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Development of A Flow Cytometry Assay for Measurement of CD4+ T Cell Proliferation

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Abstract

Development of A Flow Cytometric Assay for Measurement of CD4+ T Cell Proliferation

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Measurement of CD4+ T cell function has been widely utilized in numerous studies. In clinical medicine, in vitro tests to measure CD4+ T cell functions such as activation and proliferative capacity have been established for decades. The Ki67 molecule is a nuclear protein present exclusively in proliferating cells. Ki67 protein expression is strictly correlated to cell proliferation and to the active phases of the cell cycle, although it's functional role during cell proliferation is unclear. The purpose of this study was to define the optimal flow cytometric protocol for the detection of in vitro CD4+ T cell proliferation using Ki67. The ultimate goal is to develop a clinical-grade assay that can be used for the diagnosis and management of patients.

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LIST OF ABREVIATIONS

CD, cluster of determination.

CV, coefficient of variation.

DPBS, Dulbecco's phosphate buffered saline.

FBS, fetal bovine serum.

FSC, forward scatter.

FSC-A, forward scatter-area.

FSC-H, forward scatter-height.

HCC, hepatocellular carcinoma.

HCV, hepatitis C virus.

mAb, membrane antibody.

MDSC, myeloid-derived suppressive cells.

G-MDSC, granulocytic myeloid-derived suppressive cells.

M-MDSC, monocytic myeloid-derived suppressive cells.

PBMC, peripheral blood mononuclear cells.

PBS, phosphate buffered saline.

PHA, phytohemagglutinin.

RBC, red blood cells.

ROS, reactive oxygen species.

SSC, side scatter.

TCR, T cell receptor.

T_H1, type 1 T helper cells.

T_H2, type 2 T helper cells.

^3H-TdR, tritiated thymidine.

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Chapter I: INTRODUCTION

Measurement of CD4+ T cell function has been widely utilized in numerous of studies. Use of the CD4+ T cell count as an evaluation end point has played a significant role in studies of some antiretroviral agents, contributing to their expeditious approval by regulatory agencies [1, 2]. In transplant patients, CD4+ T cells serves as a therapeutic target, as major immunosuppressive drugs are designed to specifically inhibit T cell activation which has been implicated in rejection [3, 4]. Therefore, a reproducible laboratory assessment of CD4+ T cell function is likely to be of great clinical value in evaluating patients' immune status.

Techniques that are applied in clinical medicine as in vitro tests to measure CD4+ T cell functions, such as activation and proliferative capacity, have been established for decades. For example, measurement of lymphocyte proliferation by the use of tritiated thymidine (^{3}H-TdR) incorporation, remains a common and useful method for assessing the ability of cells to respond to immune stimuli, but it does not provide information about specific lymphocyte subpopulations that respond to various mitogens, antigens or superantigens [5, 6]. Among those techniques, CD4+ T cell proliferative capacity was previously evaluated in the clinical immunology laboratory for transplant patients by using the Cylex Immuknow Assay (ImmuKnow), which is an FDA-approved test. ImmuKnow measures adenosine triphosphate produced after stimulation of T cells with plant lectin and mitogen phytohemogglutinin (PHA) [3, 7]. However, one limitation of

the ImmuKnow assay is the 15-18h incubation time that delays results until the next day, and the other limitation is this assay measures non-specific CD4+ T-cell function [8]. In addition, several meta-analyses investigating the use of the Cylex ImmuKnow Assay to identify risks of infection and rejection post-transplantation have been performed and in the analyses, the predictive capacity of this assay has been questioned [9, 10]. So there is substantial interest from clinicians for a functional T cell assay for use in patient management [11].

The Ki67 molecule is a nuclear protein present exclusively in proliferating cells. It is present in cell nuclei during all active phases of the cell cycle (G1, S, G2, and mitosis phases), but absent from quiescent or resting cells (G0 phase), and can react with monoclonal antibodies to Ki67 [12-14]. There is no doubt that Ki67 protein expression is strictly correlated to cell proliferation and to the active phases of the cell cycle, although the functional role of Ki67 protein during cell proliferation is unknown. Data from some researches even indicate that Ki67 protein expression is an absolute requirement for progression through the cell-division cycle [14]. Numerous studies have been performed to examine the usefulness and prognostic value of immunohistochemical detection of Ki67 in various types of malignant neoplasms. As a result, Ki67 antibodies have been used as a tool to estimate the growth fraction of any human cell population, with special interest in tumor diagnostics by immunohistochemistry [14-16]. Moreover, because of the tight association between intracellular Ki67 expression and cell proliferative properties, Ki67 has also been used

an indicator of the effectiveness of vaccination by measuring the proliferation of CD4+ T cell responses [17].

With the application of flow cytometry, which allows the cell surface phenotype and other features of individual proliferating cells to be detected simultaneously, the proliferation of specific cell subsets in a cell mixture, such as CD4+ T cells among peripheral blood mononuclear cells (PBMCs), can be easily extracted using fluorescent cell surface markers and a mAb to Ki67, after membrane permeabilization [5]. Andreia Soares et al. demonstrated in their study that a Ki67 flow cytometric method can be used to detect antigen-specific CD4+ T cell proliferation after vaccination and suggested that intracellular Ki67 expression provides a specific, quantitative and reproducible measure of antigen-specific T cell proliferation in vitro [18]. Thus, this method may be particularly useful for detecting antigen-specific as well as mitogen-stimulated T-cell proliferation in diverse areas such as immunodeficiency, allergy, transplant rejection, autoimmunity, vaccination and cancer immunotherapy [19].

The goal of this project was to develop a flow cytometric methodology by using the intracellular molecule Ki67 as a proliferative marker to measure CD4+ T cell proliferation in peripheral blood mononuclear cells (PBMC). Another goal was to apply this technique to populations of both healthy and diseased patients. This project had three stages of experiments. In the first stage, experiments on PBMCs from different healthy individuals were performed to compare different cell culture time, stimuli and

extracellular as well as intracellular staining panels to obtain optimal detection of Ki67 expression in CD4+ T cells. Stage II was to confirm that expression of the intracellular Ki67 molecule as a proliferative marker for CD4+ T cells corresponded to freshly measured ex vivo-levels of myeloid-derived suppressive cells (MDSC), which are known to suppress T cell proliferation in diseased patients. In stage III, this optimized Ki67 flow cytometric method was preliminarily applied to both healthy controls and patients in different disease conditions to examine the ability of this assay to detect differences between groups. In this thesis, the principles and outlines of methods are described in “Materials and Methods”, and the detailed procedures are described in “Appendix” section.

Chapter II: MATERIALS AND METHODS

Study subjects

Healthy and diseased adult donors were recruited at the University of Washington Medical Center using IRB-approved protocols. Heparinized venous blood was collected into BD Vacutainer tubes (BD Biosciences) and immediately processed as outlined below.

PBMC isolation and in vitro stimulation

PBMC were isolated from heparinized whole blood by a standard Ficoll-Hypaque (GE Healthcare) density gradient centrifugation, washed in Hanks' Balanced Salt Solution (Ca^{2+}/Mg^{2+} free, Gibco by Life Technologies) and counted in trypan blue. $2x10^6$ isolated cells (1mL/well, 2M/mL) were expanded in cell culture media (warm RPMI 1640 with 10% Fetal Bovine Serum, L-Glutamine and Penicillin-Streptomycin) with different stimuli for 2-7 days at 37ºC with 5% CO_2 in 24-wel round bottom plate. The following stimuli were used in this experiment: anti-CD3/anti-CD28 magnetic beads at $1x10^8$/mL concentration with a final 1:1 beads/cells ratio, 100uL Phytohaemagglutinin (PHA) at 1mg/mL concentration, and plate-bound anti-CD3 and soluble anti-CD28 membrane antibodies (BD Bioscience) at a 1:1 ratio. Before being added to cell mixture, the magnetic αCD3/αCD28 coated beads were washed with DPBS (Dulbecco's Phosphate buffered saline, Gibco by Life Technologies) and re-

suspended in cell culture media back to the original volume, according to the manufacturer's instructions. To prepare plate-bound anti-CD3 antibodies, 200uL of anti-CD3 at 10ug/mL were added to a 48-well flat bottom plate to coat overnight. In the following day, coated plate wells were rinsed with 1mL DPBS one time before 500uL PBMC mixture and 5uL of anti-CD28 soluble antibodies at 1mg/mL concentration were added.

Whole blood in vitro stimulation

1mL/well of 1:10 diluted whole blood was cultured in a 24-well round bottom plate with cell culture media: RPMI1640 with 10% Fetal Bovine Serum, L-Glutamine and Penicillin-streptomycin, and stimuli for 2-7 days at 37°C with 5% CO_2. Red blood cells were lysed after culture with stimulus by using RBC Lysing Buffer 10X (BioLegend). Anti-CD3/anti-CD28 beads at $1x10^8$/mL concentration with a final 1:1 beads/cells ratio, 100uL 1mg/mL Phytohaemagglutinin (PHA) and plate-bound anti-CD3/anti-CD28 antibodies (BD Bioscience) at a 1:1 ratio were used for whole blood in vitro stimulation. As above, anti-CD3/anti-CD28 beads were washed one time prior to use. Similarly, plate wells were coated with anti-CD3 antibody overnight when using plate-bound anti-CD3/anti-CD28 antibodies as stimulus (same as with PBMC in vitro proliferation).

Proliferative response of PBMC after in vitro stimulation

The Ki67 flow cytometric assay was performed using PBMCs isolated from multiple healthy controls and patients. The cells were stimulated with or without anti-CD3/anti-CD28 beads and/or PHA and/or plate-bound anti-CD3/anti-CD28 antibodies, and incubated for multiple days within a 7-day time course. After incubation, cells were stained according to the surface marker and intracellular Ki67 staining protocols, and cell data was collected on a BD LSRFortessa flow cytometer and analyzed with Flowjo software.

PBMC immunofluorescence labeling

500uL cultured PBMC were transferred into 5mL FACS tubes (BD Bioscience) and washed with FACS buffer (Dulbecco's Phosphate buffered saline (DPBS, Gibco by Life Technologies) containing 2% fetal bovine serum) one time before labeling. Then cells were stained with fluorochrome conjugated surface mAb and incubated for 30 min at room temperature in the dark, followed by a washing step in 2% FACS buffer. Then viability dye was added and the samples incubated at 4°C in the dark, in order to separate live cells from dead cells during analysis. Cells were washed again in 2% FACS buffer. Thereafter, cell fixation/permeabilization was performed with Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Briefly, cells were fixed in 1mL 1:4 diluted Foxp3 permeabilization solution for 45 minutes in the dark and washed with 2mL 1:10 diluted washing buffer twice. APC-conjugated

Ki67 mAb was added to the samples for intracellular staining after cell fixation and cells were washed twice in FACS buffer. Finally, stained cells were maintained in DPBS with 2% paraformaldehyde at 4ºC until running on the flow cytometer.

