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Human Immune Compartment Comparisons: Optimization of Proliferative Assays for Blood and Gut T Lymphocytes

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Abstract

The accumulation of peripheral blood late-differentiated memory CD8 T cells with features of replicative (cellular) senescence, including inability to proliferate *in vitro*, has been extensively studied. Importantly, the abundance of these cells is directly correlated with increased morbidity and mortality in older persons. Of note, peripheral blood contains only 2% of the total body lymphocyte population. By contrast, the gut-associated lymphoid tissue (GALT) is the most extensive lymphoid organ, housing up to 60% of total body lymphocytes, but has never been assessed with respect to senescence profiles. We report here the development of a method for measuring and comparing proliferative capacity of peripheral blood and gut colorectal mucosa-derived CD8 T cells. The protocol involves a 5-day culture of mononuclear leukocyte populations, from blood and gut colorectal mucosa respectively, labeled with 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) and 5-bromo-2′-deoxyuridine (BrdU) and stimulated with

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anti-CD2/3/28-linked microbeads. Variables tested and optimized as part of the protocol development include: mode of T cell stimulation, CFSE concentration, inclusion of a second proliferation marker, BrdU, culture duration, initial culture concentration, and inclusion of autologous irradiated feeder cells. Moving forward, this protocol demonstrates a significant advance in the ability of researchers to study compartment-specific differences of *in vitro* proliferative dynamics of CD8 T cells, as an indicator of replicative senescence and immunological aging. The study's two main novel contributions are (1) Optimization and adaptation of standard proliferative dynamics blood T cell protocols for T cells within the mucosal immune system. (2) Introduction of the novel technique of combining CFSE and BrdU staining to do so.

Keywords

replicative senescence; GALT; CD3 T cell; CD8 T cell; proliferation assay; mucosa

Introduction

Over the life-span there is an accumulation of terminally-differentiated memory CD8 T cells with features of replicative senescence. Importantly, the abundance of these cells has been shown to correlate with increased morbidity and mortality [1]. Senescent T cells are unable to enter cell cycle, lack CD28 expression, have shortened telomeres, and show enhanced secretion of proinflammatory cytokines. These observations reflect data derived from peripheral blood, which contains only approximately 2% of the total body lymphocyte population, whereas gut-associated lymphoid tissue (GALT) is the most extensive lymphoid organ in the body and houses up to 60% of total body lymphocytes [2, 3], yet has never been assessed with respect to CD8 T cell senescence. Enhanced understanding of this major immune compartment is particularly relevant, given the progressively increasing number of older persons within the U.S. population.

The immune compartments of blood and gut may, indeed, differ in their senescence trajectories, since T cells from the gastrointestinal (GI) tract are more differentiated and antigen-experienced. In fact, rodent studies show that age-associated alterations arise in the GI mucosal immune system earlier than in the peripheral immune compartment [4]. Similarly, in HIV-1 infection (which shows features of accelerated aging), the GI tract appears to be more severely affected than the blood during the acute phase of the infection and, subsequently, shows only moderate CD4 T cell recovery compared to the peripheral blood compartment, even when antiretroviral therapy restores blood counts [5]. However, reproducible and reliable methods to assess senescent phenotypes (profiles) and proliferative potential within the human gut have not been established.

To begin to address differences within the human blood and gut immune compartments, here, we provide a detailed description of a protocol for measuring and comparing proliferative dynamics of peripheral blood- and gut-derived (specifically, colorectal mucosa) T cells. This protocol paper represents an optimization of existing methods used to study proliferative dynamics of blood-derived T cells *in vitro* and will facilitate future comparison

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of compartment-specific differences between blood and colorectal mucosa CD8 T cells, in the context of senescence among other applications. Specifically, the study's two main novel contributions are (1) Optimization and adaptation of standard proliferative dynamics blood T cell protocols for T cells within the mucosal immune system. (2) Introduction of the novel technique of combining CFSE and BrdU staining to do so.

2. Methods

2.1 Study Subjects

This study was approved by the University of California, Los Angeles Medical Institutional Review Board and each participant provided written, informed consent per the approved protocol (UCLA IRB # 11-022238 and 11-001592). This report describes some of the data gathered from 43 participants recruited over 3–4 years to study the effects of age and chronic infection on immune senescence in the blood and gastrointestinal tract (specifically, colorectal mucosa) (AG032422; PI: Effros). The participants include 21 HIV-1 seropositives (HIV-SP) (aged 23–57, median age 41.0, 19 male and 2 female) and 22 HIV-1 seronegatives (HIV-SN) (aged 25–60, median age 42.9, 20 male and 2 female).

2.2 Collection of Peripheral Blood Mononuclear Cells (PBMC)

Human peripheral blood samples were acquired by standard venipuncture immediately prior to endoscopy; 70cc of peripheral blood for the proliferation and other assays were collected in seven 10ml Heparin tubes. PBMC designated for the proliferation assay were immediately isolated by Ficoll gradient separation. Following Ficoll centrifugation, PBMC were washed with 1X PBS and resuspended in 10ml culture media (1X RPMI 1640, 15% FBS, 10mM HEPES, 2 mM glutamine, 50 IU/ml penicillin/streptomycin, 500 μg/ml Zosyn [piperacillin-tazobactam], 1.25ug/ml amphotericin B). Viable PBMC concentration was calculated via trypan blue exclusion. Five million PBMC were removed and irradiated at 50 Gy to be used as an autologous irradiated feeder PBMC population. CD3 T cell count of the remaining PBMC were obtained using TRUCount™ beads (BD Biosciences, San Jose, CA), and 10×10^6 CD3 T cells were collected from PBMC for CFSE staining and culture.

2.3 Collection of Colorectal Mucosal (gut) Mononuclear Cells (MMC)

Mucosal biopsy samples were collected as previously described [6]. Briefly, rectosigmoid biopsies were endoscopically acquired by flexible sigmoidoscopy between 10cm and 30cm from the anal verge. Biopsies were obtained by the use of large cup endoscopic biopsy forceps (Microvasive Radial Jaw #1589, Boston Scientific, Natick, MA). At each biopsy procedure, 30 specimens were collected into two 50ml tubes containing 20–25ml of RPMI medium with 7.5% fetal calf serum (FCS) (R7.5), L-glutamine, amphotericin-B (1.25ug/ml) and piperacillin-tazobactam (50ug/ml). Samples were transported to the laboratory within 2 hours of collection. Upon receipt, the transport media was aspirated and biopsies incubated in 20–25ml RPMI/7.5% FCS containing 0.5 mg/ml collagenase type II-S (sterile filtered) (clostridiopeptidase A from *Clostridium histolyticum*, Cat. #C1764, Sigma-Aldrich, St. Louis, MO) for 30 min in a 37 °C water bath, with intermittent shaking. Tissue fragments were further disrupted by forcing the suspension five to six times through a 30-cm^3 disposable syringe attached to a blunt-ended 16-gauge needle (Stem Cell Technologies,

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Vancouver, BC). The entire suspension was then passed through a 70um sterile plastic strainer (Falcon # 352350) to remove free cells and concentrate the remaining tissue fragments. Free cells were immediately washed twice in R-7.5 medium to remove excess collagenase, and tissue fragments were returned to a 50-ml conical tube. The entire procedure, including 30-min collagenase incubations, was repeated two additional times until tissue fragments were no longer intact (~ 2–3 hours duration). The isolated mucosal mononuclear cells (MMC) were combined and resuspended in 5ml of RPMI medium containing 10% FCS, amphoterin-B (1.25ug/ml) and Zosyn (50ug/ml). Absolute CD3 T cell numbers of resuspended MMC were quantified using Trucount™ beads. Based on data derived from a total of 135 donors to date, (including the samples in this study), the average recovery of MMC from 30 biopsies was 6.2×10^6 CD3 T cells per donor, with a standard deviation of 3.5×10^6 and range of 1.6×10^6–25.0×10^6. For each donor, suspensions containing 2.0×10–6–3.0×10^6 CD3 T cells were collected for CFSE staining and culture.

2.4 CFSE Staining

CFSE staining was performed as previously described [7], with assay-specific modifications to determine the final CFSE concentration. Briefly, for each donor, aliquots of whole PBMC or MMC including 10×10^6 peripheral blood CD3 T cells, 5×10^6 irradiated PBMC feeders, and $2–3\times10^6$ mucosal CD3 T cells were separated into 15ml polypropylene tubes, washed in 5ml 1X PBS, centrifuged for 10 minutes, and pellets were resuspended in 1 ml 1X PBS. Diluted 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes; Eugene, OR) was added to the resuspended pellets in the following amounts: (i) Peripheral blood T cells (2.5 μM); (ii) Irradiated PBMC feeders (20.0 μM); (iii) Mucosal T cells (5.0 μM). Tubes were then incubated for 10 minutes at 37° C, washed twice (once in 10% pure FCS (9ml PBS 1X, 1ml FCS) and once in 1% pure FCS (9.9ml PBS 1X, 0.1 ml FCS)). CFSE- treated peripheral blood and mucosal samples were then resuspended in culture media (same as PBMC culture media) at 1×10^6 CD3 T cells/ml; irradiated PBMC feeders were resuspended at 0.5×10^6 cells/ml.

