid loss of PBMCs and beads. However, the dot plots show that there is a better separation of CD3+/CD3- populations and the γδ2+ sub populations in the discriminating FL2 vs FL1 channels for antibody staining with washes compared to antibody staining without washes. In addition, there is also a small tail that bleeds into the FL-2 channel on both antibody stains with wash and no wash samples.

With these analyzed data, the next step was to determine what causes the improper separation of $CD3^{+}\gamma\delta2^{-}/\gamma\delta2^{+}CD3^{-}$ T cells from the double negative populations ($CD3^{-}\ \gamma\delta2^{-}$) and the observed tail. In order to address this problem, various controls such as PBMCs and beads (no antibody stain), in phenol red complete growth media, non-stained PBMCs in phenol red free incomplete growth media (without human serum, L-Glutamine and antibiotics) and complete growth media with phenol red (no PBMCs) were run on the FACS to determine whether there is any fluorescence present in these controls. The representative dot plots are shown in Figure 5.

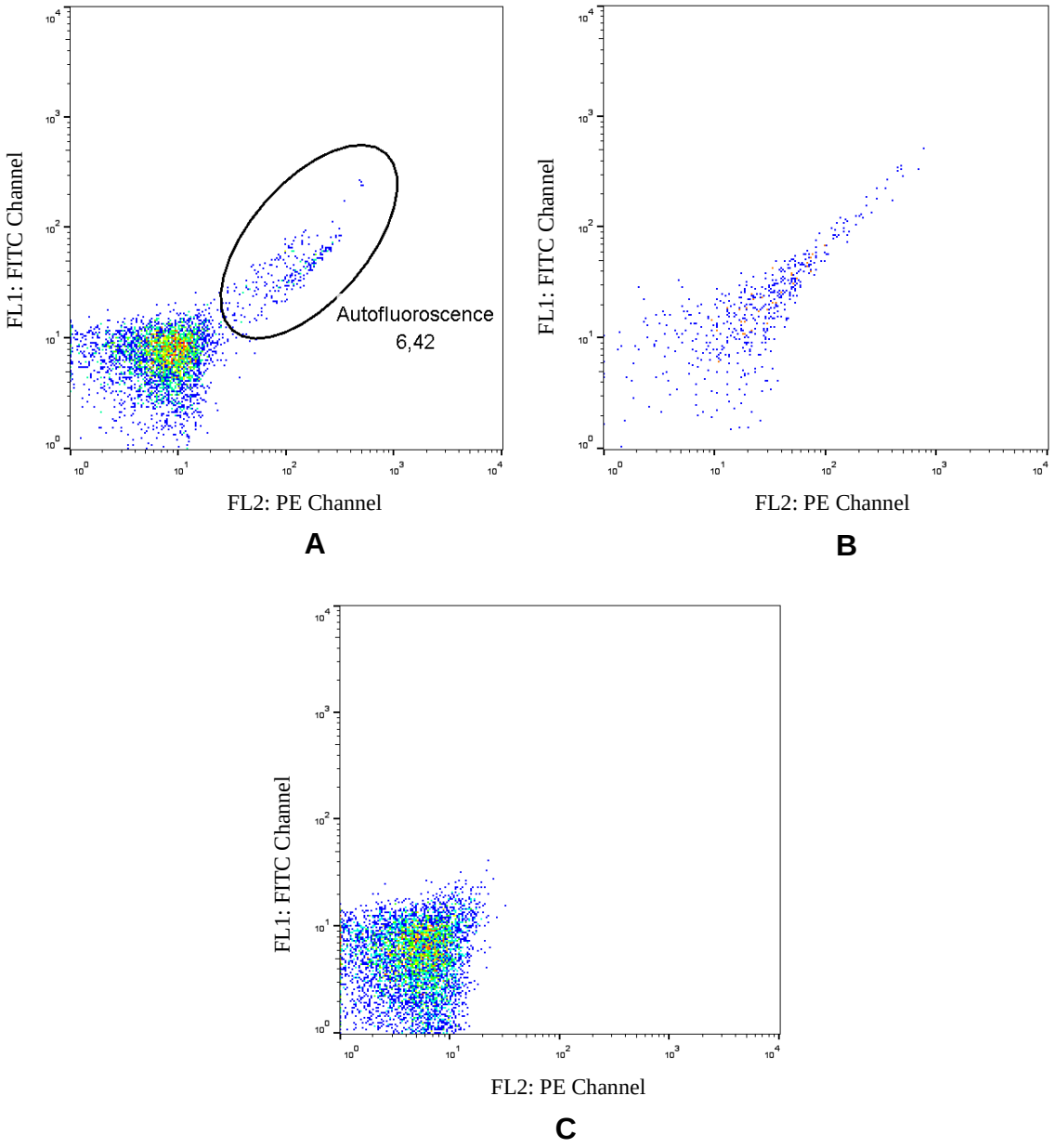


Figure 5: A. Dot plot of FL1 vs FL2 for PBMCs and beads (no stain) control. Circle is applied on the tail that bleeds into the FL2 channel and some parts of the double positive quadrant. B. Dot plot of FL1 vs FL2 for control complete growth media without PBMCs. A tail is clearly visible on both dot plots (A &B). C. Dot plot of FL1 vs FL2 for control PBMCs (no stain) with phenol red free incomplete growth media.