Whole blood immunofluorescence labeling

500uL whole blood was transferred into 5mL FACS tubes and labeled with fluorochrome conjugated surface mAb for 15 min in dark followed by 20 min incubation in the dark with 2mL FACS Lysing Solution (BD Bioscience) in order to lyse red blood cells. Samples were washed in 2mL FACS buffer. Thereafter, cell fixation/permeabilization was performed with Foxp3 Staining Buffer Set. Samples were incubated for 45 min with 1mL 1:4 diluted Foxp3 permeabilization solution for cell fixation. 2mL 1:10 diluted washing buffer was used to wash cells twice. APC-conjugated Ki67 mAb was added to samples for intracellular staining after cell fixation and cells were washed twice with FACS buffer. Finally, stained cells were fixed with 2% paraformaldehyde in PBS and kept at 4ºC until running on the flow cytometer.

Antibodies and flow cytometry

The following fluorochrome-conjugated antibodies (mAb) were used for phenotypic and intracellular antigen staining: Fixable Viability Dye eFluor 700 (BD Horizon), CD3-FITC (SK7, BD Bioscience), CD3-APC (UCHT1, Beckman Coulter),

CD4-PE (RPA-T4, Biolegend), CD8-PerCP-Cy5.5 (SK1, BD Bioscience), CD14-PerCP-Cy5.5 (MϕP9, Biolegend), CD14-FITC (MϕP9, BD Pharmingen), CD45-Pacific Blue (HI30, Biolegend), Ki67-APC (Ki67, Biolegend). Samples were acquired on a BD LSRFortessa flow cytometer (BD Biosciences, San Jose, CA).

Data analysis

PBMC data analysis

Single-stained and unstained unstimulated cells were used to calculate compensations for every run. Cell debris and erythrocytes were excluded using forward scatter versus side scatter parameters (FSC vs SSC plot). Dead cells were excluded by gating on fixable viability dye eFluor 780-negative cell (live cell) population using APC/Cy7 versus side scatter parameters, followed by monocyte exclusion with gating on the CD14-negative cell population. Then CD4-positive cells were obtained by defining a CD3-positive gate on the FITC vs SSC plot and a CD4-positive gate on the APC vs PE plot. Ki67 expression was analyzed within CD4-positive T cells using APC vs PE parameters. Data was analyzed using FlowJo software. Statistical analyses were calculated using Prism v 6.0.

Whole blood data analysis

Single-stained and unstained unstimulated cells were used to calculate compensations for every run. Single cells were obtained by gating on FSC-A vs FSC-H plot. Cell debris was excluded using forward scatter versus side scatter parameters (FSC vs SSC plot). Leukocytes were obtained by gating on the CD45-positive cell population followed by the exclusion of monocytes with CD14-negative cell population gate. The CD4-positive cell population was obtained by selecting CD3-positive cells, then the CD4-positive population. Finally, Ki67 expression was reported as the frequency of Ki67-positive cells among CD4-positive cell population on the APC vs PE plot. Data was analyzed by FlowJo software. Statistical analyses were calculated using Prism v 6.0.

Statistical analysis

Statistical analysis was performed using Pearson's correlation (r) to measure linear correlation. $P<0.05$ was considered significant.

Chapter III: RESULTS

I. Assay Optimization

In order to obtain the best Ki67 expression among CD4+ T cells from in vitro mitogen stimulation, assay conditions, such as cell type, stimuli type and concentrations, cell culture time, reagents, etc., were optimized using blood samples from healthy volunteers.

Optimal cell permeabilization solution determination. For Ki67 flow cytometric assay, an important point to remember is that this method utilizes fluorochrome conjugated monoclonal antibodies for both surface markers and an intracellular marker, Ki67 antigen. As a result, the membrane permeabilization step, or membrane permeabilization buffer, which permits the access to the intracellular compartment without destroying cell surface markers, is critical [14]. One buffer considered for use was 70% ethanol, which was used in several publications, and also suggested by Biolegend, the manufacturer of the APC fluorochrome conjugated monoclonal Ki67 antibody. The other candidate was eBioscience Foxp3 Staining Buffer Set, which has been used in many studies to permeabilize cell membrane for the staining of Foxp3, an intracellular transcription factor in regulatory T cells [20-22].

To determine the effect of the two permeabilization buffers, 70% ethanol and Foxp3 Staining Buffer set, we compared the expression of Ki67 in unstimulated PBMCs as well as αCD3/αCD28 bead-stimulated PBMCs cultured for 48 hours. Ki67

expression in unstimulated CD4+ T cells permeabilized with either 70% ethanol or Foxp3 kit was lower than 1%. But in αCD3/αCD28 magnetic bead-stimulated cells the expression of Ki67 in CD4+ T cells demonstrated an obvious difference: only 6% of CD4+ T cells expressed Ki67+ when permeabilized with 70% ethanol, whereas 33% of CD4+ T cells expressed Ki67+ when permeabilized with the Foxp3 Buffer (Fig.1). This experiment was repeated 2 times with consistent results. Therefore, it was decided that the eBioscience Foxp3 Staining Buffer set should be used as the cell permeabilization buffer for intracellular Ki67 staining in this protocol.

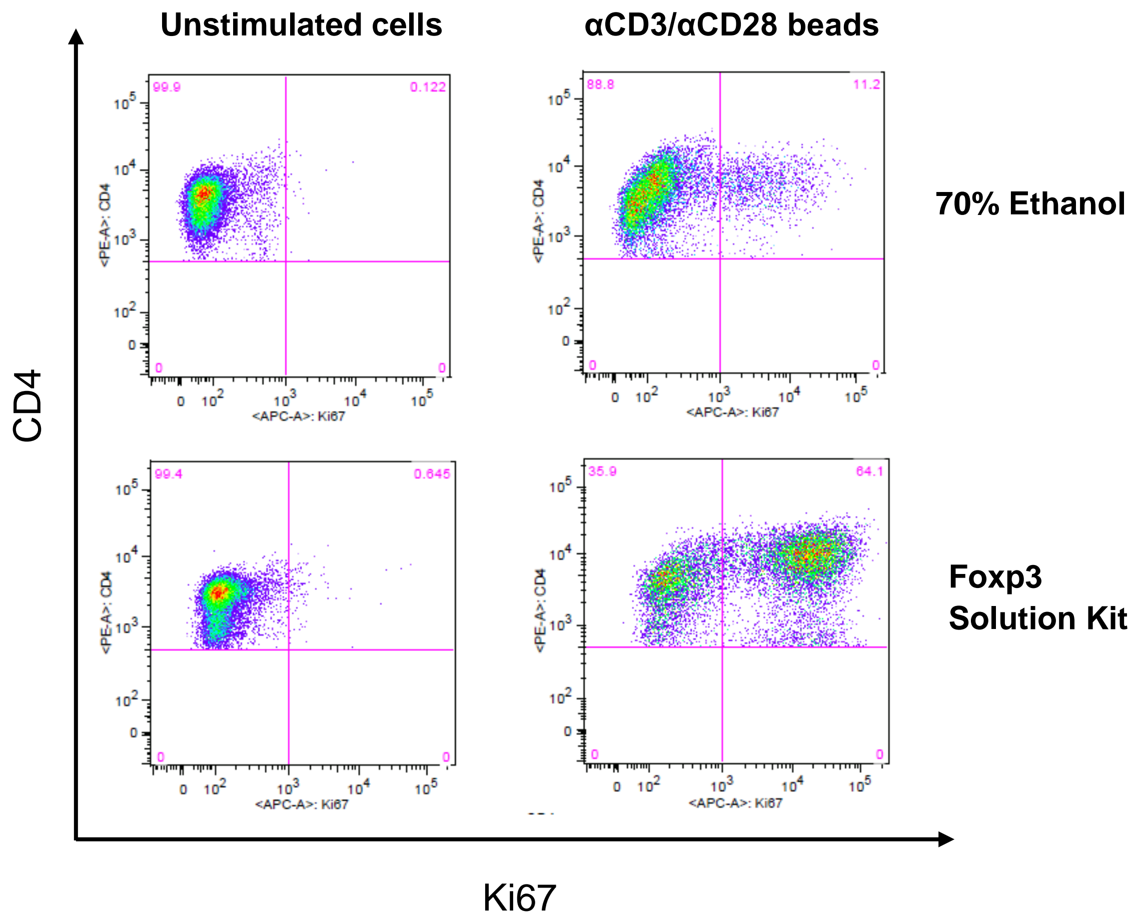


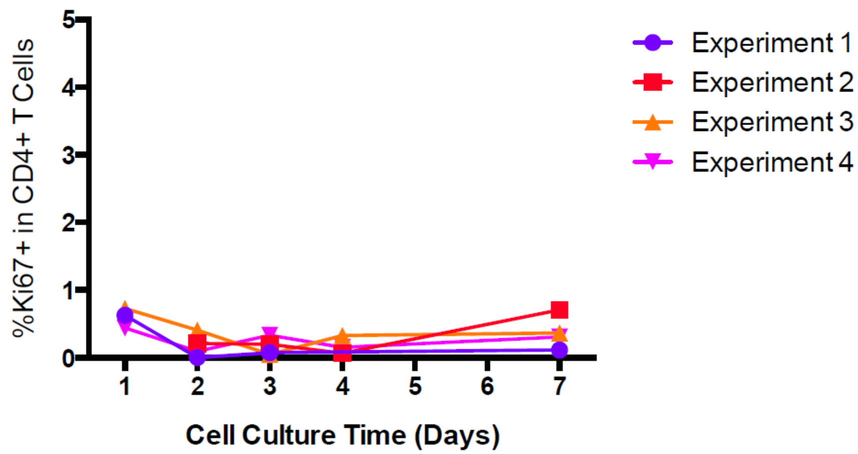
Figure 1. Expression of Ki67 by intracellular staining after cells permeabilization with Foxp3 solution or 70% ethanol.