2.5 Cell Cultures

CFSE stained peripheral blood cells and mucosal cells were plated in 48-well flat-bottom culture plates (Corning, NY) in 1ml culture medium at a concentration of 1×10^6 CD3 T cells/ml. For each participant's samples, both stimulated and unstimulated control wells were included in all assays. All wells also included 0.5×10^6 irradiated (50 Gy) autologous PBMC feeder cells. Two additional wells, containing 0.5×10^6 stimulated and unstimulated irradiated feeder PBMC, respectively, were established as further controls to verify that no feeder PBMC were being included in the live CD3 T cell gate.

5 μl T cell activation microbeads (anti-CD2/3/28; Miltenyi Biotec; Auburn, CA) were added to each of the stimulated culture wells (0.5×10^6 microbeads/ml culture). For all conditions, 0.1 mg 5-bromo-2′-deoxyuridine (BrdU) (Becton Dickinson Immunocytometry Systems (BDIS); San Jose, CA), 0.1 μg Darunavir (NIH AIDS Reagent Program; Germantown, MD), and 25 IU rIL-2 (NIH; Germantown, MD) were added to each culture well prior to incubation with blood/gut T cell subsets.

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Cultures were incubated for 5 days at 37° C, 5% CO_2. On day 3, 0.5 ml culture medium was replaced with fresh medium, and 25 IU rIL-2 was added. On day 5, the entire contents of the 1ml cultures for PBMC, MMC, and irradiated feeder cells were collected for antibody staining and flow cytometry. As will be discussed in Section 3 below, given the limited starting cell numbers, several parameters of the above protocol were tested in a step-wise, staged effort in in order to optimize the cellular proliferation assays of blood and gut samples, the main goal of this paper. These parameters include stimulation method, initial cell concentration, culture kinetics, culture volume, and whether or not to include irradiated autologous feeders.

2.6 Trucount™ and Absolute T-Cell Counting

2.6.1 Day 1 Trucount™—To standardize the starting T-cell count for the proliferation assay, Trucount™ absolute counting was performed on MMC and PBMC. A volume of 20 µl of BD Multitest antibody cocktail (BD Biosciences, San Jose, CA) containing CD3-fluoroscein (FITC), CD8-phycoerythrin (PE), CD45-Peridinin Chlorophyll protein (PerCP), CD4-allophycocyanin (APC) and 50 ul of either the PBMC or MMC cell suspension were added to BD Trucount™ tubes, which contained lyophilized pellets of fluorescent beads. After a 15 minute incubation at room temperature in the absence of light, 450 ul of 1× BD FACS lysing solution was added, mixed and incubated for an additional 15 minutes. The stained cells were immediately analyzed on a BD FACSCalibur flow cytometer. Lyse/No-Wash settings on this instrument were determined each day using FACSComp software and BD Calibrite beads. The mucosal T-cell counts utilized a tight lymphocyte CD45 versus side scatter gate to help exclude debris that often contaminated the lymphocyte gate [6]. An additional T-cell gate was used to help exclude contaminating events that stained at a 45 degree angle as displayed in a CD4 versus CD8 bivariate plot. The 45 degree staining pattern indicated non-specific staining of dead cells and debris. As discussed in Section 2.6.3, initial experiments utilized 7AAD to confirm exclusion of dead cells. We were aware that a fraction of dead cells may have slipped though (as we were limited by number of fluorescent channels) but assumptions were made that the variable would be equally distributed about subject's samples and that population would not confound the subset of cells studied for proliferative potential. The level of debris was highly variable across samples. Back-gating the CD3 stained mucosal T-cells back through the CD45-versus-side scatter plot indicated that the vast majority of the T-cells (~95%) were included in our original CD45-versus- SSC gate [8, 9]. The PBMC did not contain excess debris, and gating was performed according to the manufacturer's procedures for whole blood analysis.

2.6.2 Day 5 Trucount™—The 5-day cultured cells were harvested, washed and resuspended in 200µl of PBS staining buffer (1X phosphate buffered saline with 2% heat-inactivated newborn calf serum). A 100 µl aliquot of cells was stained for 15 minutes at room temperature with saturating amounts of CD3-PE, CD4-APC and CD8-phycoerythrin-cyanine dye Cy7 tandem (PE-Cy7). The cells were washed once and resuspended in 500µl of staining buffer. The cells were transferred to Trucount tubes just prior to acquisition on a FACSCanto cytometer (BD).

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FACSCanto acquisition stop counters were initially set at 20,000 viable CD3 cells. A minimum of 3000 Trucount beads were collected for counting purposes. List mode files were collected and analyzed using FACSDiva software to determine the total T-cell counts for each culture condition. The voltage for the fluorescence detector used to measure CFSE was initially set so that the median fluorescence of the non-dividing T- cell population was positioned at channel 25,000. The instrument threshold was set at channel 200 of the CFSE detector to exclude the non CFSE-labeled debris; we used a forward scatter threshold to exclude small Trucount beads that could contribute to erroneous counts.

2.6.3 Absolute T-cell counting

Gating sequence: List mode data were first gated for viability, using a forward versus side scatter gate (Supp. Fig. 1i). During initial efforts to optimize this protocol for mucosal T cells, 7AAD was used to confirm exclusion of dead cells. Going forward, we relied on our initial gating strategy to eliminate the majority of debris/dead cells. Due to limited cell numbers and laser channels, a viability marker was purposefully not included in our protocol; however, in other studies/future iterations, it would be a beneficial, additional control. Next, the cells were gated using a CFSE-FITC versus CD3-PE display (Supp. Fig. 1ii). This gate excluded non T cells, feeder cells and additional contaminating non-viable T-cells. A CD4-APC versus CD8-PE-Cy7 display gated on the viable T-cells was then used to enumerate the CD4 and CD8 T cells (Supp. Fig. 1iii). Finally, a Trucount™ beads gate was set on beads with high fluorescence, using a plot of APC-CY7 versus PE-Cy7 (Supp. Fig. 1iv). The numbers of CD4, CD8, CD3 and Trucount™ beads were recorded for calculation of absolute counts following manufacturer's recommended protocol [(*T-cell count/Bead count) × (Total beads/100 µl cells) = Absolute T-cell count*].

2.7 BrdU staining and flow cytometry

A 100 µl aliquot (the leftover volume of the original 200 µl after 100 µl had been taken for Day 5 Trucount™ staining—See Section 2.6.2) of the remaining cells from each culture condition were used to detect the presence of BrdU in combination with CFSE measurements. The cells were processed using the BD Pharmingen APC BrdU Flow Kit following the manufacturer's single day staining procedure directions, with the modification that CD3-PE and CD8-PECy7 were added at the same time as the anti-BrdU-APC. A minimum of 10,000 lymphocytes with the light scatter qualities of viable cells were analyzed.

To establish suitable assay conditions for the proliferation of PBMC and MMC cultures, we analyzed bivariate plots of CFSE-FITC (x-axis) versus BrdU-APC (y-axis). Similar to Trucount™ gating (Supp. Fig. 1), cells were first gated for viability using a forward versus side scatter feed, followed by gating on CD3-PE versus CFSE-FITC, and then CD8 T cells were isolated using CD8-PE-Cy7 on viable CD3 T cells (Supp. Fig. 2i–iii). The scatter gate allowed exclusion of smaller cells with high side scatter, and the CD3 gate excluded CD3 positive feeder cells that were off-scale with respect to CFSE fluorescence. The quadrant markers of the bivariate plot were used to differentiate proliferating ($CFSE^{lo}BrdU^{+}$) cells from non-proliferating $CFSE^{hi}$ T-cells (Supp. Fig. 2iv).

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2.8 Statistical Analysis

All quantitative, matched pairs data were analyzed using a two-side, two-tailed Student's paired *t* test. *p* values <0.05 were considered significant.

3. Results

3.1 CFSE concentration

CFSE is an intracellular fluorescent dye that is often used to measure peripheral blood T lymphocyte proliferation in response to stimulation, both *in vitro* and *in vivo* [7, 10]. During labeling, CFSE is able to stably incorporate into cells via covalent coupling to intracellular proteins with a high fluorescence intensity, low variance, and low toxicity; following activation, intracellular CFSE concentration is halved with each cellular division [7, 11]. Thus, staining T cells with CFSE prior to culturing allows differentiation of non-divided ($CFSE^{hi}$) CD3 T cells from divided ($CFSE^{lo}$) CD3T cells, and calculation of the number of divisions undertaken by each cell, either visually or quantitatively, by CFSE dilution.