These data show that there is fluorescence present in the control tubes with phenol red, even without any antibody staining or cells (Figure 5A-B) compared to phenol red free incomplete growth media (5C). The complete growth media (RPMI-1640 with 25 mM HEPES+ 5% human serum+ 1% PenStrep+ 1% L-Glutamin) used to re-suspend the PBMCs have Phenol Red, which is a pH indicator of the cell culture medium. The phenol red dye has fluorescent properties that may interfere with target fluorophores of interest, which may end up causing autofluorescence [32]. Hence, we suggest that it is always best to use RPMI free of phenol red for PBMC and bead assays that do not involve any washes after antibody staining, in order to eliminate the background fluorescence interfering with PE and FITC fluorophores.

In addition to Phenol red media, other issues that arise with improper separation is also the fact that gating of lymphocytes, monocytes and some cells to the right in the dot plots of FSC vs SSC. Since, monocytes do not express CD3 markers it is not appropriate to gate the monocytes as this would only decrease the percentage of $CD3^+$ populations and also cause an improper separation between the $CD3^-$ and $CD3^+$ populations when gating the monocytes and other cells. Hence, the gating should only be on the lymphocyte populations in the dot plots of FSC vs SSC to generate better separation between the two populations.

Next, the assay was repeated with the same conditions (ab stain no wash) but, the isolated PBMC cell pellet was re-suspended in media that contained phenol red free RPMI without components of L-Glutamine, human serum and antibiotics (incomplete growth media). Furthermore, the gate was only set on lymphocytes in a dot plot of FSC vs SSC also to generate a better separation between $CD3^+$/$CD3^-$ populations and to evaluate the absolute counts of the lymphocytes in the PBMCs and PBMC subsets from different donors.

Absolute counts of PBMCs and PBMC subsets

The addition of CaliBrite PE beads at a known concentration to tubes containing PBMCs and antibody staining without any washes enabled the calculation of the absolute numbers of the PBMCs and PBMC subsets. Also, another factor to be considered is that there are no washes and hence, no loss of PBMCs and beads that need to be accounted for. The dot plots used for the absolute count calculations are shown in Figure 6.

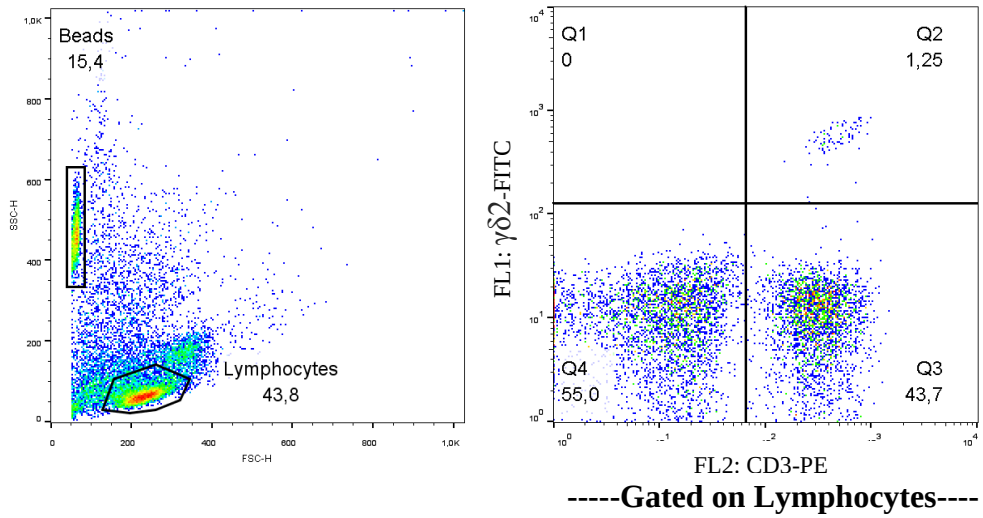


Figure 6: PBMCs and beads stained with antibodies without any washes. Gate is set on lymphocytes only. The total number of events acquired in FACS acquisition was 15,000 for data analysis. The known bead concentration in a 50uL bead suspension was 1.16 x 10^5 beads/mL

Calculation:

Lymphocyte Count = (6571/2304) x 1.16 X 10^5 beads/mL = 3.40 x 10^5 cells/mL

CD3+ T Lymphocyte Count = (2874/2304) x 1.16 X 10^5 beads/mL = 1.45 x 10^5 cells/mL