PBMC were either not stimulated or stimulated with αCD3/αCD28 beads for 48 hours, fixed, and acquired on a flow cytometer within 48 hours. Unstimulated cells are shown in the left panels and αCD3/αCD28 bead-stimulated cells are shown in the right panels. The x-axis is Ki67-APC and y-axis is CD4-PE. Each dot represents a cell. Each permeabilization solution was used for a pair of unstimlated + αCD3/αCD28 bead-stimulated PBMC to compare the expression of Ki67 in CD4+ T cells. Percentages of Ki67+ among CD4+ T cells in unstimulated cells after permeabilized by 70% ethanol or Foxp3 solution kit were 0.1% versus 0.6%. In αCD3/αCD28 bead-stimulated PBMC, the percentages of Ki67+ in CD4+ T cells permeabilized in Foxp3 solution or 70% ethanol were 64.1% versus 11.2%.

Optimal cell culture time determination. The ultimate goal of this study was to develop a flow cytometric assay for measuring expression of Ki67 in CD4+ T cells and apply it for the prognostic evaluation of patients' immune function. Since in vitro stimulation is necessary for cell proliferation and expression of Ki67, cell culture time was a critical factor in this assay. Soares et al. demonstrated that the expression of Ki67 in CD4+ T cells from PBMC reached its peak at day 3 (72 hours) and started to decrease at day 4 (96 hours) after stimulation with αCD3/αCD28 beads in their study [18]. So we quantitated the expression of Ki67 in CD4+ T cells from PBMC of four different healthy controls cultured with both αCD3/αCD28 beads and PHA over a 7-day time course. Our data showed the expression of Ki67 in unstimulated CD4+ T cells was lower than 1% during the 7 days (Fig.2A). But after stimulation with either αCD3/αCD28 beads or PHA, the Ki67 expression of CD4+ T cells showed a logarithmic increase (Fig.2B, Fig.2C). Even though the peak of Ki67 expression was at day 4, the highest expression *rate* of Ki67 increase was within the first 3 days, especially around day 2 and day 3 (48-72 hours) (Fig.3). Using αCD3/αCD28 bead-stimulation, we compared the rate of increase of Ki67 expression, which represents the rate of in vitro proliferation, at day 2 and day 3. We found the greatest rate was observed at around day 2, represented by the slope of lines (Fig.2B). After 3 days culture (72 hours culture), the increase in Ki67 expression in CD4+ T cells slowed and gradually reached its peak. After day 4, the expression of Ki67 either remained the same as or was lower than the day 3 level (Fig.3). In order to ensure that cells were in a linear phase of proliferation when we measure Ki67 expression, and to limit the time

necessary to obtain the result, we decided to use 2 days (48 hours) culture or less for this assay.

A



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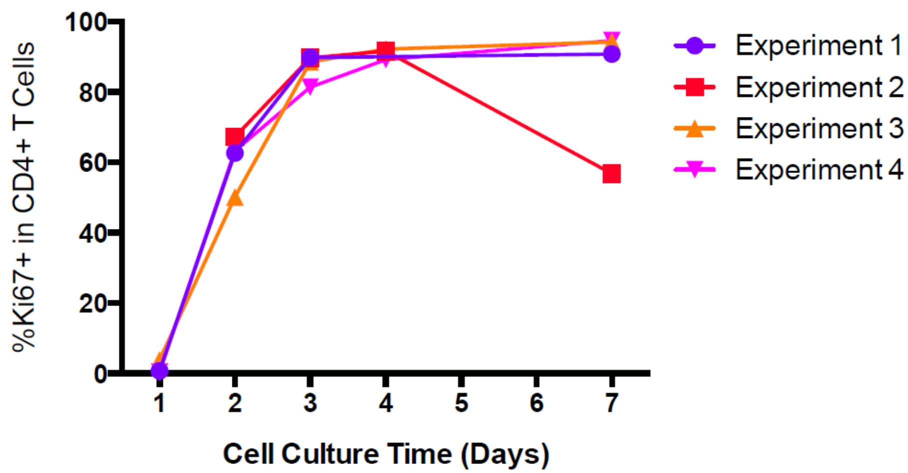

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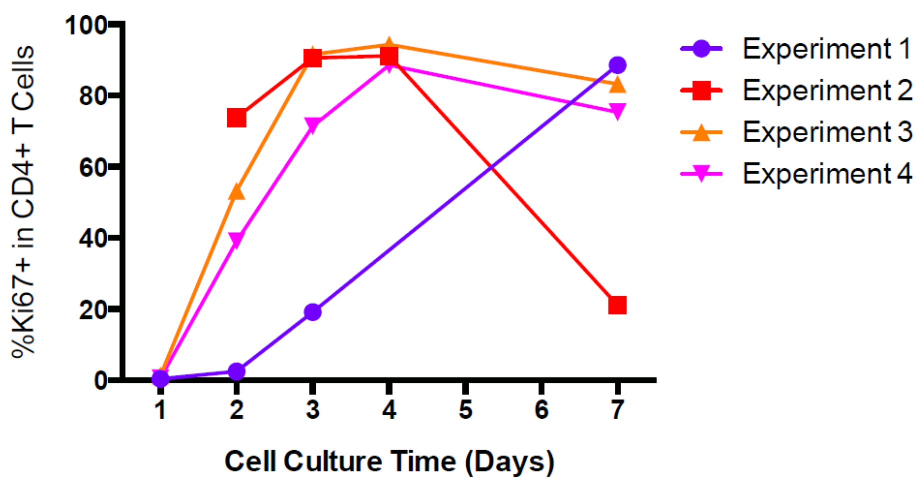


Figure 2. Change in Ki67 expression in CD4+ T cells using different stimuli over a 7-day cell culture period.

For each experiment, three replicates of PBMCs obtained from the same healthy control

were cultured for 7 days. Each experiments utilized a different healthy control subject. PBMC were stained to obtain Ki67+ CD4+ percentages on selected days. The x-axis shows cell culture time and the y-axis shows the frequency of Ki67+ in CD4+ T cells. In each graph, different symbols represent the percentages of Ki67+ in CD4+ T cells obtained from different healthy controls. (A) Low expression of Ki67+ in CD4+ T cells was obtained from unstimulated PBMCs in four different experiments with different healthy controls over 7 days cell culture. (B) Expression of Ki67 in CD4+ T cells in PBMCs stimulated by αCD3/αCD28 beads through 7 days. (C) Expression of Ki67 in CD4+ T cells in PBMCs stimulated by PHA through 7 days in different healthy controls.

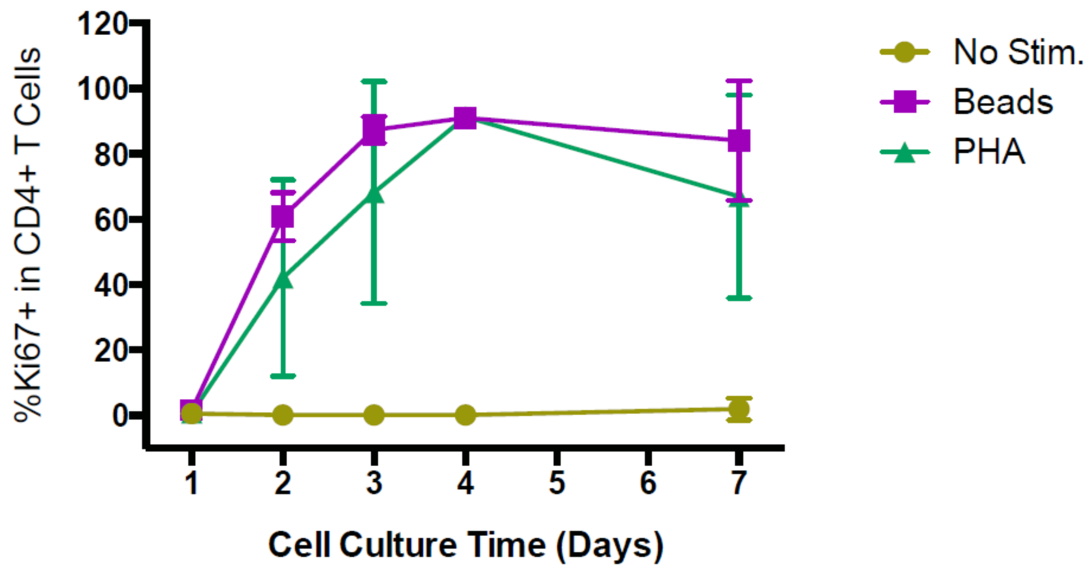


Figure 3. Change of Ki67 in CD4+ T cells frequencies in PBMCs over 7-day cell culture.

Three replicates of PBMCs obtained from the same healthy control were cultured for 7 days. Cells were stained to obtain Ki67+ CD4+ percentages on selected days. The x-axis shows cell culture time and the y-axis shows the frequency of Ki67+ in CD4+ T cells. Different symbols and colors represent percentages of Ki67+ in CD4+ T cells obtained from PBMC groups cultured with different stimuli. Yellow dots are unstimulated cells, purple squares are αCD3/αCD28 beads stimulated cells while green triangles are PHA stimulated cells. Each symbol represents the average percentage of Ki67+ in CD4+ T cells of four different healthy controls and the error bars in each group are one standard deviation away from the average. The same experiment was repeated on PBMCs from three other different healthy controls with similar results.

Cell stimulus optimization. In order to achieve optimal Ki67 expression in CD4+ T cells, the best stimulus for in vitro lympho-proliferation needed to be determined. There are several cell stimulating agents used in cell expansion protocols including phytohaemagglutinin (PHA), and anti-CD3/anti-CD28 monoclonal antibodies immobilized on magnetic beads [23-25]. We compared the efficacy of αCD3/αCD28 and PHA in stimulating Ki67 expression. A number of different concentrations of αCD3/αCD28 beads:cells were compared to determine the optimal ratio for stimulation of Ki67 expression in CD4+ T cells (data not shown), and the best stimulation of Ki67 expression was obtained with a 1:1 ratio. The amount of αCD3/αCD28 beads used in this assay was $2x10^6$, which is a 1:1 beads/cells ratio, and 20ug of PHA was used for each cultured well of $2x10^6$ cells. The expression of Ki67 was quantitated in different healthy control samples.

After 48 hours of culture, the expression of Ki67 in CD4+ T cells was evaluated in 3 replicates each of unstimulated, αCD3/αCD28 bead- and PHA-stimulated PBMCs. The frequency of Ki67+ in CD4+ T cells in unstimulated PBMC was less than 1% (mean=0.2%), in αCD3/αCD28 coated magnetic beads-stimulated PBMC was 61% and in PHA-stimulated group was around 42% (Fig.4). In addition, the coefficient of variation was much higher in the PHA-stimulated group (CV=71.4%) than αCD3/αCD28 coated magnetic bead-stimulated group (CV=12.2%) (Fig.4). What's more, the data from cells cultured for 7 days also showed that αCD3/αCD28 bead-stimulattion led to a more consistent proliferative response in CD4+ T lymphocytes

than PHA did (Fig.2B, Fig.2C). Therefore, in order to achieve the most consistent and reliable Ki67 result from PBMC, αCD3/αCD28 bead stimulation was chosen for the Ki67 flow cytometric assay. Our results are consistent with other published studies suggesting that optimal CD4+ T cell proliferation can be obtained using αCD3/αCD28 coated magnetic beads [26].