To distinguish proliferating and non-proliferating cells both from each other and from background, an optimal CFSE concentration is needed for pre-incubation. While we confirmed previously established efficacy of staining PBMC (using 10×10^6 CD3 T cells) with 2.5 μM CFSE [12] (data not shown), limitations in biopsy-derived gut (MMC) cell numbers existed (due to IRB-safety limits and need for concurrent other assays); thus, only $2–3 \times 10^6$ gut derived CD3 T cells from each subject were available for culture/proliferation assay. The efficacy of exposing $2–3 \times 10^6$ gut derived CD3 T cells prior to culture with the same 2.5 μM CFSE concentration used for PBMC was evaluated in MMC from two subjects (Fig. 1). Following stimulation with 5 μl anti-CD2/3/28 microbeads, results on Day 5 showed that while this CFSE concentration (2.5 μM) identified an undivided ($CFSE^{hi}$) CD3 T cell population, some $CFSE^{lo}$ CD3 T cells (presumably those with the highest number of cell divisions) had CFSE concentrations too low to be distinguished from background. Doubling the initial CFSE concentration in gut-derived cultures to 5.0 μM increased CFSE levels to a point where all dividing and non-dividing CD3 T cells were in a detectable range above background, with no apparent toxicity issues (Fig. 1, Table 1). Based on these results, our final protocol involved staining of 10×10^6 blood-derived CD3 T cells with 2.5 μM CFSE and $2–3\times10^6$ gut derived CD3 T with 5.0 μM CFSE for each 5 day culture.

3.2 BrdU as a second proliferation marker

Preliminary CFSE studies on T cells from the gut showed that, in many cases, rather than distinct CFSE peaks between proliferative generations, there was a single, broad population with decreasing CFSE intensity, presumably coinciding with cellular proliferation (Fig. 2A). Additionally, the demarcation between proliferating and non-proliferating populations was often difficult to determine (Fig. 2A). This phenomenon was variable, with some cultures exhibiting distinct peaks, and others broad peaks that were difficult to distinguish. To confirm that CFSE dilution was, in fact, due to cellular division, we elected to add BrdU, a thymidine analogue that is incorporated into the DNA of dividing cells, as a second proliferation labeling marker.

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Adding this second marker allowed us to more accurately differentiate and quantify non-dividing ($CFSE^{hi}BrdU^{-}$) from dividing ($CFSE^{lo}BrdU^{+}$) populations. Fig. 2B illustrates representative bivariate plots of day 5 (D5) cultures, including stimulated and unstimulated control wells, from a single donor for blood-derived and colorectal mucosa (gut) derived CD8 T cells.

Five day proliferation assays following stimulation with 5 µl anti-CD2/3/28 microbeads on blood and gut samples from 29 subjects were conducted using dual CFSE and BrdU staining. To assess the additive benefit of the second marker, we focused on the proportion of $CD8^{+}CFSE^{lo}BrDU^{-}$ cells, which would have been registered as proliferating had CFSE been the sole marker being used. If no intracellular BrdU was detected, these cells could now be confirmed as "non-proliferating". On day 5 in stimulated cultures, $CFSE^{lo}BrdU^{-}$ cells accounted for, on average, 3.3% of blood-derived and 9.8% of gut-derived $CD8^{+}$ T cells ($p<0.005$) (Fig. 2C). In the unstimulated control cultures from these same subjects, $CFSE^{lo}BrdU^{-}$ cells accounted for, on average, 3.3% of blood-derived and 7.8% of gut-derived $CD8^{+}$ T cells ($p<0.005$) (data not shown) These data indicate a high proportion of the $CFSE^{lo}BrdU^{-}$ population is not proliferating in response to microbead stimulation, as it is seen in both unstimulated and stimulated cultures. These observations underscored the importance of including BrdU, as a second stain, particularly in gut cultures, to help define true stimulation-induced proliferation and eliminate $CFSE^{lo}$ "false positive" proliferating cells.

3.3 Optimal mode of mucosal T cell stimulation to assess proliferative potential

Building on the prior methods above, three commonly used techniques to stimulate peripheral blood T cells were compared to evaluate stimulation of mucosal mononuclear cells (MMC) T cells: (1) Miltenyi anti-CD2/3/28 Ab beads (2) immobilized OKT3 + CD28 Ab and (3) bispecific CD3/CD4 Ab (selectively stimulates CD8 T cells) (see Fig 3B). On day 5 after Miltenyi bead stimulation (N=4), the percentages of live gated CD3+ cells that were activated ($CFSE^{lo}$) were 84%, 83%, 60% and 72% (mean: 75%), (Fig. 3A). Immobilized OKT3 was tested on MMC from three donors; only one donor's sample showed detectable proliferation, (17% of live gated cells (Fig. 3A). Bispecific Abs stimulation of MMC (N=2) showed 42% and 54% (mean: 48%) activated live gated CD3+ cells (Fig. 3A). In a side-by-side comparison of Miltenyi beads and bispecific Ab from the same 2 donors, Miltenyi bead stimulation resulted in a higher percentage of stimulated CD3+ cells (mean 66% vs. 48%, data not shown) and was selected for our protocol going forward. An additional advantage of the Miltenyi reagent over the bispecific (CD3/CD4) Ab is that the former resulted in both CD4 and CD8 T cell stimulation, whereas the latter selectively activated only the CD8 T cell subset (data not shown), consistent with previous experiments [13]. Although our current studies focus on the CD8 T cell subset, we felt it advantageous to have unbiased activation of both subsets both to mimic a more physiologically relevant environment and to allow for further future studies of CD4 T cell proliferative dynamics.

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3.4 Optimal bead:cell ratio

Miltenyi anti-CD2/3/28 Ab stimulation beads have been used previously in our studies on human peripheral blood CD3 T cells [14–16]. Following the manufacturer's recommendation of a 1:2 bead:cell ratio (Miltenyi T Cell Activation/Expansion Kit Protocol, #130-091-441), during initial assay development, we had evaluated bead:cell ratios of 1:2 and 1:4 in a volume of 1ml using gut-derived CD3 T cells from one donor. Results showed that stimulation ratios of 1:2 and 1:4 did not differ significantly in proliferative results between days 3 and 7 (Day 3 $CFSE^{lo}$ CD3 T cells: mean CFSE 9,818 for the 1:2 ratio vs. 10,423 for the 1:4 ratio MFI; Day 7: mean CFSE 643 for the 1:2 ratio vs. 567 for the 1:4 ratio MFI) (data not shown). After refining other parameters of the assay, we repeated our evaluation of whether different bead:cell ratios altered the percentage of quantified non-dividing MMC CD3 T cells. Three bead:cell ratios (1:4, 1:2, 1:1) were used on gut samples from the same donor. No significant differences were seen on day 5 post-stimulation; the percentage of non-dividing gut derived CD3 T cells was 4%, 3.5% and 3.2%, respectively; the percentage of dividing gut-derived CD3 T cells was 75.6%, 77.4% and 79.3%, respectively (Fig. 4). Based on these experimentally defined parameters over 7 day observation periods, subsequent method development retained the manufacturer's recommendation of a 1:2 bead:cell ratio for both PBMC and MMC.

3.5 Kinetics of the proliferative response

A time course experiment was performed to determine optimal culture duration for analysis of T cell proliferation of blood- and gut-derived T cells. Blood and gut samples from three donors were evaluated for proliferation parameters on days 3, 5 and 7. For blood-derived T cell cultures, T cell numbers and proliferative generations steadily increased in all three donors' cultures from Day 3 to Day 7 (data not shown), consistent with published data [14, 17].

The results for the gut-derived cultures were less uniform. We analyzed the CD3 T-cell number fold-change (defined as the number of live CD3 T cells/initial number of live CD3 T cells) at Day 3, 5 and 7 in cultures from 3 subjects. The peak mean fold-change occurred at Day 5 (0.47 fold-change) as compared to Day 3 (0.33 fold-change) and Day 7 (0.41 fold change) (Fig. 5A). Interestingly, on D5 of the corresponding blood-derived cultures, the mean T cell fold-change was 0.64 (data not shown), considerably higher than the mean T cell-fold-change of 0.47 from gut-derived cultures. This observation of peripheral blood cultures having a higher fold change in T cells on D5 is addressed further in the discussion.

Within just the CD8 T cell population, the average percentage of proliferating ($CFSE^{lo}BrdU^{+}$) cells levels off at Day 5 (Day 3, 37%; Day 5, 54%; Day 7, 56%) (Fig. 5B). In addition, there was high donor-to-donor variation in proliferative kinetics, especially from Day 5 to Day 7 (Fig. 5B), similar to that seen between Day 5 to 7 in the overall total CD3 T cell population. Based on the data from these initial experiments, day 5 was chosen as the optimal culture duration, as it (i) yielded the highest mean CD3 fold-change, (ii) was the time point at which the percentage of proliferating $CD8^{+}$ T cells plateau, (iii) showed less 'between subject' variability, and (iv) represented a time frame of uniform proliferative growth.