γδ2+ T cells Count = (82/2304 x 1.16) X 10^5 beads/mL = 4.13 x 10^3 cells/mL

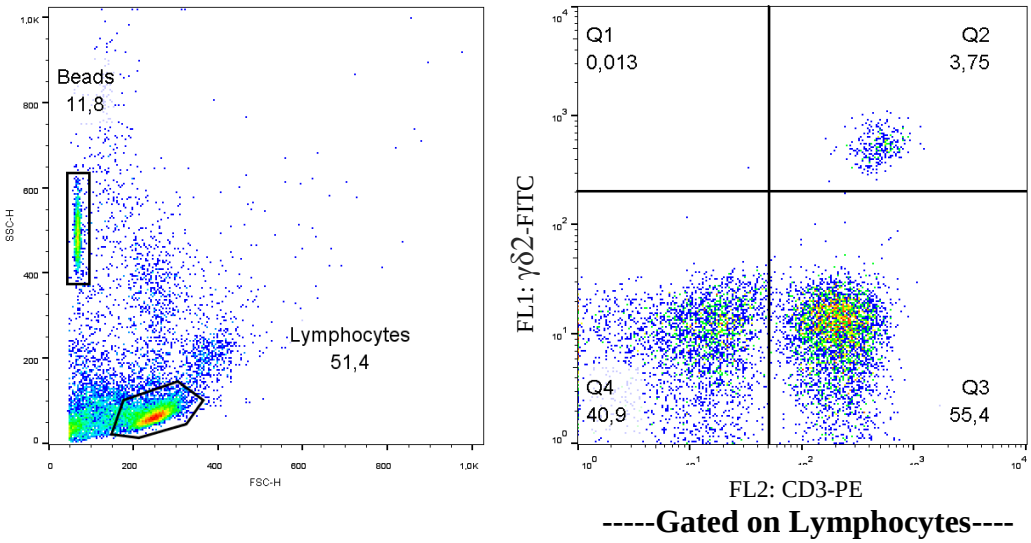
Since, there is no loss in the PBMCs and beads in antibody staining without any washes, by referencing bead counts we can estimate the counts of lymphocytes in the PBMCs, $CD3^+$ and $\gamma\delta2^+$ cell counts. The rationale behind the denominator (Bead events) and counting equations is that the PBMC and PBMC subset compositions are counted by referencing the known number of beads in FACS analysis. This is mainly done in FACS analysis to eliminate the conventional and time-consuming manual microscopy counting. There is also a better separation of the $CD3^+/CD3^-$ populations and the $\gamma\delta2^+$ populations in the FL2 vs FL1 channels for antibody staining without any washes with phenol red free incomplete growth media and lymphocytes only gating.

Reproducibility assay for absolute counts

The assay with antibody stain and no washes was repeated with the same conditions as described above with two different donors. Calculations of PBMC: bead ratios and absolute counts are shown in Table 5 and the dot plots are shown in Figure 7.

Table 5: The number of events, ratios of PBMCs: beads, and absolute counts for PBMCs, CD3+ T Lymphocytes and γδ2+ T cells for two donors. Bead count in a 50uL diluted bead suspension was 9.35 x 10^4 beads/mL.

Name	#Events	Ratio of Lymphocytes: beads	Absolute Counts
Dn1	15000	4.35	
Beads	1772		
PBMCs (lymphocytes only)	7709		406,767.00
CD3+ lymphocytes	4269		225,255.00
CD3- γδ2-	3150		N/A
γδ2+ T cells	289		15,249.00
Dn2	15000	2.64	
Beads	3228		
PBMCs (lymphocytes only)	8518		246,726.00
CD3+ lymphocytes	6424		186,073.00
CD3- γδ2-	1570		N/A
γδ2+ T cells	524		15,178.00



-----Gated on Lymphocytes----

Donor 1

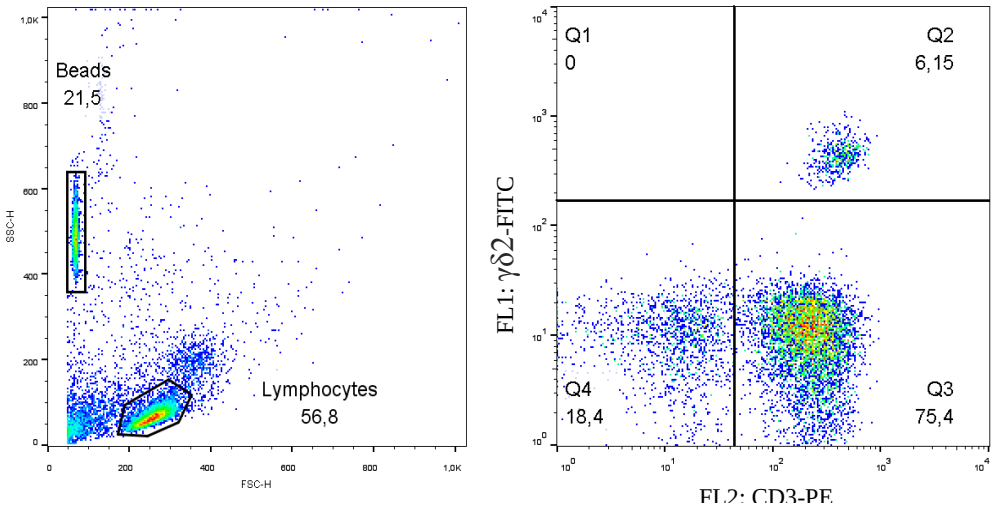


Figure 7: PBMCs and beads surface stained with antibodies without any washes for two different donors. Gates are set on lymphocytes only for both donors. The total number of events acquired in FACS acquisition was 15,000 for data analysis.

Hence, based on these data it is clear that there is better separation between the $CD3^-$ and $CD3^+$ populations for PBMCs re-suspended in phenol red free media (Figures 6 and 7) than in PBMCs re-suspended in phenol red media (Figure 3). However, for reproducibility assays only phenol red free media was used, and comparison experiments of media with phenol red were not performed simultaneously due to the limitation of PBMC isolations done on one day.