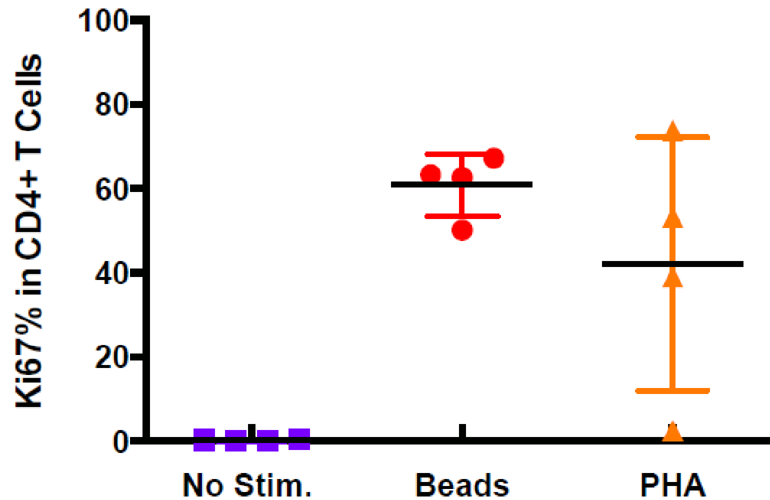


Figure 4. Expression of Ki67 in CD4+ T cells stimulated by αCD3/CD28 coated magnetic beads and PHA after 48 hour culture.

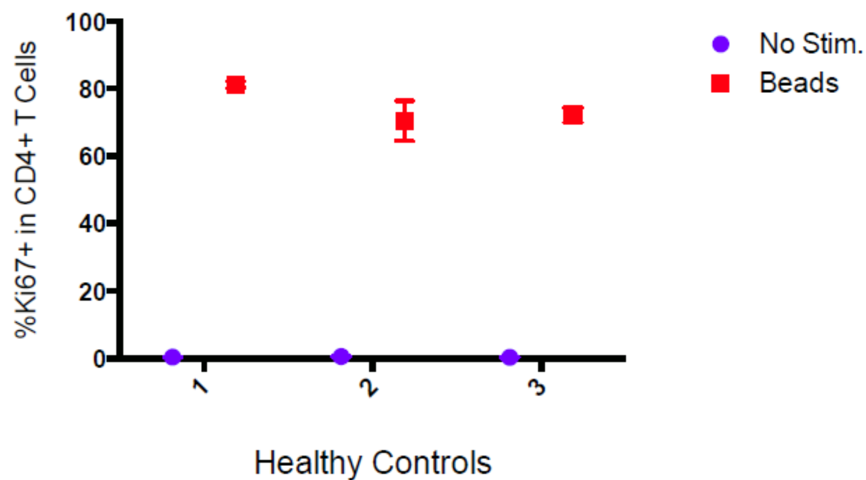
Three replicates of PBMC obtained from the same healthy control sample was either not stimulated or stimulated by αCD3/αCD28 beads or PHA. The mean of these results is shown as a single symbol. This experiment was repeated three additional times with different healthy controls on different dates. The x-axis shows different cell stimuli and the y-axis is the frequency of Ki67+ in CD4+ T cells. Different symbols represent PBMC treated with different stimuli. Blue squares represent the frequency of Ki67+ in CD4+ T cells obtained from the unstimulated PBMC group, red circles from the αCD3/αCD28 bead-stimulated PBMC group and orange triangles from the PHA-stimulated PBMC group. The black line in each group represents the mean percentage of Ki67+ in CD4+ T cells acquired from four different experiments and the error bars in each group shows one standard deviation from the mean value. Unstimulated PBMC groups showed a mean of 0.18% (± 0.17%) of Ki67+ in CD4+ T cells from four different healthy control samples. The average percentage of Ki67+ in CD4+ T cells stimulated with αCD3/αCD28 beads was 60.8% (±7.4%), while 42.1% (±30.1%) of Ki67+ in CD4+ T cells was acquired from PBMC groups stimulated with PHA.

43 hours vs 48 hours cell culture time comparison. We also compared the expression of Ki67 in CD4+ T cells after stimulation for 43 hours and 48 hours. The major reason to compare these two time points was because 43 hours is more practical in the real clinical testing setting, due to the time when patient blood samples are received and when PBMC processing from whole blood and staining cells after culture can be performed on a regular basis. In this comparison, we tested PBMC obtained from three different healthy donors on different days. PBMC were tested in triplicate for both unstimulated and αCD3/αCD28 bead-stimulated conditions, and cultured for either 43 hours or 48 hours. Compared to unstimulated cells, Ki67 expression was high among CD4+ T cells cultured for both 43 hours and 48 hours (Fig.5A, Fig.5B). The mean frequencies of Ki67+ in CD4+ T cells from three different donors cultured for 43 hours and 48 hours were compared to determine the difference and feasibility of completing the assay at 43 hours vs 48 hours. The expression of Ki67 in CD4+ T cells stimulated by αCD3/αCD28 beads for 43 hours (74.6%) was highly comparable to that of cells cultured for 48 hours (77.8%) (Fig.6). Therefore, we determined that 43 hours, a more practical time point than 48 hours, could be used for the cell stimulation period in clinical testing.

Repeatability and reproducibility evaluation. For a method designed to be applied clinically, which is potentially a prognostic assessment, its repeatability (intra-assay variation) and reproducibility (inter-assay variation) are pivotal for generating a convincing and precise result. Thus, we evaluated if this flow cytometric assay for

measuring expression of Ki67 in CD4+ T cells in PBMC is repeatable and reproducible. To evaluate assay repeatability, we determined the expression of Ki67 in CD4+ T cells stimulated by αCD3/αCD28 beads among three replicates in three different experiments of different healthy donor samples after either 43 or 48 hours of culture. The mean and one standard deviation of Ki67+ in CD4+ T cell frequencies was calculated based on data obtained from the three different experiments as shown in Fig.5. The coefficients of variation (CV) were <10% for both 43 and 48 hour stimulated cell groups in all experiments (except for one outlier), which indicated that this Ki67 flow cytometric assay was highly repeatable (Table 1). We also examined the reproducibility of the Ki67 flow cytometric assay by testing Ki67 expression in CD4+ T cells with αCD3/αCD28 beads stimulation in 5 separate experiments of the same frozen PBMC (Fig.6). Excellent reproducibility of this Ki67 flow cytometric assay was found with the frequency of Ki67 expression in CD4+ T cells at 74.5% (± 5.2%) for αCD3/αCD28 bead stimulation and a coefficient of variation for reproducibility at 7.0% (Table 2). Therefore, flow cytometric measurement of Ki67 in CD4+ T cells is highly repeatable and reproducible under the experimental conditions described.

A



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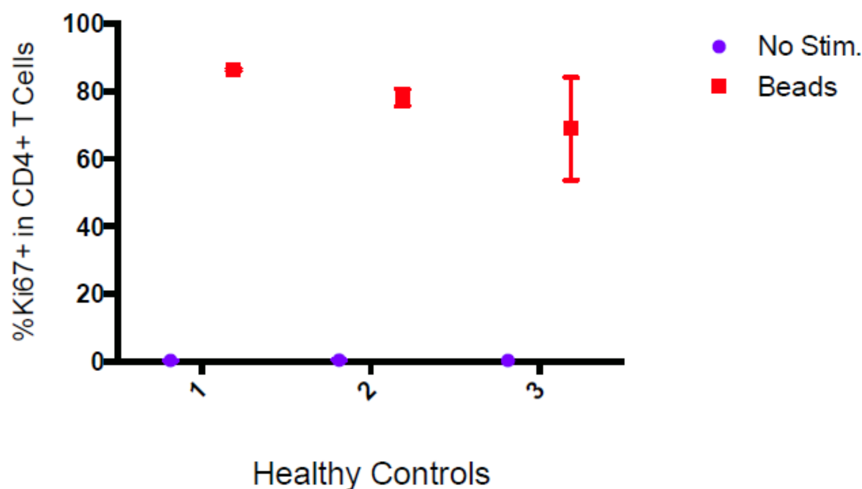


Figure 5. Repeatability of measuring Ki67+ in CD4+ T cells after αCD3/αCD28 bead-stimulation for 43 hours or 48 hours.

Three experiments were conducted on 3 different healthy control samples on different days. Data from triplicate wells of unstimulated and αCD3/αCD28 bead-stimulated conditions are shown. The x-axis indicates the different healthy donors and the y-axis indicates the frequencies of Ki67+ in CD4+ T cells. Blue squares represent the average percentage of Ki67+ in CD4+ T cells in PBMCs without stimulation while red circles represent the average Ki67+ percentage for CD4+ T cells in αCD3/αCD28 bead-stimulated PBMCs. Error bars indicate one standard deviation from the mean of Ki67+ percentage in CD4+ T cells. (A) Comparison of Ki67+ in CD4+ T cell frequencies after 43 hours of stimulation. (B) Comparison of Ki67+ in CD4+ T cell frequencies after 48 hours of stimulation.

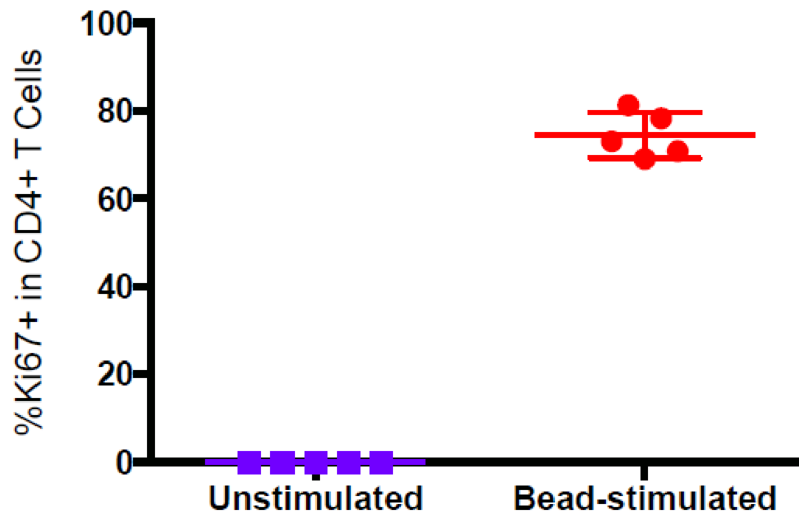


Figure 6. Reproducibility of Ki67 expression in CD4+ T cells among frozen PBMC after αCD3/αCD28 bead-stimulation for 43 hours.