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3.6 Number of cells in the initial culture and inclusion of autologous irradiated feeder PBMC

Initiating cultures—Having established stimulation, labeling and culture duration parameters, we sought to optimize the initial culture size of CD3 T cells present in mononuclear cell suspension. We tested 2 different initial culture sizes, 0.5×10^6 T cells (N=13 donors) and 1.0×10^6 T cells (N=30 donors). An assay was determined to be technically 'successful' if it yielded a sufficient number of CD3 positive events in unstimulated and stimulated samples following BrdU treatment to produce viable proliferating and non-proliferating populations in 2 dimensional gating of CFSE vs. BrdU. Based on assessments of stimulated and unstimulated blood and gut samples from the 43 donors, this threshold was estimated to be approximately 1500 events. For blood-derived cultures, cell input of 0.5×10^6 and 1×10^6 CD3 T cells in 48-well flat bottom plates resulted in 100% assay success, as defined by the above criterion (data not shown). For gut, cultures starting with 0.5×10^6 T cells yielded a 54% success rate at Day 5 and cultures starting with 1×10^6 T cells yielded a 70% success rate (Data not shown). In unsuccessful cultures (i.e., <1500 events in BrdU tube), no viable CD3 T cell MMC populations were seen at Day 5 (reasons unknown). These data helped us define starting cultures as requiring 1.0×10^6 cells for PBMC and MMC.

Inclusion of feeders—In short-term cultures of blood-derived T cells, inclusion of irradiated autologous feeders was shown to facilitate T cell survival and proliferation [18, 19]; this procedure has not yet been tested for freshly acquired, gut tissue-derived cells. To determine whether feeders would enhance proliferative parameters in the culture conditions being used for this assay, we compared bead-stimulated PBMC and MMC cultures from 7 subjects, with and without 0.5×10^6 autologous irradiated feeder PBMC. Prior to culture, irradiated feeders were incubated for 10 minutes with a very high concentration of CFSE (20 μM CFSE per 5×10^6 feeder PBMC), so that they could be gated out during analysis on Day 5, due to off-scale high CFSE levels. For the blood-derived cultures, the ratio of surviving CD3 T cells at Day 5 with feeders versus non-feeders was 1.14 (N=7) in the stimulated fraction, and for gut derived cultures the ratio was 1.05 (n=7) (Supp. Fig. 3A). Despite only modest enhancements in cell viability, we chose to include 0.5×10^6 autologous irradiated PBMC feeder cells stained with 20.0 μM CFSE per 5×10^6 feeder cells in our assay.

To ensure that CD3 positive feeders were not being included in our D5 analysis, for each donor, we included two feeder cell control wells, one bead-stimulated and one non-stimulated, to confirm that no feeders were present in the CD3 CFSE gate. This approach was taken for every culture as part of the D5 TRUcount portion of the protocol (Section 2.6.3, Supp. Fig. 1ii). Supp. Fig. 3B is a representative bivariate plot of unstimulated and stimulated feeder control wells gated on markers CFSE-FITC vs. CD3-PE, confirming that irradiated feeders labeled with off-scale high CFSE are not entering the live CD3 CFSE gate. Overall, given the only modest enhancement seen, in large scale studies it may be more efficient to not include feeders, particularly since their use necessitates extra gating steps during flow cytometry.

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Note: During pilot studies, in addition to tests labeling feeders with high (20 μM) levels of CFSE, we also tested an additional feeder stain (Claret). These tests demonstrated no surviving CD3 positive feeders were included in D5 analysis and there was no toxicity to non-feeder CD3 cells following inclusion of 5×10^6 irradiated, autologous feeders stained with 20 μM CFSE (data not shown). Given that there was no benefit in using the Claret stain as opposed to the higher CSFE concentration, we opted to utilize the latter approach in order to minimize complexity during D5 flow cytometric analysis.

4. Discussion

One critical gap in our ability to more fully characterize the effects of aging on the human immune system is the absence of a reproducible method to evaluate proliferative dynamics of human peripheral blood- and gut-derived lymphocyte populations. Here, we provide the first detailed protocol for evaluating the proliferative potential of T cells from the human gut, the largest lymphoid organ in the body. The protocol was optimized for freshly-acquired, biopsy-derived human mucosal T cells using existing proliferation assays for peripheral blood immune cells. The protocol was developed in a necessarily sequential, iterative, evolving manner, using limited number of mucosal cells, addressing the following issues: stimulation methods, labeling parameters, initial cell concentrations, culture kinetics, culture size, and whether or not to include irradiated autologous feeders. The current protocol and similar assays will be critical in future compartment comparisons between blood and intestinal tissue, for example, in studies on aging or autoimmunity. Moreover, given emerging data on the importance of individual variations within the gut microbiome, evaluation of the mucosal immune responses within the gut (compared to the less-reactive blood compartment) will become increasingly important clinically [20, 21].

Our optimization of the proliferation assay required modification of the usual CSFE protocol, since the demarcation between proliferating and non-proliferating CD3 T cell populations was often difficult to determine in mucosal cultures using CFSE alone. The method of simultaneous staining with both CFSE and BrdU emerged, in part, from an earlier kinetic experiment examining T cell turnover *in vitro* using CFSE and BrdU as proliferation markers [11]. Although that study did not actually employ both markers simultaneously, it did include side-by-side comparisons to establish the relative precision of each method and the concentrations that were non-toxic to T-cells [11]. Adding BrdU simultaneously with CFSE has proven to be an invaluable tool in differentiating T-cell populations with low CFSE that is due to cellular division, as opposed to poor staining or other unknown reasons.

Interestingly, utilizing CFSE and BrdU dual staining, we observed that on Day 5 cultures, gut mucosa-derived cells consistently had a much higher $CFSE^{lo}BrdU^{-}$ “false positive” population (so named because these cells would be regarded as proliferating if using CFSE alone) than blood-derived cells. This was true in both stimulated and unstimulated cultures. Gut-mucosa-derived cells also had significantly greater variance in the frequency of $CFSE^{lo}BrdU^{-}$ cells in both stimulated and unstimulated cultures (As illustrated in Fig. 2C). It is unknown why this “false positive” population is more prevalent and variable in colorectal mucosa-derived cultures as compared to than blood-derived cultures from the same donor. Possible explanations meriting further study include increased ‘between

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subject' variability in MMC versus PBMC. An additional contributory factor may be stimulation and/or relative hypoxia/activation effects related to MMC sample preparations (several hours). Importantly, as this "false positive" population is similar in both stimulated and unstimulated blood and gut- derived cultures, the findings suggest that CD3 downregulation upon activation is not a major concern in either compartment; if it were, one would expect to see greater discrepancies.

As mucosal-derived cultures have neither high cell yields nor consistently tight, CFSE proliferation bands, it was critical to verify the technical reliability of each experiment in order to confidently interpret the data. Initially, we were concerned that in cultures with poor quantitative CD3 yields at day 5, the number of live CFSE/BrdU stained T cells might be insufficient to accurately measure proliferative dynamics. We therefore set an arbitrary threshold where data would only be valid if there were greater than 1500 CD3+ T cell events in stimulated and unstimulated BrdU-treated aliquots at day 5 following staining and flow cytometry. We chose this threshold after analyzing several donors' cultures and determining 1500 CD3+ events was the lowest threshold which could definitively identify viable populations of proliferating and non-proliferating cells.

In developing this protocol, we also carefully considered the various modes of T cell stimulation. Previous work stimulating and culturing mucosal T cells has involved use of a CD3:CD4 (blocking) bispecific antibody to preferentially stimulate CD8 T cells [6, 13], mainly for HIV research. Although we considered and initially tested bispecific antibodies, we sought to optimize a protocol that would stimulate all CD3+ T cells, to better duplicate physiological conditions and also to allow study of proliferation dynamics of multiple T cell subsets. We intentionally avoided the use of peptide antigens from persistent viruses known to be present in most of the adult population, since a substantial portion of human T cells that are specific for these viruses are poorly proliferative [22]. In addition, in order to evaluate the global proliferative potential of CD3 T-cells, we intentionally focused on three activation methods that stimulate via the T cell receptor. We ultimately selected Miltenyi beads, as this method resulted in the highest number of live gated T-cells and highest % of proliferating T-cells at day 5, especially for the mucosal cultures. Additionally, in contrast to bi-specific antibodies, this stimulation method demonstrated no bias towards proliferation of CD4 or CD8 T cells at day 5.

Interestingly, we found that concentrations of OKT3+CD28 Ab (1 μg/ml OKT3, 20 μg/ml CD28 Ab) that are routinely employed for peripheral blood T cell activation failed to adequately stimulate MMC. A possible reason for this is that residual cellular debris in the mucosal suspension interferes with ligand binding in this system. Alternatively, the relative concentration of available anti T cell antibody may be greater in the bead-conjugated vs. the plate-bound form at concentrations tested. A final point that may be relevant is that the experiments comparing different stimulation methods incorporated only CFSE, as opposed to CFSE *and* BrdU. Therefore, it is possible these experiments slightly *overestimatde* final percentage of proliferating cells, as they include cells that would be $CFSE^{lo}BrdU^{-}$ under the finalized protocol.