In an additional experiment for the PBMC and bead assays, PBMC suspension exposed to complete growth media with phenol red and complete growth media without phenol red on the day of isolation, and on day 4 was performed with antibody stain with washes and without washes to investigate the tail observed in Figures 3 and 5. However, a tail was observed for both conditions as a minor tail was seen on day 0 and a major tail was seen on day 4 during FACS analysis. This tail was further investigated using the controls phenol red free complete growth media and the same media stained with antibody. A major tail bleeding to the FL-2 channel was visible in FACS analysis for both controls with stain and without stain. Phenol red free "complete growth media" contains 5% human serum, 1% PenStrep and 1% L-Glutamine. Hence, it was noted that human serum and other components of the phenol red free complete growth medium are sources of autofluorescence. Complete growth media was used in this assay because the medium was suitable to keep the PBMCs alive until analysis after 4 days. Due to these effects of background fluorescence caused by the components of the "complete growth medium", the absolute count calculation of PBMCs and PBMC subsets was only limited to the day of isolation and were not evaluated in expansion and immunophenotyping assays, but only the percentages derived from FACS analysis were taken into consideration.

Various assays have been developed to estimate the absolute counts of PBMCs, NK and NK T cells. Kim et al., and Donnenberg et al., 2007 described a cell counting method by the use of flow count fluorospheres [1]. In their study, they state that fluorospheres were added to the

PBMCs after intracellular staining and washes prior to FACS acquisition. However, it is not as clear as to how the cell counts can be estimated if there are any PBMCs lost in the process of intracellular staining and washes. On the other hand, Oberg et al., 2014 used a plausible method to count the number of PBMCs and PBMC subsets by directly staining whole blood samples with BD TrueCount™ beads and MoAbs (CD45 PE-Cy7/γδ2-PE) by red blood cell lysis and FACS analysis [29]. On the other hand, our assay for absolute counts of CD3 T Lymphocytes and γδ2 T cells is simple and cost effective compared to the cited literature.

Expansion assay data analysis

FACS assays were performed to determine the proliferation index of the expanded PBMCs after 10 days in comparison to the day of PBMC isolation. The dot plots for monitoring the expansion of γδ2 T cells after 10 days (compared to day 0) are shown in Figure 8 and percentage total calculations for $CD3^+/\gamma\delta2^+$ T cells on day 0 and day 10 are shown in Table 6.

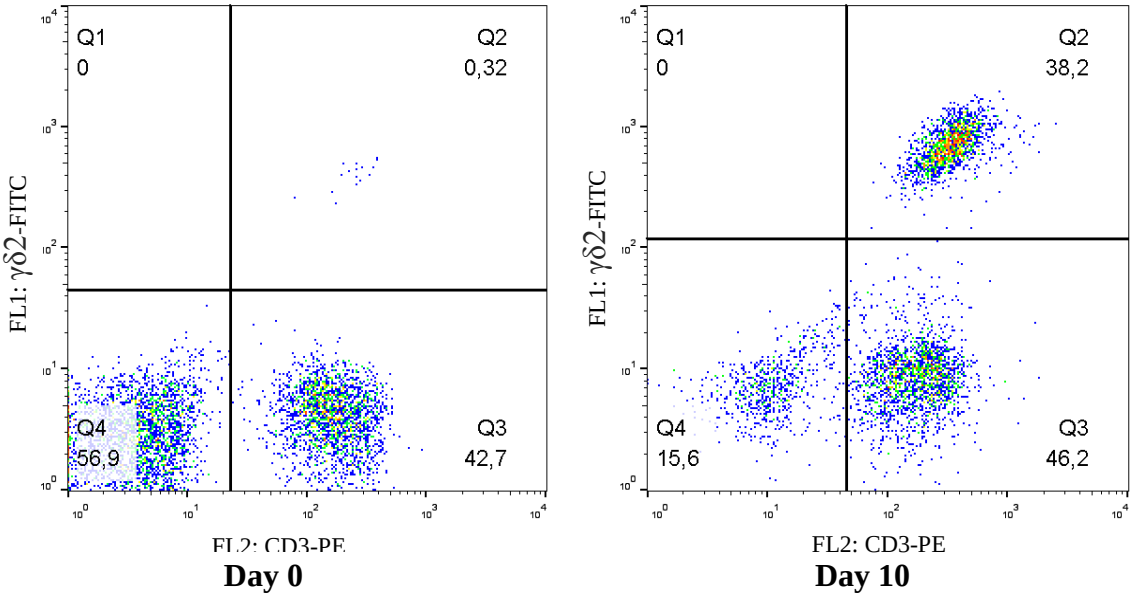


Figure 8: Representative dot plots of FL1 vs FL2 for isolated PBMCs from one donor on day 0 and expanded PBMCs on day 10. These cells were surface stained with antibodies and washed before FACS acquisition. Total number of events acquired was 10,000 events.

An increase in the population of $CD3^+\gamma\delta2^+$ T cells is clearly visible on day 10 in comparison to day 0 (Figure 8). The increase in the total $CD3^+\gamma\delta2^+$ T cell events and percentages are illustrated in Table 6 below.