Five experiments were conducted on frozen PBMC obtained from the same healthy control sample at different times by the same person. The x-axis indicates cell groups and the y-axis indicates the frequency of Ki67+ in CD4+ T cells. Blue squares represent the percentage of Ki67+ in CD4+ T cells without stimulation while the red circles represent the Ki67+ percentage in CD4+ T cells in αCD3/αCD28 bead-stimulated PBMCs. The mean frequency of Ki67+ in CD4+ T cells was 0.03% (± 0.01%) in unstimulated PBMC *versus* 74.5% (± 5.2%) for αCD3/αCD28 bead-stimulated PBMC.

Table 1. Repeatability of the Ki67 flow cytometric assay

Repeatability (Coefficient of Variation, %)			
	HC-1	HC-2	HC-3
43 hours	1.18	3.17	2.94
48 hours	0.50	8.47	22.11

The difference of expression of Ki67 in CD4+ T cells among three replicates within one experiment was compared after 43 and 48 hours of stimulation. Coefficient of variation (%CV) represents the difference in Ki67+ percentages in CD4+ T cells among replicates in the same experiment, completed at the same time. Shown are the data from 1 representative experiment; experiments were repeated three times using PBMCs from different healthy donors with similar results.

Table 2. Reproducibility of the Ki67 flow cytometric assay

Reproducibility		
Experiment	Unstimulated	αCD3/αCD28 beads
1	0.02	73.0
2	0.04	69.0
3	0.03	81.3
4	0.02	78.3
5	0.04	70.8
Mean	0.03	74.5
Standard Deviation	0.01	5.2
Coefficient of Variation	34.3%	7.0%

The difference in expression of Ki67 in CD4+ T cells among different experiments (inter-experiments) was compared. Expression of Ki67 in CD4+ T cells in frozen PBMC obtained from the same healthy donors was stimulated with αCD3/αCD28 beads in 5 different experiments. The coefficient of variation, was calculated from the mean and standard deviation of the frequencies of Ki67+ in CD4+ T cells in the absence of stimulus or with αCD3/αCD28 bead stimulation.

Ki67 expression in PBMC vs whole blood. Most studies of lympho-proliferation use purified peripheral blood mononuclear cells (PBMCs) and conventional proliferation assays [27]. However, compared to PBMC, whole blood would be a much easier source of material to use since purifying steps are not required. Another major advantage of using whole blood is that the cells have been less manipulated and pre-activated ex vivo [27]. Therefore, we compared the expression of Ki67 in CD4+ T cells by stimulating both whole blood and PBMC from the same subjects and culturing for 2 days. Even though we confirmed that αCD3/αCD28 beads work well on PBMC to stimulate expression of Ki67, expression of Ki67 in CD4+ T cells stimulated by αCD3/αCD28 coated magnetic beads from whole blood was poor to nonexistent despite testing multiple concentrations of beads (Data not shown). In order to achieve the best comparison from each sample, we used αCD3/αCD28 beads at a 1:1 beads/cells ratio, a 1:1 ratio of plate-bound αCD3/αCD28 antibodies and 100μg PHA as the stimuli. Similar frequencies of Ki67+ in CD4+ T cells from PBMC were stimulated by αCD3/αCD28 coated magnetic beads (70.3%) and plate-bound αCD3/αCD28 antibodies (69.5%), but PHA stimulated less expression of Ki67 in CD4+ T cells (35.8%) (Fig.7). In whole blood, PHA stimulation generated a higher expression of Ki67 in CD4+ T cells (45.4%) than plate-bound αCD3/αCD28 antibodies (13.2%), and αCD3/αCD28 beads stimulated the least expression of Ki67 in CD4+ T cells (0.2%), which was comparable to that of unstimulated whole blood (0.1%) (Fig.8). However, it should be noted that Ki67 expression in whole blood varied with the concentration of PHA used, and higher PHA concentrations were required with whole blood than that

used to stimulated PBMC (data not shown). Therefore, we turned to plate-bound αCD3/αCD28 antibodies to see if identical stimulation conditions could be used to stimulate both PBMC and whole blood, thereby enabling a true comparison of assay results. Figure 9A shows the frequencies of Ki67+ in CD4+ T cells from PBMCS and whole blood obtained from the same healthy donor after stimulation with plate-bound αCD3/αCD28 antibodies for 43 hours, while Figure 9B shows a compilation of data from 3 different healthy control samples. All 3 different experiments showed that PBMCs stimulated by plate-bound αCD3/αCD28 antibodies more consistently produced high expression levels of Ki67 in CD4+ T cells (65.7%±3.5%) than that in stimulated whole blood (7.2%±5.0%). Based on the results of these comparisons, we selected peripheral blood mononuclear cells (PBMCs) as the source material to be used for the Ki67 flow cytometric assay.

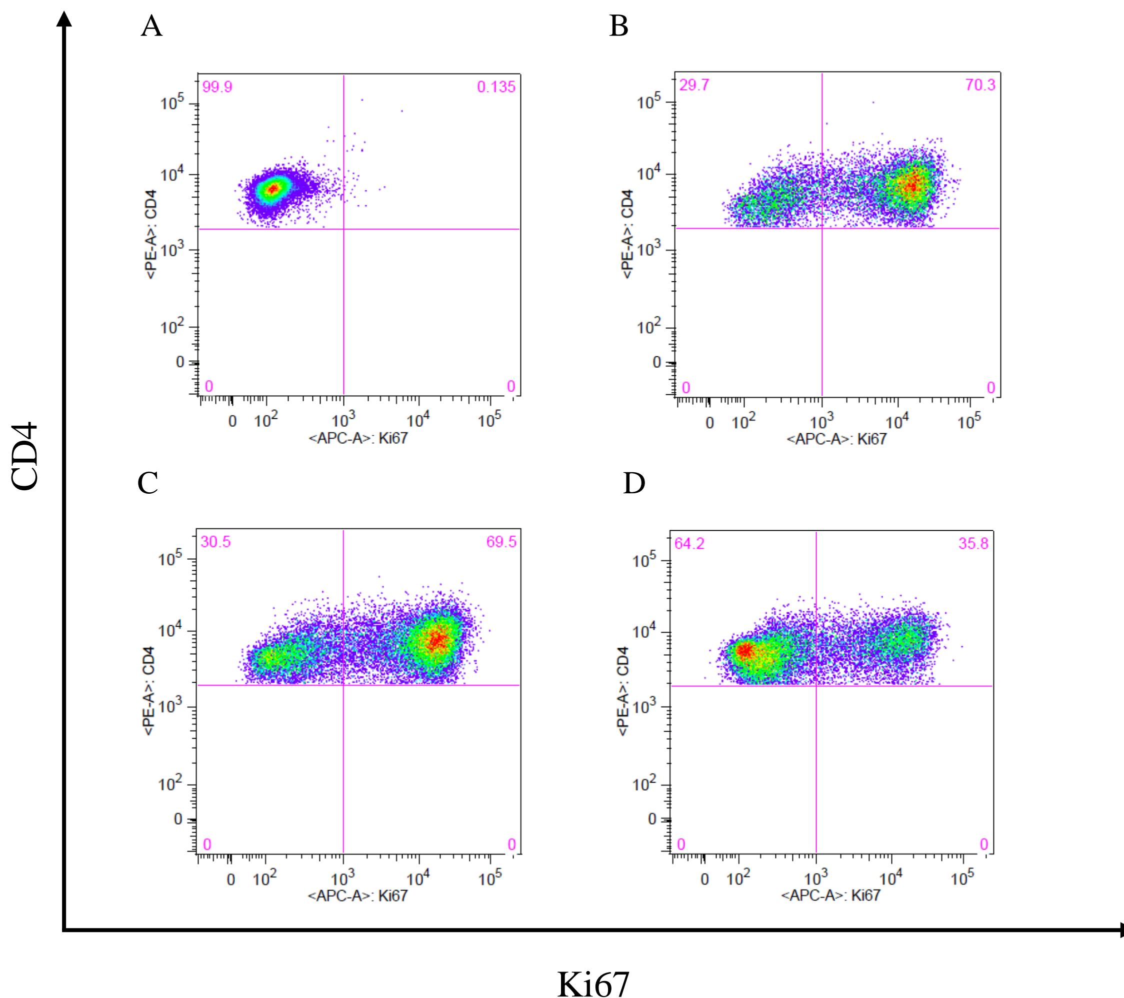


Figure 7. Expression of Ki67 in CD4+ T cells within PBMCs after in vitro proliferation with different stimuli.

Shown is a representative example of Ki67 expression in CD4+ T cells treated with different stimuli within the same experiment. The x-axis is Ki67 and the y-axis is CD4. Each dot represents a cell. Fresh PBMC were cultured with (A) medium only (unstimulated, 0.1% Ki67+ in CD4+ T cells), (B) αCD3/αCD28 coated magnetic beads (70.3%), (C) plate-bound αCD3/αCD28 antibodies (69.5%) and (D) PHA (35.8%) for 43 hours. The same experiment was repeated another two times using PBMCs obtained from different healthy donors, with similar results.