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An essential feature of our protocol was the inclusion of unstimulated wells, which were critical for both quality control and for determining culture viability. The CSFE fluorescence distribution of the unstimulated cells was important to verify satisfactory loading of the cells, and also as a guide for determining the fluorescence associated with non-proliferating cells. Interestingly, based on both Trucount™ and BrdU assays, unstimulated mucosal T cell cultures consistently contained a cluster of proliferating T cells which was only rarely seen in the blood-derived T cell. Fig. 2B illustrates this observation, as the blood-derived unstimulated control well has 0.5% proliferating CD8 T cells ($CFSE^{lo}BrdU^{+}$) and the gut-derived has 8.5%.. This observation is consistent with previous studies indicating mucosal T cells are generally in a more activated state than peripheral blood T cells [23, 24].

In development of this protocol we utilized mononuclear cells taken from HIV SN and HIV SP subjects. Although HIV seropositivity is not relevant to the methodological description, one of our intentions moving forward is to utilize this protocol to study blood and gut T cell proliferative dynamics in the context of HIV disease. Indeed, HIV seropositivity itself is often used as a model to study chronic antigenic stimulation and the effect of such stimulation on proliferative potential, among other parameters of immunosenescence [16, 25, 26]. Therefore, we wanted to validate that the methodology worked on blood and gut T cells from HIV SN and SP subjects. Generally, peripheral blood and gut T cells from HIV SN and SP cells had equivalent outcomes at D5 utilizing the optimized protocol outlined in this manuscript (data not shown). Further studies ongoing in our laboratory will focus on comparing proliferative dynamics of blood versus gut cells in HIV SN *and* SP persons, to determine what effect HIV infection has (if any) on proliferation.

In the course of our assay development, we made several unexpected observations. First, CSFE-stained stimulated gut mucosal T cells did not routinely exhibit the tight, divisional bands seen for peripheral blood, but, rather, often showed broadly overlapping histograms, where divisions could not be qualitatively judged. One possible explanation for this observation might be variable protein carryover in the mucosal suspension after tissue digestion, which could potentially interfere with CFSE uptake, resulting in high variability of CFSE content per cell. We also found that the apparently low CD3 T cell yield on day 5 (often below 1.00, even in activated culture, indicating loss of live CD3 cells) following stimulation was due, in part, to an overestimate of the day 0 starting cell number, which had been markedly reduced during the extensive CSFE staining/washing procedure. Importantly, however, we determined the cell loss due to CSFE washes (~50% cells lost) was equivalent for blood- and gut-derived samples, as determined by a day 0 post staining/washing CD3 T cell Trucount™ on 2 donors (data not shown). Finally, we noticed that within each subject, the 5-day live T cell count of the gut is significantly less than that of the blood. Indeed, blood-derived cultures had almost three-fold the live CD3 T cells as gut-derived cultures (average fold-change 0.94 vs. 0.36, $p<0.005$, data not shown) This observation suggests a fundamental difference in the two compartments that may reflect functional differences related to the respective compartments *in vivo*. Indeed, other projects underway in our laboratory comparing blood and gut T cells, utilizing this protocol and phenotypic analyses, indicate gut cells are generally more antigen experienced and differentiated, which could explain why gut cells quantitatively have lower cell yields following activation.

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In sum, this report outlines the first detailed protocol for evaluating proliferative dynamics of blood versus colorectal mucosal T cells *in vitro*, optimizing previous methods developed for blood derived cells. Additionally, although our focus is CD8 T cells, since this protocol involves global stimulation of CD3+ T cells, it allows for analysis of proliferative dynamics of CD4 T cells as well. Ultimately, this assay, along with additional functional and phenotypic tests, will allow immunologists and clinicians to better understand how a variety of disease processes (e.g., aging, chronic HIV infection) affect immune performance in this vital immune organ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

GALT	gut-associated lymphoid tissue
PBMC	peripheral blood mononuclear cells
MMC	mucosal mononuclear cells
CSFE	5-(and 6)-carboxyfluorescein diacetate succinimidyl ester
BrdU	5-bromo-2′-deoxyuridine

Literature Cited

1. Dock JN, Effros RB. Role of CD8 T Cell Replicative Senescence in Human Aging and in HIV-mediated Immunosenescence. Aging and disease. 2011; 2(5):382–397. [PubMed: 22308228]
2. Banerjee M, et al. Immunohistochemical analysis of ageing human B and T cell populations reveals an age-related decline of CD8 T cells in spleen but not gut-associated lymphoid tissue (GALT). Mechanisms of ageing and development. 2000; 115(1–2):85–99. [PubMed: 10854631]
3. Mowat AM, Viney JL. The anatomical basis of intestinal immunity. Immunological reviews. 1997; 156:145–66. [PubMed: 9176706]
4. Koga T, et al. Evidence for early aging in the mucosal immune system. Journal of immunology. 2000; 165(9):5352–9.
5. Shacklett BL, Anton PA. HIV Infection and Gut Mucosal Immune Function: Updates on Pathogenesis with Implications for Management and Intervention. Current infectious disease reports. 2010; 12(1):19–27. [PubMed: 20174448]
6. Shacklett BL, et al. Optimization of methods to assess human mucosal T-cell responses to HIV infection. Journal of immunological methods. 2003; 279(1–2):17–31. [PubMed: 12969544]
7. Parish, CR., et al. Use of the intracellular fluorescent dye CFSE to monitor lymphocyte migration and proliferation. Coligan, John E., et al., editors. 2009. Current protocols in immunologyChapter 4: p. Unit4 9

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8. Detrick, B., Hamilton, RG., Folds, JD. Manual of molecular and clinical laboratory immunology. 7th. Washington, D.C.: ASM Press; 2006. p. xxvip. 1340
9. Loken MR, et al. Establishing optimal lymphocyte gates for immunophenotyping by flow cytometry. Cytometry. 1990; 11(4):453–9. [PubMed: 1693112]
10. Gett AV, Hodgkin PD. A cellular calculus for signal integration by T cells. Nature immunology. 2000; 1(3):239–44. [PubMed: 10973282]
11. De Boer RJ, Perelson AS. Quantifying T lymphocyte turnover. Journal of theoretical biology. 2013; 327:45–87. [PubMed: 23313150]
12. Newton P, et al. T cell extravasation: demonstration of synergy between activation of CXCR3 and the T cell receptor. Molecular immunology. 2009; 47(2–3):485–92. [PubMed: 19767105]
13. Yang OO, et al. Differential blood and mucosal immune responses against an HIV-1 vaccine administered via inguinal or deltoid injection. PloS one. 2014; 9(2):e88621. [PubMed: 24558403]
14. Parish ST, Wu JE, Effros RB. Sustained CD28 expression delays multiple features of replicative senescence in human CD8 T lymphocytes. Journal of clinical immunology. 2010; 30(6):798–805. [PubMed: 20721608]
15. Graham LS, et al. Oxidized lipids enhance RANKL production by T lymphocytes: implications for lipid-induced bone loss. Clinical immunology. 2009; 133(2):265–75. [PubMed: 19699688]
16. Chou JP, et al. Accelerated aging in HIV/AIDS: novel biomarkers of senescent human CD8+ T cells. PloS one. 2013; 8(5):e64702. [PubMed: 23717651]
17. Fauce SR, et al. Telomerase-based pharmacologic enhancement of antiviral function of human CD8+ T lymphocytes. Journal of immunology. 2008; 181(10):7400–6.
18. Puck TT, Marcus PI. A Rapid Method for Viable Cell Titration and Clone Production with Hela Cells in Tissue Culture: The Use of X-Irradiated Cells to Supply Conditioning Factors. Proceedings of the National Academy of Sciences of the United States of America. 1955; 41(7): 432–7. [PubMed: 16589695]
19. Amel Kashipaz MR, et al. Human autologous mixed lymphocyte reaction as an in vitro model for autoreactivity to apoptotic antigens. Immunology. 2002; 107(3):358–65. [PubMed: 12423312]
20. Ahern PP, Faith JJ, Gordon JI. Mining the Human Gut Microbiota for Effector Strains that Shape the Immune System. Immunity. 2014; 40(6):815–823. [PubMed: 24950201]
21. Alexander KL, Targan SR, Elson CO 3rd. Microbiota activation and regulation of innate and adaptive immunity. Immunological reviews. 2014; 260(1):206–20. [PubMed: 24942691]
22. Appay V, et al. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nature medicine. 2002; 8(4):379–85.
23. Schieferdecker HL, et al. T cell differentiation antigens on lymphocytes in the human intestinal lamina propria. Journal of immunology. 1992; 149(8):2816–22.
24. Poles MA, et al. A preponderance of CCR5(+) CXCR4(+) mononuclear cells enhances gastrointestinal mucosal susceptibility to human immunodeficiency virus type 1 infection. Journal of virology. 2001; 75(18):8390–9. [PubMed: 11507184]
25. Kalayjian RC, et al. Age-related immune dysfunction in health and in human immunodeficiency virus (HIV) disease: association of age and HIV infection with naive CD8+ cell depletion, reduced expression of CD28 on CD8+ cells, and reduced thymic volumes. The Journal of infectious diseases. 2003; 187(12):1924–33. [PubMed: 12792869]
26. Papagno L, et al. Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection. PLoS biology. 2004; 2(2):E20. [PubMed: 14966528]

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Highlights

- Optimization and adaptation of standard proliferative dynamics blood T cell protocols for T cells within the mucosal immune system.
- Introduction of the novel technique of combining CFSE and BrdU staining to do so.
- This method will allow study of compartment-specific differences in T cell subsets
- Importance of these comparisons in the context of T cell replicative senescence and associated morbidities.