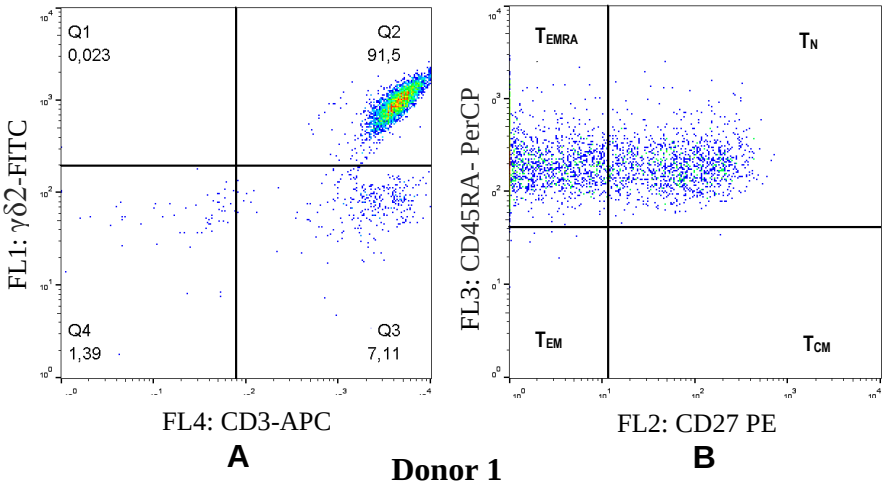
Table 6: The total number of events and percentage total of gated events for lymphocytes and γδ2+ T cells (highlighted in bold) for day 0 and day 10. A notable increase in the percentage of γδ2+ T cells (37.88%) on day 10 compared to day 0.

Name	# Events	Statistic (%)
Dn1_day 0	10000	
Lymphocytes	5278	52.8
CD3+ lymphocytes	2256	42.7
γδ2+ T cells	**17**	**0.32**
CD3-γδ2-	3005	56.9
Dn1_day 10	10000	
Lymphocytes	4312	43.1
CD3+ lymphocytes	1993	46.2
γδ2+ T cells	**1647**	**38.2**
CD3-γδ2-	672	15.6

Hence, γδ2+ T cells can be expanded when isolated PBMC suspensions with complete growth media are stimulated with HMB-PP and IL-2, and are incubated at 37 ^{O}C in an atmosphere of 5% CO_2 for 10 days. These data support the cited literature that states, the rapid and selective expansion of γδ2 T cells are triggered by phosphoantigens and cytokines [6].

Flow cytometry for immunophenotyping

Immunophenotyping assays were performed on isolated PBMCs, expanded PBMCs and purified γδ2 T cells to analyze the phenotypic differentiation of γδ2 T cells based on the cell surface markers CD27 and CD45RA. The four major subpopulations that these assays were looking for included Naïve ($CD27^+CD45RA^+$), Effector Memory ($CD27^-CD45RA^-$), Central Memory ($CD27^+CD45RA^-$) and Terminally Differentiated Effector Memory ($CD27^-CD45RA^+$). The data analysis for purified γδ2 T cells of expanded PBMCs from two donors are shown in Figure 9.



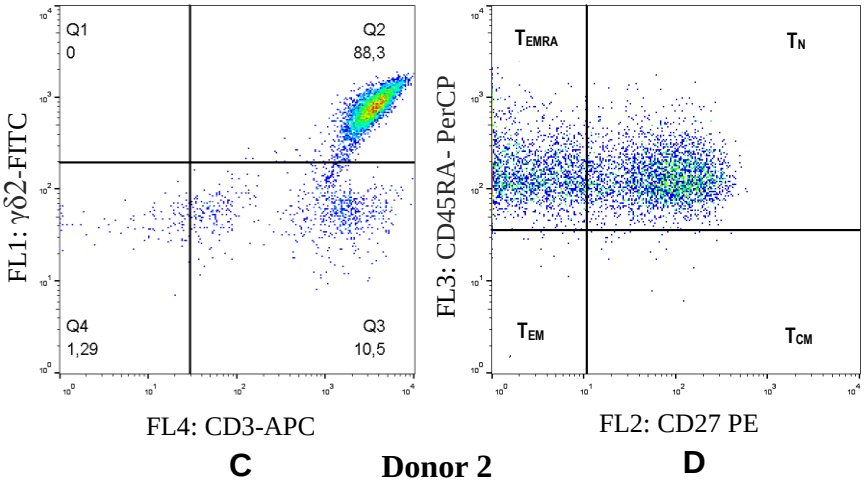


Figure 9: Analysis dot plots of γδ2 T cells with four color staining. FL1 vs FL4 dot plots shown for two donors (A, C). Based on the FL1 and FL4 double positive quadrant ($CD3^+$ $\gamma\delta2^+$), FL3 vs FL2 dot plots were analyzed (B, D) for both donors. A quadrant is set on a dot plot of FL3 vs FL2, to discriminate between the four different subpopulations of the $\gamma\delta2^+$ T cells. $CD45RA^+CD27^-$ (T_{EMRA}) events are displayed in the upper left quadrant and $CD45RA^+CD27^+$ (T_N) are displayed in the upper right quadrant.

Based on these data (Figure 9), it is notable that not all four phenotypes of γδ2 T cells could be detected. However, there are abundant populations of T_{EMRA} and T_N subsets and extremely low or no populations of T_{EM} and T_{CM} subsets expressed in γδ2 T cells for both donors. The spectral overlap between all four channels were manually compensated with the single color isotype control MoAbs and compensation was further validated with FMO staining controls. Hence, spectral overlap or improper separation are not probable causes for the results shown above. The specific cell surface markers used in this assay were CD3, γδ2, CD45RA and CD27 and not markers with expression of any natural killer receptor NKG2D and pattern recognition receptors [4, 5]. On the other hand, Dieli et al., 2003 and Caccamo et al., 2005 discussed the four subsets of γδ2 T cells are dependent upon inter-individual variability [9, 10]. The number of donors used in these assays were two donors and based on inter-individual variability of these donors, the subsets possibly could have not been identified.