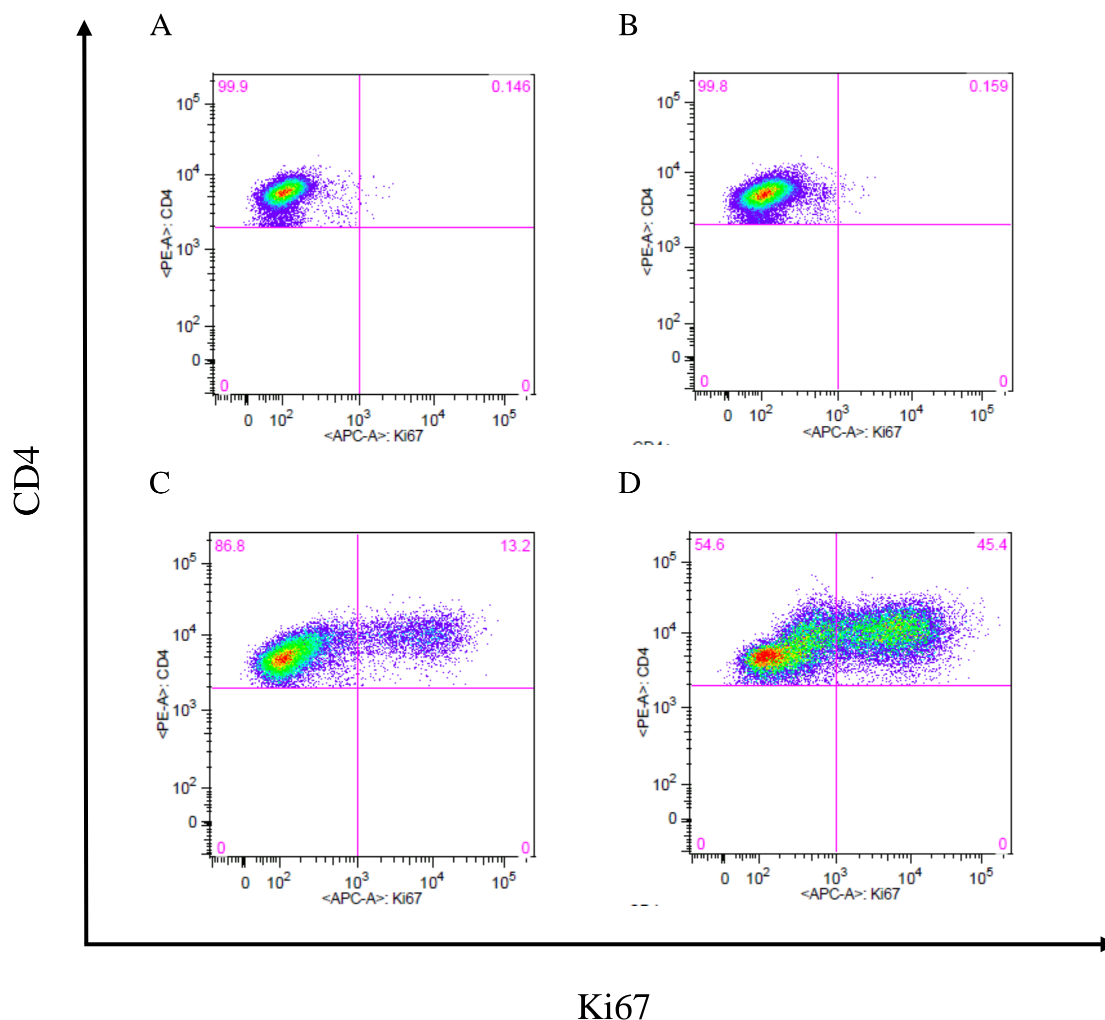
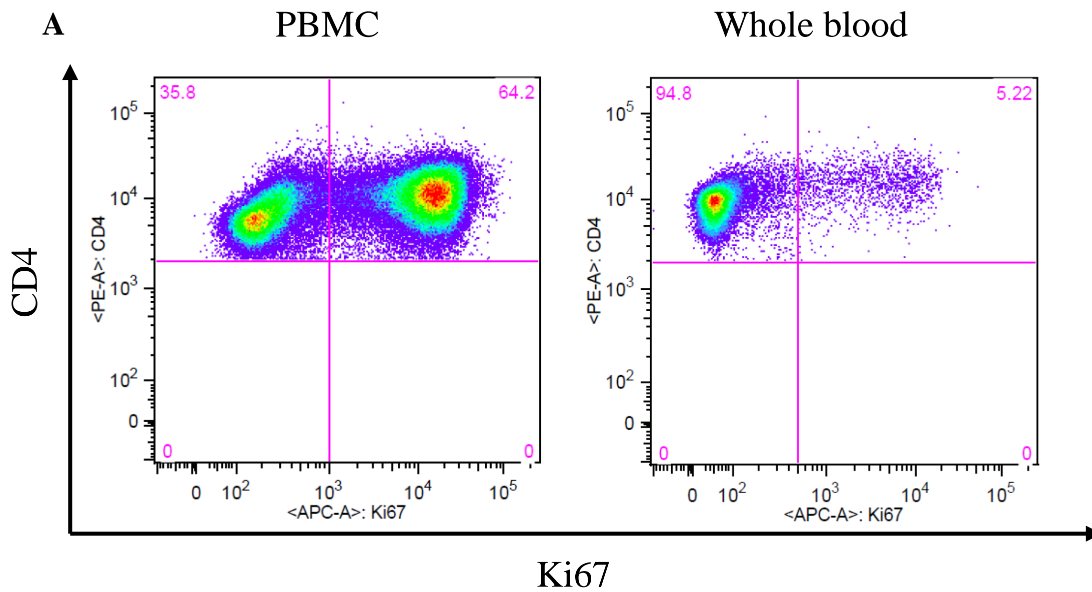


Figure 8. Expression of Ki67 in CD4+ T cells within whole blood after in vitro proliferation with different stimuli.

Shown is a representative example of Ki67 expression in CD4+ T cells treated with different stimuli within the same experiment. These data were obtained from the same sample and on the same day as that shown in Figure 7. The x-axis is Ki67 and the y-axis is CD4. Each dot represents a cell. Diluted whole blood was cultured with (A) medium only (unstimulated, 0.1% Ki67+ in CD4+ T cells), (B) αCD3/αCD28 coated magnetic beads (0.2%), (C) plate-bound αCD3/αCD28 antibodies (13.2%) and (D) PHA (45.4%) for 43 hours. The same experiment was repeated another two times using whole blood obtained from different healthy donors, with similar results.



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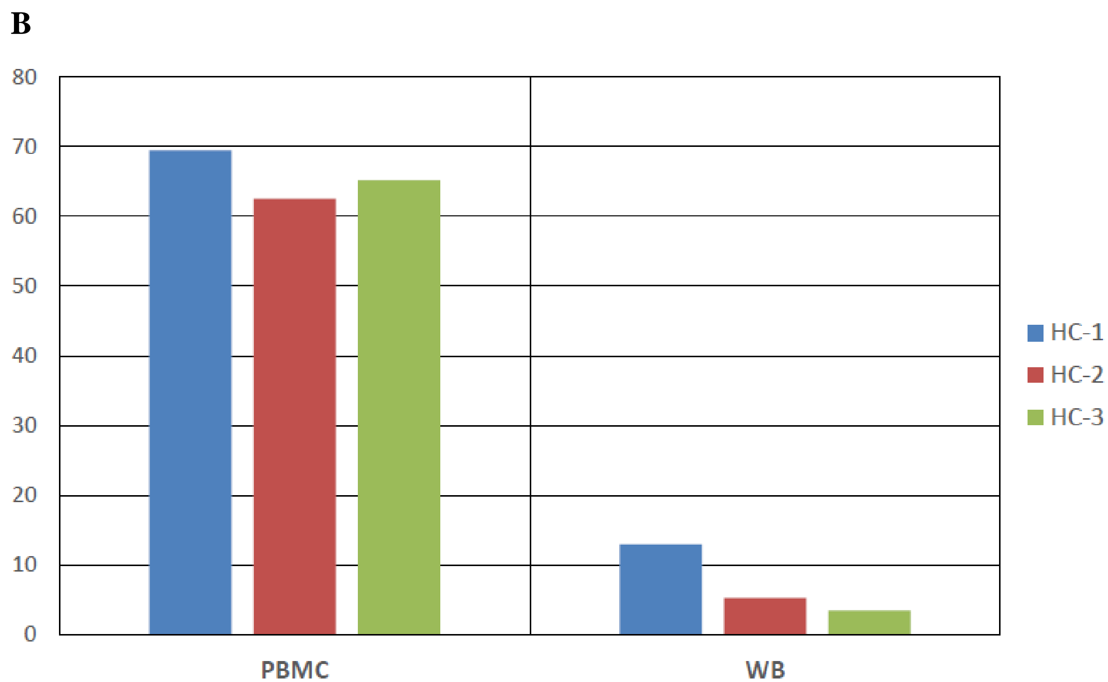

Figure 9. Expression of Ki67 in CD4+ T cells within PBMC and whole blood.
(A) A representative example shows the expression of Ki67 in CD4+ T cells. The x-axis is Ki67-APC and the y-axis is CD4-PE. Each dot represents a cell. PBMC and whole blood obtained from the same healthy donor and tested at the same time were used to compare the frequency of Ki67+ in CD4+ T cells after stimulation with plate-bound αCD3/αCD28 antibodies for 43 hours. In this example, the frequency of Ki67+ in CD4+ T cells was 64.2% in PBMC and 5.2% in whole blood, with all culture conditions being the same. (B) The x-axis indicates the different source material used and the y-axis shows the frequency of Ki67+ in CD4+ T cells. Each bar shows the results from a different healthy donor. The average frequency of Ki67+ in CD4+ T cells

in PBMC stimulated by plate-bound αCD3/αCD28 antibodies was 65.7% (±3.5%), while in whole blood the average frequency was 7.2% (±5.0%).

II. Clinical results

A trend toward an inverse correlation between the expression of Ki67 in CD4+ T cells and the frequency of M-MDSC. Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of myeloid cells that play a major role in the regulation of immune responses in many pathological conditions. These cells have a common myeloid origin, relatively immature state, common genetic and biochemical profiles, and most importantly, the ability to inhibit T cell proliferation. The fact that MDSCs can inhibit different types of T cell responses is widely accepted [28]. Human MDSCs were initially defined as HLA-DR$^{-/low}$CD33^{+} or CD14^{-}CD11b^{+} cells [29], with both phenotypes identifying cell populations with T cell suppressive activity. In this project, MDSCs were identified as Lin^{-} HLA-DR^{-} CD16$^{low/-}$ CD33^{+} CD11b^{+} cells. There are two major subsets of MDSC phenotypes which are monocytic MDSCs (M-MDSCs) with expression of CD14$^{+/dull}$ CD15^{-} [30, 31] and granulocytic MDSCs (G-MDSCs) with expression of CD14^{-} CD15^{+} [32, 33]. In recent years, it has become increasingly clear that MDSCs play a critical role in the regulation of different types of inflammation, and the role of MDSCs as an important negative regulator of immune responses has been observed in many pathological conditions including cancer. In some cancer settings such as melanoma, M-MDSCs appear to play a central role in creating an immunosuppressive environment [28].