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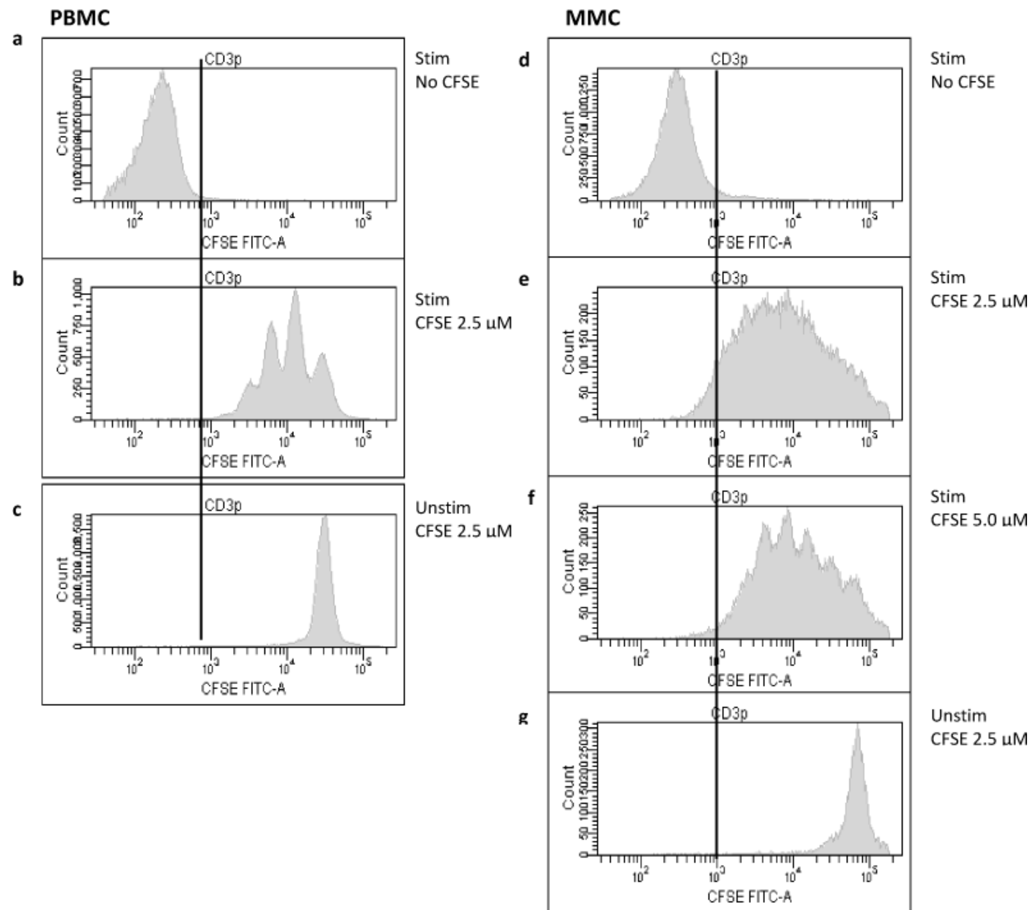
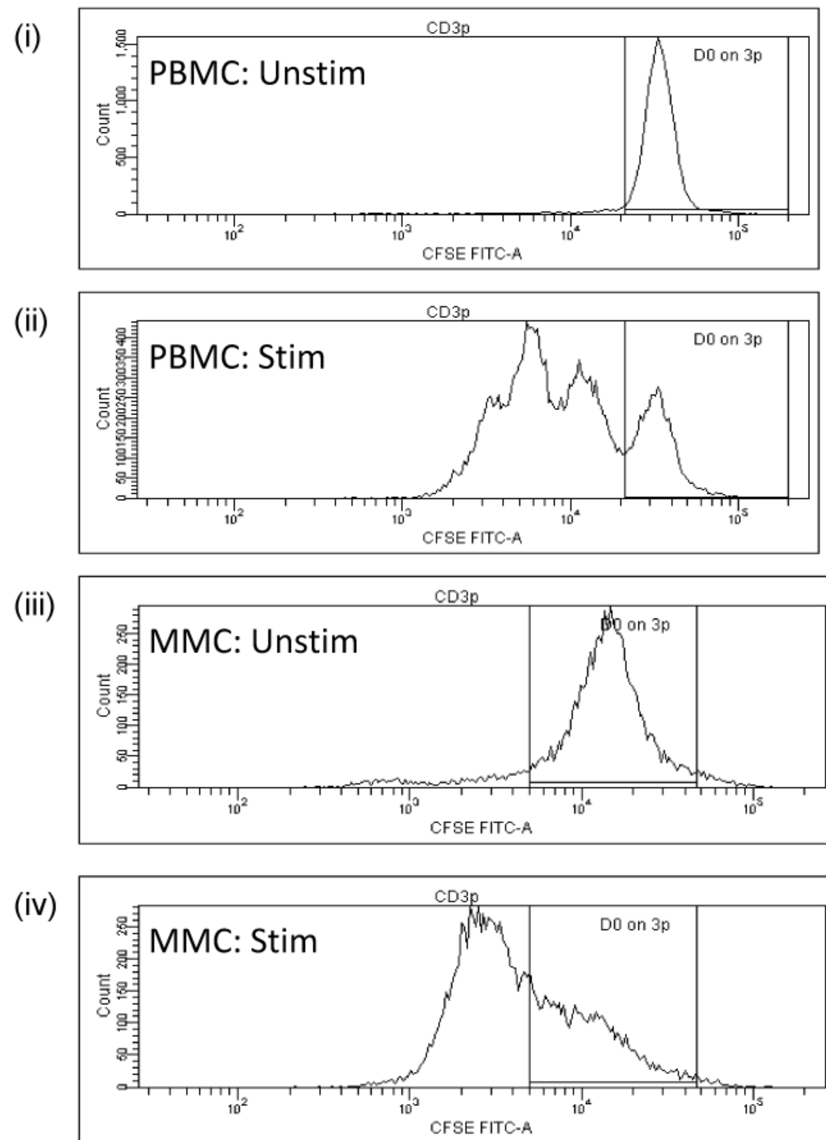


Figure 1. Staining 10×10^6 blood-derived T-cells (PBMC, Left panel) with 2.5 μM CFSE and 2–3×10^6 gut-derived T-cells (MMC, Right panel) with 5.0 μM CFSE provides an optimal CFSE range where on Day 5 (D5), all non-replicating and replicating cells are in the flow cytometer's detection range and not in background range

Representative donor samples (n=2) were tested to determine an optimal CFSE concentration for PBMC and MMC. Panels a-g show results from one representative donor (a) PBMC stim (No CFSE), (b) PBMC stim (CFSE 2.5 μM), (c) PBMC unstim (CFSE 2.5 μM), (d) MMC stim (No CFSE), (e) MMC stim (CFSE 2.5 μM), (f) MMC stim (CFSE 5.0 μM) and (g) MMC unstim (CFSE 2.5 μM) compartments. All culture stimulations were performed using 5 μl anti-CD2/3/28 microbeads.