Monitoring of effector cell activity

In order to identify and assess the cytotoxicity of effector cells by their activity in the presence of target cells, effector cells were co-incubated with target cells at four different *E:T ratios.* In Figure 10, γδ2 T cells are identified as CD69 (early activation marker) activated in the presence of HT-29 target cells. Hence, monoclonal antibodies specific for cell surface markers can be used in the flow cytometry based cytotoxicity assay to monitor the effector cell activity and function during the killing of target cells. The activity of $CD69^+$ was measured in the presence and absence of HT-29 targets and Mean Fluorescent Intensity (MFI) was obtained from the geometric mean in the FL-3 channel of $CD69^+$ gated cells in FACS data analysis. The representative bar graph with MFIs are shown below (Figure 10).

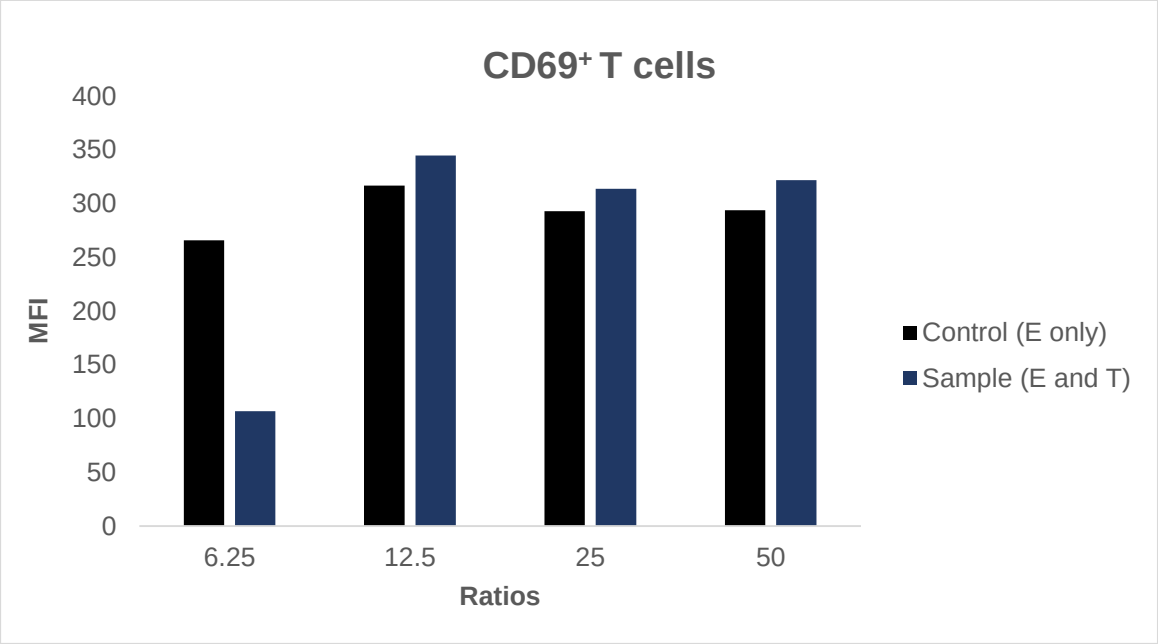
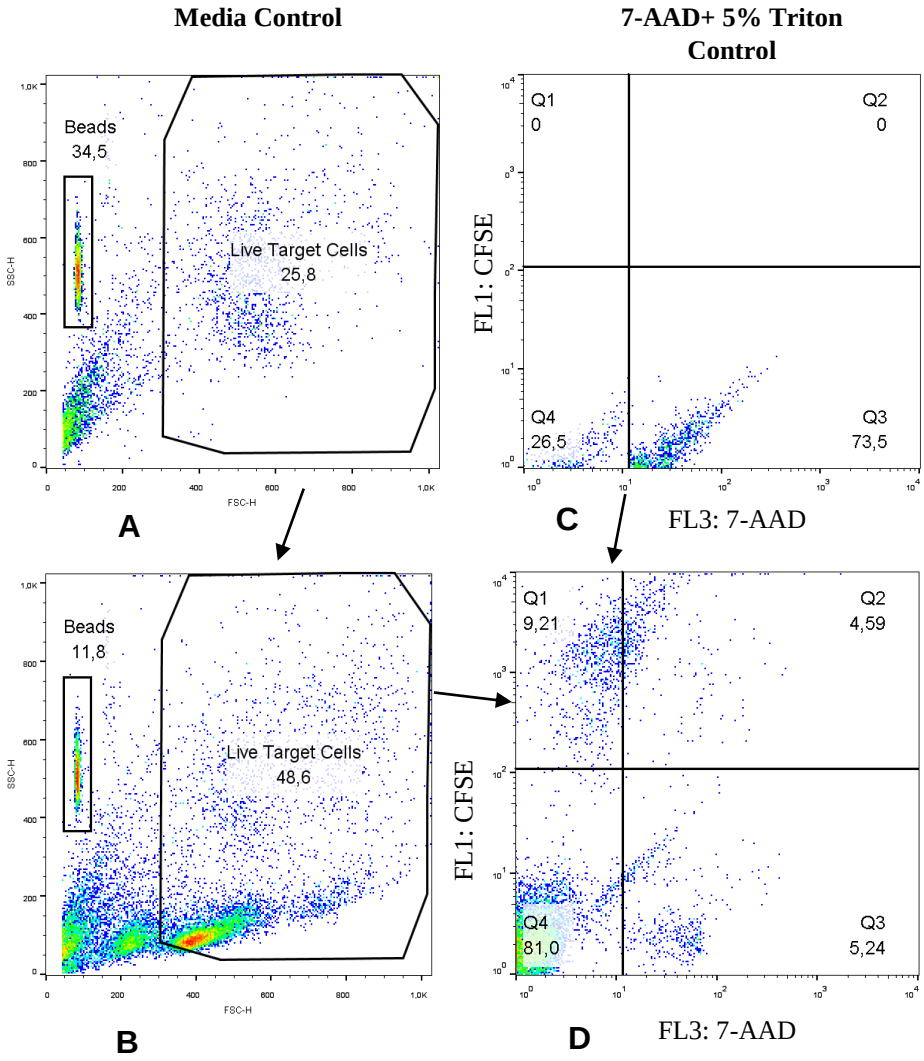