In order to confirm that the expression of Ki67 in CD4+ T cells can be used as a proliferative marker of in vitro lympho-proliferation, we hypothesized that there should

be a relationship between these two biomarkers: if the frequency of MDSCs was relatively higher in patient samples, the percentage of Ki67+ in CD4+ T cells should be relatively lower, and thus suppression of proliferation or immune response of CD4+ T cells would be relatively greater. A total of 8 patients with chronic HCV infection were recruited for this project. PBMCs isolated from whole blood of HCV-positive patients' samples were cultured to measure the expression of Ki67 in CD4+ T cells after being stimulated by αCD3/αCD28 coated magnetic beads for 43 hours in vitro. Whole blood samples from the same individuals were used to measure the frequencies of MDSCs as well as M-MDSCs and G-MDSCs at the same time. We found that there was a strong trend toward an inverse correlation between the frequency of Ki67+ cells in CD4+ T cells after stimulation and the frequency of M-MDSCs, with a Pearson r of -0.68 (Fig. 10). This correlation coefficient was nearly significant, with a p value of 0.06. We also examined the relationship between the frequency of Ki67+ cells in stimulated CD4+ T cells and the frequency of total MDSCs as well as G-MDSCs. Neither correlation was significant (data not shown), suggesting that among these different MDSC populations, the M-MDSC population is likely to be the most functionally important for immunosuppression in chronic hepatitis C infection.

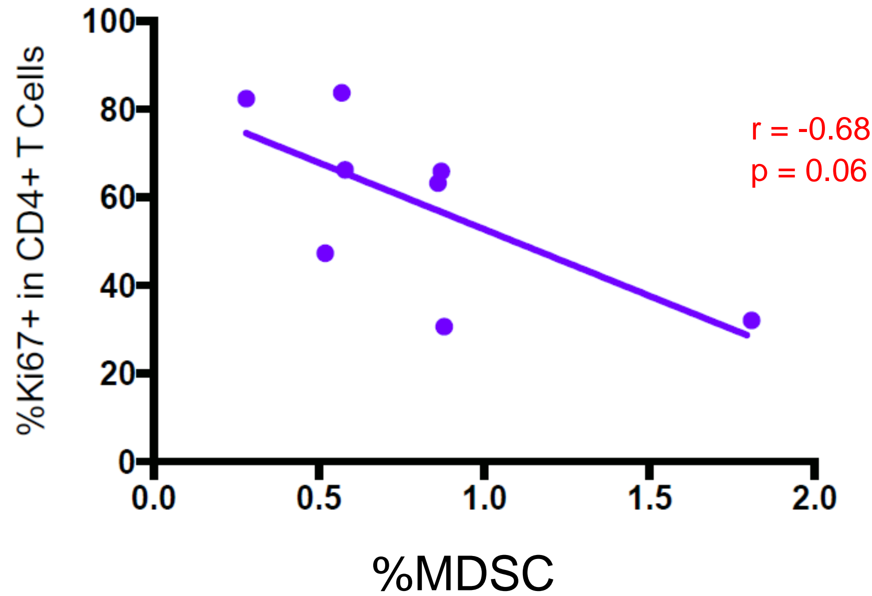

Figure 10. Inverse correlation between the expression of Ki67 in CD4+ T cells and M-MDSC frequency.

A total of 8 patients with chronic HCV infection were included in this analysis. The x-axis is the frequency of M-MDSCs in CD45+ cells and the y-axis is the percentage of Ki67+ in CD4+ T cells. The frequency of Ki67+ cells in CD4+ T cells in PBMCs was inversely correlated with the frequency of Monocytic MDSCs (Lin- HLA-DR^- $CD16^{low/-}$ $CD33^+$ $CD11b^+$ $CD14^+$ $CD15^-$ cells) in CD45+ cells (Pearson r = -0.68, P value = 0.06, α = 0.05).

Expression of Ki67 in CD4+ T cells in healthy donor samples vs diseased patient samples. In order to evaluate the effectiveness of this assay in assessing the function of diseased patients' immune systems, we compared the proliferative responses of CD4+ T cells, as shown by expression of Ki67, between chronically HCV-infected patients and healthy controls. PBMCs were obtained under informed consent from 11 patients for this project (Table 3). Fresh PBMCs obtained from 6 different healthy controls were also included in this analysis. At first, we grouped all patients together to compare the stimulated expression of Ki67 in CD4+ T cells with that of healthy controls. Figure 11 shows the expression of Ki67 in CD4+ T cells in unstimulated and αCD3/αCD28 bead-stimulated PBMCs after 43 hours of cell culture. The unstimulated cells from both patient and healthy control groups consistently showed extremely low Ki67+ percentages in CD4+ T lymphocytes, with a mean of 0.09% and standard deviation of 0.11% of Ki67+ cells in CD4+ T cells from the patients group and a mean of 0.5% and standard deviation of 0.36% of Ki67+ cells in CD4+ T cells from the healthy control group. When comparing αCD3/αCD28 bead-stimulated results from patients and healthy controls, the frequencies of Ki67+ cells among CD4+ T cells demonstrated more variability in the patient vs control group, with a patient mean of 62.8% and a standard deviation of 18.6% and a healthy control mean of 67% and standard deviation of 13.1%. Based on data shown in Figure 11 and Table 4, the expression of Ki67 in CD4+ T cells after stimulation was not significantly different between patients and healthy controls.

However, when patients were categorized based on their disease status as subgroups of chronic HCV infection, an interesting difference in the expression of Ki67 in CD4+ T cells was observed. Patients were separated into four groups: chronic HCV infection without cirrhosis, chronic HCV infection with liver cirrhosis, HCV infection with liver cirrhosis but currently on antiviral treatment and chronic HCV infection with untreated hepatocellular carcinoma (HCC) (Table 3, Fig.12). Among those four groups of patients, HCC patients were found to have a mean frequency of 31.3% Ki67+ in CD4+ T cells after being stimulated by αCD3/αCD28 beads, which was much lower than that of the other three HCV-positive patients' groups (HCV without cirrhosis: 71%; HCV with cirrhosis: 65.2%; HCV with cirrhosis currently on treatment: 73%) as well as healthy controls (67%) (Table 5). However, there was no difference in Ki67 expression in CD4+ T cells after stimulation between the other three patient groups and the healthy control group.

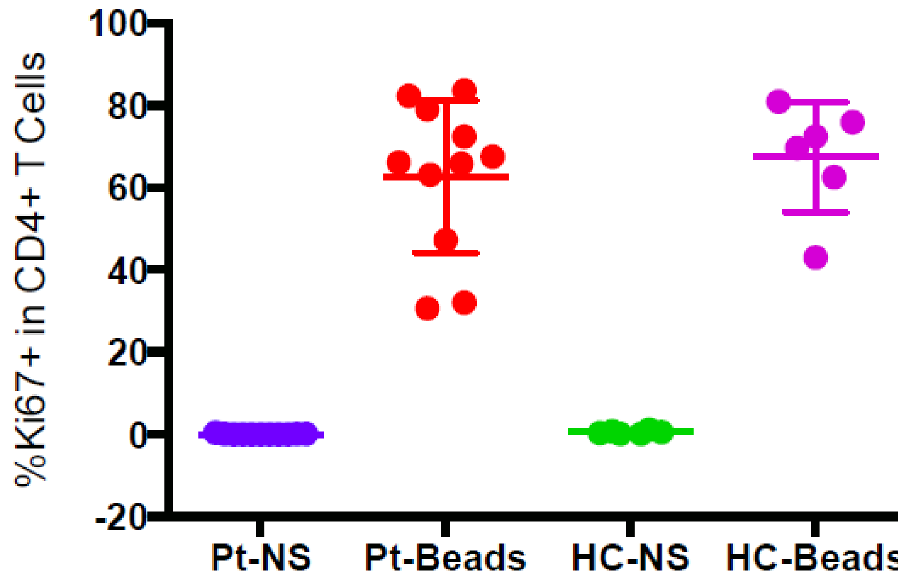

Figure 11. Expression of Ki67 in CD4+ T cells in PBMCs from patients and healthy control samples.

PBMCs obtained from 11 patient and 6 healthy control samples were cultured either in the absence of stimuli or with αCD3/αCD28 coated magnetic beads for 43 hours. Labels for the different patient groups and conditions are shown on the x-axis: Patient-No stimulation (Pt-NS), Patient-Bead stimulated (Pt-Beads), Healthy control-No stimulation (HC-NS), Healthy control-Bead stimulated (HC-Beads). The y-axis is the frequency of Ki67+ in CD4+ T cells. The middle line represents the mean value for the group. The error bars represent one standard deviation and are shown as the distance between mean and the top or bottom lines for each group. Healthy controls were fresh PBMC obtained from different healthy individuals.

Table 3. Numbers and disease status of patients tested.

Diseased Status of Patients	Number
Chronic HCV infection without cirrhosis	3
Chronic HCV infection with cirrhosis	3
Chronic HCV infection with cirrhosis currently on treatment	3
Chronic HCV infection with untreated hepatocellular carcinoma (HCC)	2
Sum	11

Four categories of patients with different liver disease status were consented for this project.

Table 4. **Mean and standard deviations of the frequencies of Ki67+ in CD4+ T cells in patient and healthy control groups.**

	Unstimulated PBMCs		αCD3/αCD28 beads stimulated PBMCs	
Frequency (%)	Patients	Healthy Controls	Patients	Healthy Controls
Mean	0.1	0.5	62.8	67.0
Standard deviation	0.1	0.4	18.6	13.1

Fresh PBMCs obtained from 11 patients and 6 healthy controls were cultured either without stimulus or with αCD3/αCD28 coated magnetic beads for 43 hours. Assays were conducted in triplicate for each sample tested.