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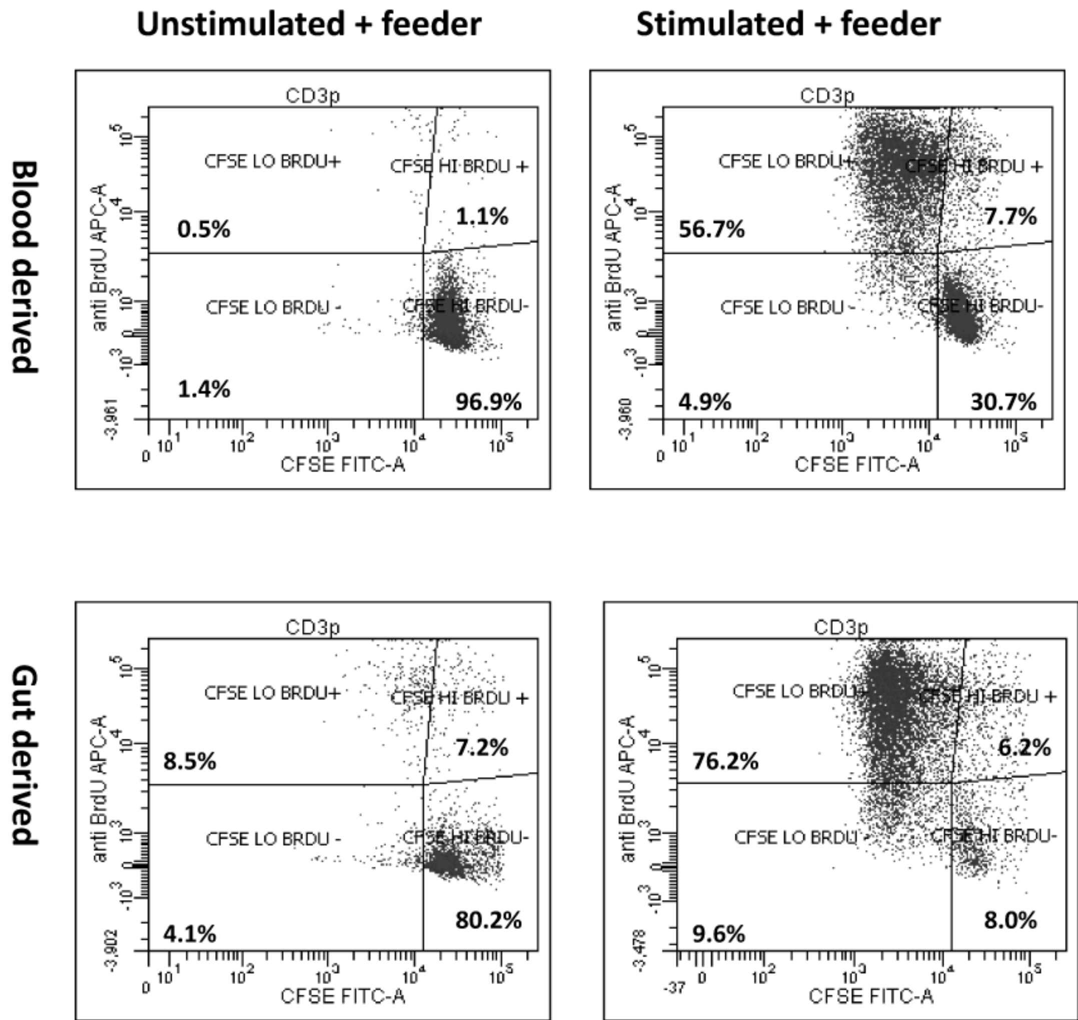

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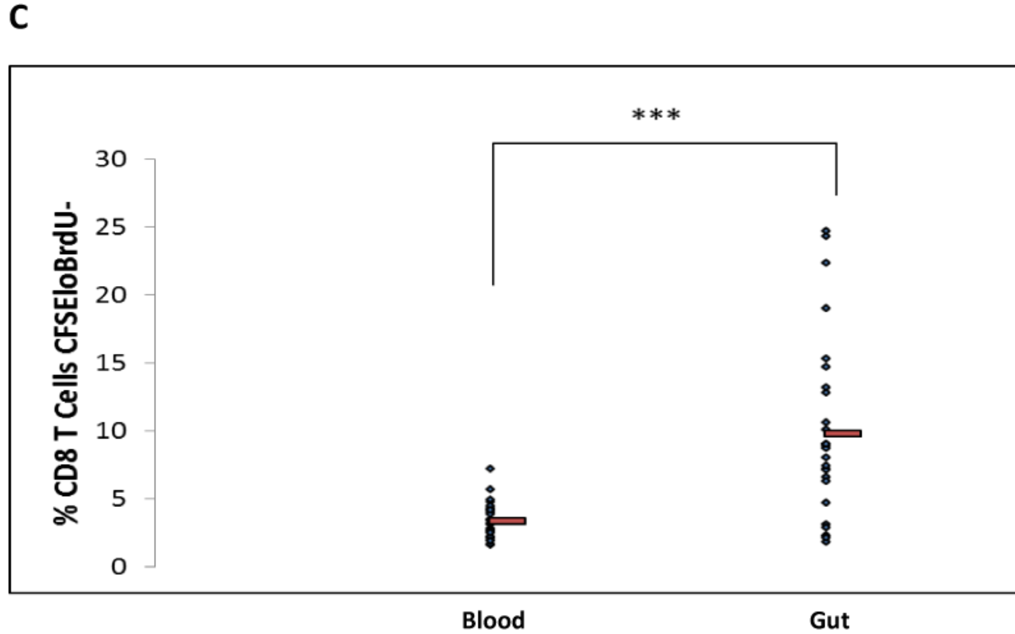

Figure 2. BrdU facilitates enhanced demarcation of proliferating vs. non-proliferating MMC and permits exclusion of any false positive CFSE^{lo} cells that are BrdU^{-}

A. CFSE histogram of live CD3 T-cells following a representative 5 day culture of (i) PBMC unstim, (ii) PBMC stim, (iii) MMC unstim, (iv) MMC stim cultures. **B**. Representative bivariate plots from blood and gut derived cultures (stimulated culture and unstimulated control) of CFSE-FITC (x-axis) versus BrdU-APC (Y-axis) gating used to enumerate replicating (CFSE^{lo}BrdU^{+}) verus non-replicating (CFSE^{hi}BrdU^{-}) CD3+ populations, and illustrating (CFSE^{lo}BrdU^{-}) non-replicating populations. All cultures include 0.5×10^{6} irradiated autologous PBMC feeders. **C**. Mean % of live blood- and gut-derived CD3+8+ cells that are (CFSE^{lo}BrdU^{-}) following bead stimulation and 5 day culture (n=29); ***, $p<0.005$ (two-side, two-tailed Student's paired *t* test). All culture stimulations were performed using 5 μl anti-CD2/3/28 microbeads.

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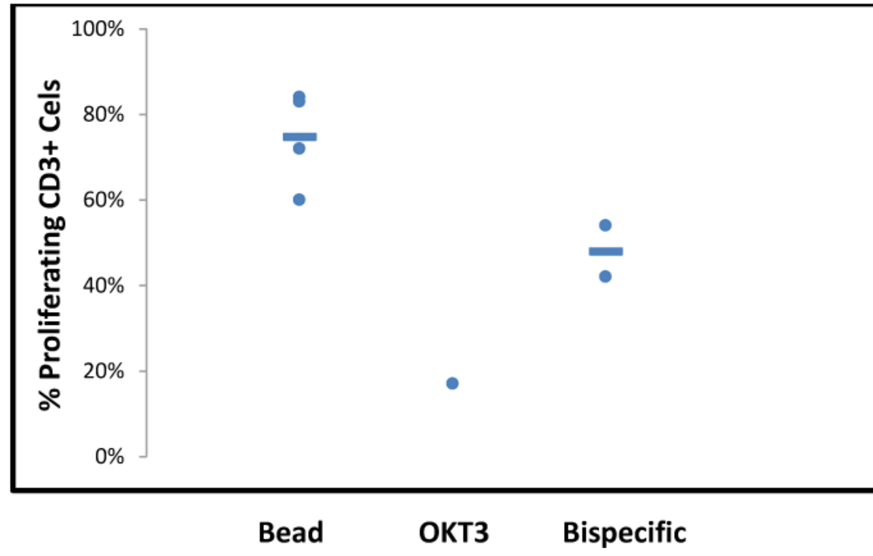

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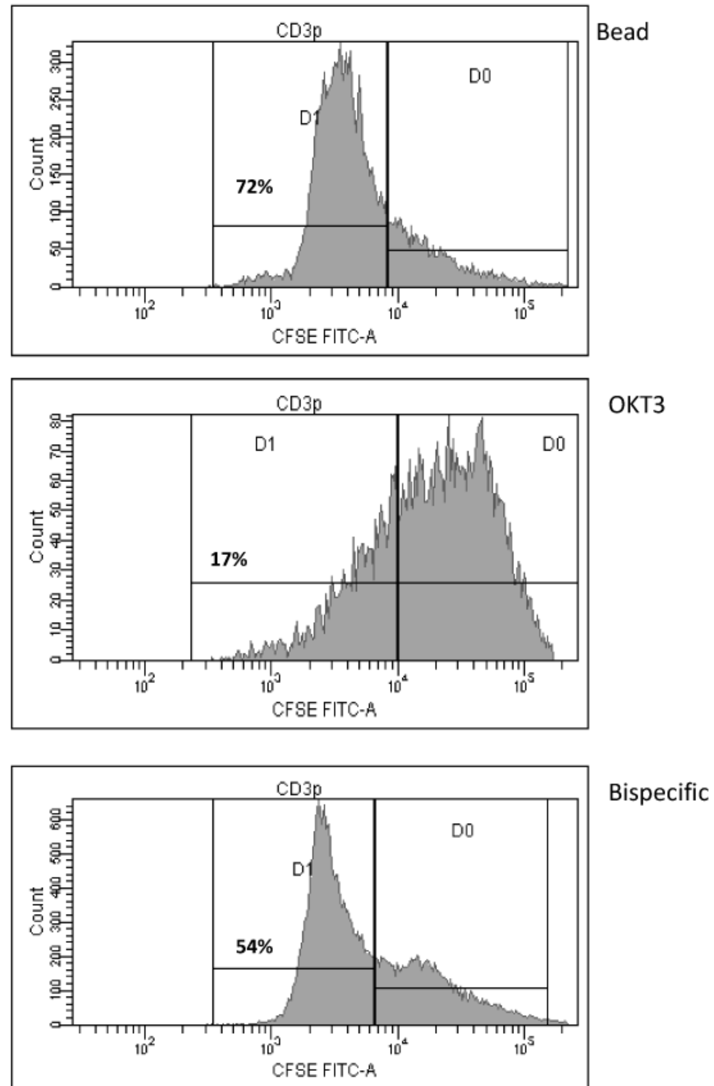