Figure 10: The MFIs for the CD69$^+$ T-cell activity in the presence and absence of target cells for four different ratios. Data represent the MFI for one experiment obtained from one donor performed in parallel with the cytotoxicity assay in separate FACS tubes. In here, γδ2 T cells are identified by their cell surface marker expression of CD69$^+$ T-cells. The controls (black) contain effector cells with ratios 6.25:0, 12.5:0, 25:0 and 50:0. The samples (blue) contain effector and target cell ratios of 6.25:1, 12.5:1, 25:1 and 50:1.

These data show that the MFIs of the effector cells have increased slightly for ratios 12.5, 25 and 50 at the presence of target cells. However, at a ratio of 6.25 there is a decrease in the MFI for sample compared to the control. This is an outlier in this experiment and possible reasoning behind this result could be errors caused by the incorrect addition of effector and target cells into tubes, lower number of events recorded at FACS acquisition and errors in FACS data analysis. The *E:T* ratios of 12.5:1 and 50:1 both have 9.52% increase in the MFI, whereas *E:T* ratio of 25:1 only has a 7.17% increase in the presence of target cells. Higher the concentration with lower volume of effector cells co-incubated with a constant concentration and volume of target cells, may have an higher impact on effector cells activated in the presence of targets, and thus have highest MFI values even at lower *E:T* ratios of 12.5:1 (Figure 10).

FCC assay data analysis and quantification of specific lysis

Flow cytometry based cytotoxicity (FCC) assays were performed to measure the cytotoxic activity of γδ2 T cells towards HT-29 targets. Target cells, were stained with Carboxyfluorescein succinimidyl ester (CFSE), prior to effector cell addition. CFSE is a fluorescent cell labeling dye which is retained within cells and covalently couples, via its succinimidyl group to intracellular lysine residues and other amine groups [30]. Also due to this stable linkage, once trapped within cells the CFSE dye is not transferred to effector cells when they are co-incubated. After the incubation 7-amino actinomycin D (7-AAD) is added in order for the uptake of dead target cells and enters the cytoplasm of apoptotic or necrotic cells [31]. The discrimination between the effectors and targets as described in the gating strategies in the materials and methods section are shown in the dot plots in Figure 11.



Example: Donor 1- *E:T* = 6.25:1

Figure 11: Gating strategies used for the FCC assay with representative dot plots. Target cells are stained with CFSE and dead target cells are 7-AAD positive. A. CFSE stained target cells incubated with media only for four hours. Gate set on live target cells on a dot plot of FSC vs SSC and applied to all dot plots of FSC vs SSC in samples with different ratios. B. Dot plot of FSC vs SSC for sample with *E:T* ratio 6.25:1. Beads are gated for the absolute count measurements and the total number of beads acquired were 2000. C. Quadrant applied to the 7-AAD$^+$ events on the lower right show maximum lysis with control 5% Triton on a dot plot of FL1 vs FL3. The same quadrant is applied to the sample dot plot (D), and CFSE$^+$/ 7-AAD$^-$ (upper left) events show remaining target cells. 7-AAD$^+$/ CFSE$^-$ (lower right) events show the dead target cells.

The absolute cell number and % specific lysis were calculated using the following formulas.

$$\text{Absolute cell number} = \frac{\text{CFSE}^+\ \text{7-AAD}^-\ \text{Events x actual bead counts}}{\text{Bead Events}}$$

$$\%\ \text{Specific Lysis} = \frac{\text{Live target cells in media control} - \text{Live target cells remaining in sample}}{\text{Live target cells in media control}}$$

Note: second formula is derived based on the absolute cell number calculations.

The calculations made based on above formulas are shown in Table 7.

Table 7: The $CFSE^{+}/7\text{-}AAD^{-}$ events and calculations for absolute number of remaining live targets in sample and % specific lysis are shown for different *E:T* ratios. The bead count was 2.0 x 10^4 beads/ mL. Absolute number of live targets in media control was 9720.

E:T ratios	$CFSE^{+}/7\text{-}AAD^{-}$ events for live targets in sample	Absolute number of live targets in sample	% Specific lysis
6.25:1	756	7560	22.22
12.5:1	703	7030	27.67
25.0:1	632	6320	34.98
50.0:1	523	5230	46.19

As the *E:T* ratios increase there is a gradual decline in the absolute number of remaining live targets in sample and increase in the percentage of specific lysis (Table 7).These results can be explained in terms of increase in the number of effector cells do in fact increase the % lysis of target cells. A line chart that shows the effects of % specific lysis tested on four different *E:T* ratios are shown in Figure 12.

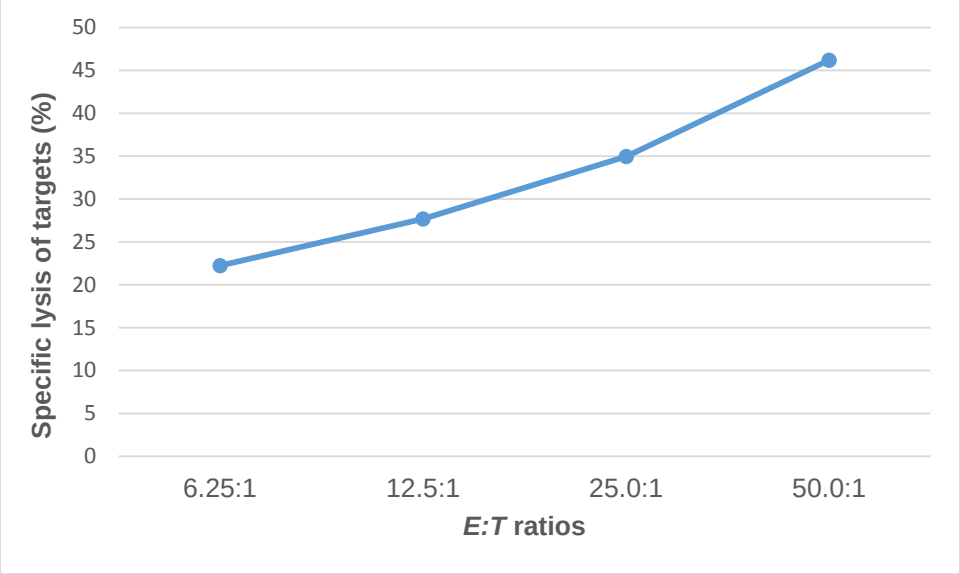


Figure 12: Specific lysis (%) measurements on four different *E:T* ratios using purified γδ2 T cells from expanded PBMCs as effectors and HT-29 as targets. Data obtained from one donor.

In this Flow cytometry based cytotoxicity assay, the number of live targets in media control and remaining live targets in sample were only measured for % specific lysis calculations. Media control contains the total number of targets stained with CFSE and incubated only with complete growth media. On the other hand, samples contain effectors co-incubated with target cells with

different *E:T* ratios. After the addition of effectors to targets, and the remaining number of live target cells as a result of effector cell addition, can provide evidence as to how many target cells have been lysed by the effector cells. Hence, the difference in the number of live targets in control and remaining live targets after effector cell addition divided by the number of live targets in media control (denominator) will give the % specific lysis impacted by effector cells. This is also a suitable measurement to estimate the efficiency of effector cell lysis when they are presented with target cells. Also, for these calculations $CFSE^{+}$/7-AAD^{-} events (Figure 11.D), are only taken into consideration.

Conclusions

There is a significant loss of both PBMCs and beads when they are surface stained and washed. Hence, the more feasible method for avoiding PBMC and bead loss is to perform the cell surface staining without any washes. In order for surface staining without washes to accurately work, the isolated PBMC pellet needs to be re-suspended in a media free of phenol red and other complete growth medium components to eliminate the background fluorescence interfering with target fluorophores of interest. Also, proper gating of lymphocyte populations is required to detect specific markers (CD3) that are only found in lymphocytes and not monocytes, to determine the absolute number of CD3 T lymphocytes and γδ2 T cells. A known bead concentration to add to the PBMC assay tubes, as a form of reference to calculate the absolute number of cells is the other most integral part of these assays. Hence, we have optimized a new bead and flow cytometry based technique to evaluate the counts of lymphocytes in PBMCs and PBMC subsets by the addition of cost effective fluorophore beads to PBMC assay tubes by cell surface staining without any washes.

The absolute count evaluations were not performed for proliferation index determination and immunophenotyping assays due to the components in the complete growth medium that were found to be sources of background fluorescence. The four subsets based on cell surface marker expression of CD45RA and CD27 phenotypes could not all be identified. A flow cytometry based cytotoxicity (FCC) assay has been completed by the detection of both effector cell activity ($CD69^{+}$) and cytotoxicity of γδ2 T cells in the presence of HT-29, target cells. The reproducibility and intra-assay variability of these FCC assays were not assessed. In order to develop and assess the full functionality of this assay, repeated experiments with different kinetics such as different *E:T* incubation times (4h-24h), different *E:T* ratios (10:1,75:1,100:1) and triplicate measurements from different donors should be performed.

Therefore, we have developed a flow cytometry and bead based assay to calculate the absolute number of γδ2 T cells in a mixed cell population and the development of flow cytometry based cytotoxicity assays are still in progress. Future research, should aim to include functional FCC assays that simultaneously detect the phenotypes of γδ2 T cells, viability of target cells and also the formation of target-effector cell conjugates. In addition, if there are current limitations in the use of color combinations for multi-color FCC assays by the use of FACS caliburs with 2 lasers that can only detect four colors, it would also be appropriate to use instruments such as LSR II with four lasers that can detect up to 18 colors.

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