Figure 3. Comparing gut CD3T- cell proliferation using three TCR-mediated stimulation methods, Miltenyi beads demonstrated the highest % proliferating cells on day 5

MMC were stained with 5 μM CFSE and cultured for 5 days following CD3 stimulation via three different methods, at the recommended concentrations; (1) Miltenyi anti-CD2, 3, 28 Ab beads (5μl/ml) (n=4), (2) OKT3 (1μg/ml) + CD28 Ab (20μg/ml) (n=1), (3) Bispecific CD3/CD4 (blocking) Ab (1μl/ml) (n=2). At day 5, cells were stained with CD3-PE and %CD3+ cells that had proliferated ($CFSE^{lo}$) was determined. **(A)** Comparison of % proliferating ($CFSE^{lo}$) cells using Miltenyi beads (n=4), OKT3 (n=1) and Bispecific Ab (n=2). **(B)** Representative 1D flow histogram demonstrating proliferating ($CFSE^{lo}$) versus non-proliferating ($CFSE^{hi}$) CD3+ cell populations of gut derived cells for each stimulation

method (bead method 72% proliferating, OKT3 17% proliferating, bispecific ab 54% proliferating).

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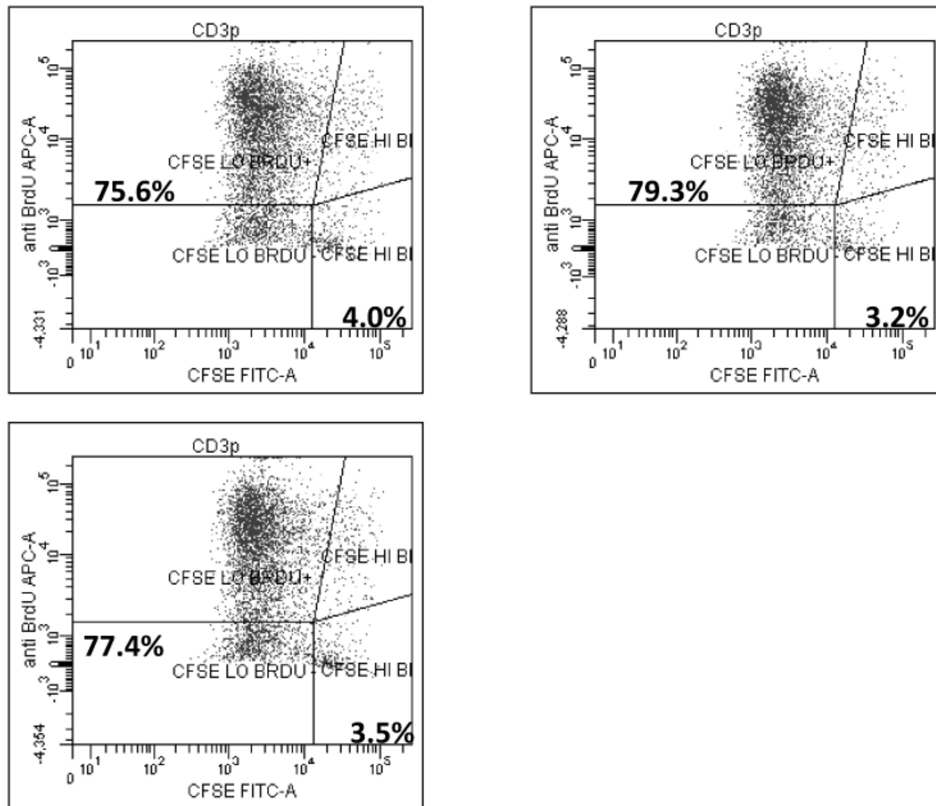

Figure 4. Stimulation with Miltenyi anti-CD2,3,28 Ab microbeads was tested with bead:cell ratios of 1:4, 1:2 and 1:1 on MMC from a single donor (n=1)

On Day 5 post-stimulation the percentage of non-dividing ($CFSE^{hi}BrdU^{-}$) gut derived CD3 T cells was 4%, 3.5% and 3.2%, respectively; the percentage of dividing ($CFSE^{lo}BrdU^{+}$) gut-derived CD3 T cells was 75.6%, 77.4% and 79.3%, respectively, indicating no major quantitative difference in proliferative dynamics.

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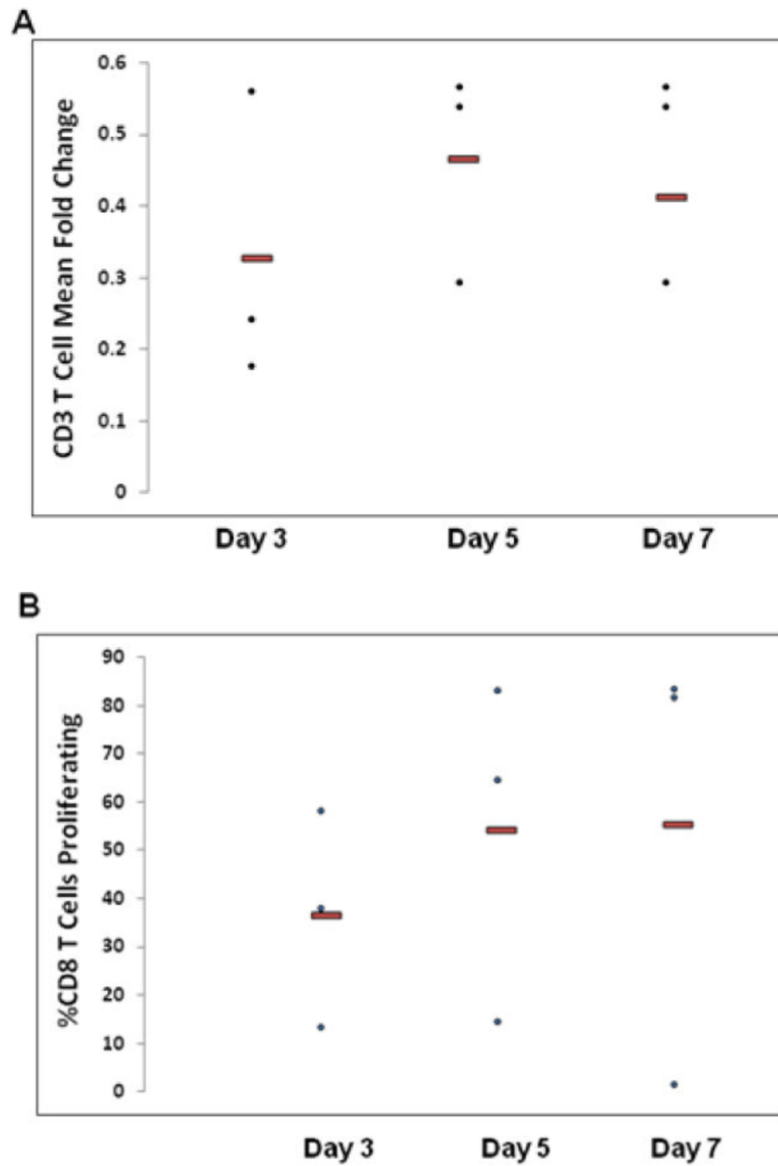

Figure 5. Five days was chosen as the optimal culture duration, as it yielded the highest mean CD3 fold-change, was the time point where % proliferating CD8 T cells plateau, and represents a time frame of uniform proliferative growth

Proliferative dynamics were evaluated on days 3, 5 and 7 and the CD3 fold-increase was determined. **A**. Average CD3 T cell fold-change (Day "X" culture size/Day 1 culture size) for gut cultures as determined by TRUcount™ method at Day 3, 5, 7 (n=3). **B**. Average % of live CD8 T cells replicating ($CFSE^{lo}BrdU^{+}$) in gut-derived cultures at Day 3, 5, 7 (n=3).

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Table 1

Staining 2–3×10^6 gut-derived T-cells (MMC) with no CFSE, 2.5 μM CFSE, and 5.0 μM CFSE indicates no toxicity as shown by D5 T Cell Fold Change (D5 number of live CD3 T cells/initial number of live CD3 T cells) with CFSE doses range 0–5.0 μM (n=2). All samples were stimulated with 5 μl anti-CD2/3/28 microbeads.

	CFSE stain (μM)	D5 T cell fold change
Donor 1 (MMC)	No CFSE	0.72
	CFSE 2.5 μM	0.81
	CFSE 5.0 μM	0.81
Donor 2 (MMC)	No CFSE	0.27
	CFSE 2.5 μM	0.30
	CFSE 5.0 μM	0.27