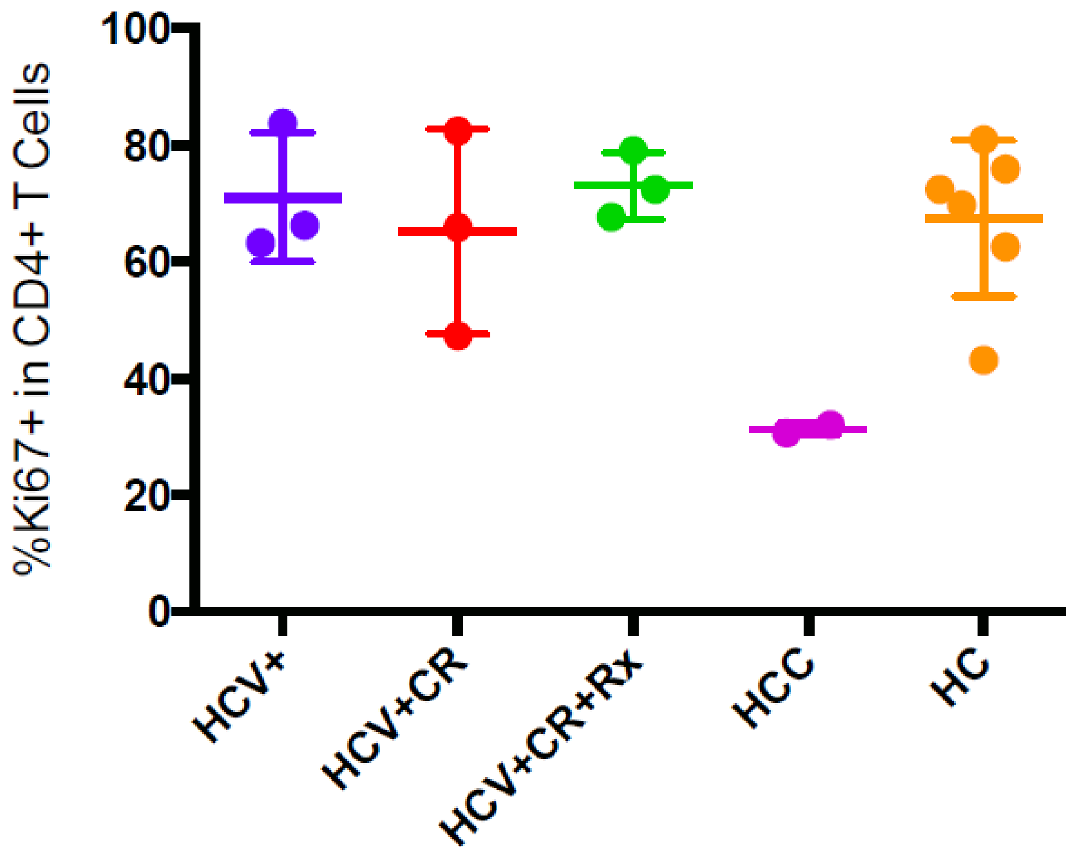

Figure 12. Expression of Ki67 in CD4+ T cells in PBMCs was compared for 4 patient subgroups and healthy controls.

The x-axis indicates the different patient groups and the y-axis is the frequency of Ki67+ in CD4+ T cells. PBMC obtained from patients or health donors, were cultured either in the absence of stimuli or with αCD3/αCD28 coated magnetic beads for 43 hours. Results are shown as circles in different colors. The middle line represents the mean value for the group. The error bars represent one standard deviation and are shown as the distance between middle line and top or bottom lines of each group, representing one standard deviation. A total of 11 patients were separated into 4 categories based on their different disease status (HCV+, HCV+CR (cirrhosis), HCV+CR+Rx (cirrhosis with treatment), HCC) while the last group consisted of 6 healthy donors (HC).

Table 5. Mean and standard deviation of the frequencies of Ki67+ in CD4+ T cells in four different patient groups as well as in healthy controls.

Patient groups	Mean (%)	Standard deviation (%)
Chronic HCV infection only	71.0	11.1
Chronic HCV infection with liver cirrhosis	65.2	17.6
Chronic HCV infection with liver cirrhosis but currently on treatment	73.0	5.7
Hepatocellular carcinoma (HCC)	31.3	1.0
Healthy controls	67.0	13.1

Patients were categorized based on different liver disease status as well as treatment. Fresh PBMCs were obtained from consented patients and healthy controls. PBMCs from each sample were cultured and tested in triplicate with αCD3/αCD28 coated magnetic beads for 43 hours. Mean and standard deviations of frequencies of Ki67 in CD4+ T cells were calculated based on data acquired from each sample.

Chapter IV: DISCUSSION

Measuring the expression of Ki67 as a proliferative marker in specific CD4+ T cells by flow cytometry could be a promising technique for evaluating the immune function of patients and a useful tool for prognostic assessment of immune suppression for some patients.

Assay optimization

As partially described in Chapter III: Assay optimization, we conducted many experiments to optimize the Ki67 flow cytometric assay before applying it to measure CD4+ T cell proliferation in patient samples. During this optimization phase, we identified that autofluorescence from monocytes complicated the analysis of our data, leading us to add anti-CD14 to exclude monocytes from the analyses (data not shown). We also added a viability marker to decrease noise from dead or dying cells, and adjusted the fluorochrome used for the Ki67 antibody to make it brighter. The experiments conducted to determine the best permeabilization buffer to use for the assay were described here. We only tested 70% ethanol vs Foxp3 buffer because 70% ethanol was recommended by the vendor of Ki67 antibodies, and used in other publications. However, we did not exclude the possibility that other concentrations of ethanol might work better than 70% ethanol or the Foxp3 Staining Buffer set. We did not perform an exhaustive analysis of other permeabilization methods because 1) we needed to balance the need for high levels of signal in healthy control samples with the potential loss of surface antigen signal using harsher permeabilization methods and 2)

we were able to obtain reasonably high levels of Ki67+ in CD4+ T cells results in our healthy controls using a commercially available reagent (Foxp3 Staining Buffer).

For assay optimization, αCD3/αCD28 coated magnetic beads at a 1:1 beads/cells ratio were determined to be the best stimulus for achieving the optimal Ki67 expression in CD4+ T cells among PBMC. We came to this conclusion after testing multiple different beads/cells ratio of αCD3/αCD28 beads, such as 1:1, 1:2, 1:5, 1:10, and found that the best Ki67 expression in CD4+ T cells was obtained with a 1:1 beads/cells ratio. On the other hand, the concentration of PHA we used to compare with αCD3/αCD28 beads was 20μg, and we did not test different concentrations of PHA for the PBMC source material because our previous experience suggested that PHA stimulation might yield more variable results than with aCD3/αCD28 beads. We therefore focused on identifying the best bead concentration to use in our assay.

After conducting multiple 7 day time course experiments, we also established 43 hours to be the optimal duration of cell stimulation for our assay.We recognize that different strength of cell stimulus could affect the optimal cell culture time. For example, a stronger cell stimulus could result in a greater increase of Ki67 expression in CD4+ T cells within 24 hours, instead of our 43 hours.

When we conducted the comparison experiment of PBMC *versus* whole blood, a 1:1 ratio of αCD3/αCD28 beads, a 1:1 ratio of plate-bound αCD3/αCD28 antibodies,

and 100μg PHA were used for cell stimulation. The concentrations of these plate-bound antibodies was determined after different concentrations of cell stimuli were tested. In addition, multiple concentrations of PHA were tested on whole blood prior to selecting the 100μg concentration. Ki67 expression in CD4+ T cells was also compared in whole blood with red blood cells lysed before and after cell stimulation with αCD3/αCD28 beads. Although greater Ki67 expression in CD4+ T cells was achieved in the pre-lysed RBC assay than the post-lysed RBC assay of whole blood, the frequency of Ki67+ in CD4+ T cells was still much lower than that in PBMCs.

A trend toward inverse correlation between Ki67 expression in CD4+ T cells and the frequency of M-MDSCs

Myeloid-derived suppressor cells (MDSCs) play a major role in the regulation of immune responses in cancer and many pathological conditions associated with chronic inflammations. It appears that, at least in cancer, monocytic MDSCs (M-MDSCs) may play a central role in the development of immune suppression. [27]. Many studies have also shown that most chronic infections are associated with an expansion of M-MDSCs, and M-MDSCs have a suppressive effect on CD4+ T cell proliferation [27, 33-35]. Now there is enough evidence demonstrating that G-MDSCs and M-MDSCs use different mechanisms of immune suppression [36]. Many reports indicate that M-MDSCs exert their suppressive activity in an antigen-independent manner. In contrast, G-MDSCs are largely dependent on reactive oxygen species (ROS) for their mechanism of action,

which requires closer and more prolonged cell-cell contact, and occurs during antigen-specific interactions [37-39]. According to our data, there was an inverse correlation between the frequency of M-MDSCs and increased expression of Ki67 in CD4+ T cells. This relationship indicates that the use of Ki67 in CD4+ T cells as a proliferative marker for in vitro lympho-proliferation is valid, and hereby, may be useful as a prognostic index for patients who have suppressed immune function. Although the inverse correlation between Ki67 expression in CD4+ T cells and the frequency of M-MDSC was relatively high at $r = -0.68$, the p-value of 0.06 was not quite significant. One possible reason that the p-value was not <0.05 could be that the sample size was not large enough to generate a significant p-value ($n=8$). This is a statistical issue and could be resolved by collecting more data from the same type of patients in the future.

Expression of Ki67 in CD4+ T cells in patient vs healthy control samples

Differences in patient selection can impact the results and interpretation of data [40]. In Fig. 11, there was no significant difference in the expression of Ki67 in CD4+ T cells between αCD3/αCD28 bead-stimulated patient and healthy control groups. Even though the average frequency of Ki67+ in CD4+ T cells for the entire patient group was similar to that of healthy control group, the frequency of Ki67+ cells in CD4+ cells for diseased patient was highly variable. However, we noticed that when we grouped patients based on their different disease subgroup status, obviously different results

were observed. Patients with chronic HCV infection plus untreated hepatocellular carcinoma showed a much lower frequency of Ki67+ cells in CD4+ T cells after stimulation than other patient groups, including the healthy control group. It has been shown that in cancer, not only functional exhaustion and elimination but also cell anergy can influence the impairment of T cell function [41]. Therefore, it follows that much less expression of Ki67 in CD4+ T cells was stimulated in this group of patients because of their severely impaired function and proliferative capacity. On the other hand, compared to healthy control group, the other three patient groups demonstrated similar or even higher percentages of Ki67+ in CD4+ T cells. These patient groups were all chronically infected with HCV infection and were without cirrhosis, had cirrhosis, or had cirrhosis but were currently on treatment. The Ki67 expression in CD4+ T cells in these patient groups was much higher than what we expected since some studies had shown HCV positive patients had a worse immune function than HCV negative patients [42-44]. Even though chronic HCV infection is known to cause impaired T cell responses, leading to T cell dysfunction through functional exhaustion, the mechanism for HCV-mediated T cell dysfunction is yet to be defined [41, 45]. However, one thing that needs to be remembered is that αCD3/αCD28 beads provide a very strong mitogenic stimulus, much stronger than a peptide or protein antigens. So it is likely that while the antigen specific response of T cells to HCV antigens may have been impaired, the non-specific response was not affected by HCV infection. In addition, the mean percentage of Ki67+ in CD4+ T cells in cirrhotic patient group was 8% less than the patient group without cirrhosis and patient group with cirrhosis but currently on

treatment. Although this is a small difference, it is possible that this difference could increase with more data. We speculate that the reason for this lower expression of Ki67 in CD4+ T cells might be impaired lymphocyte reactivity due to liver cirrhosis. Besides, cirrhotic patients have worse liver function than patients without cirrhosis, which could indirectly impact the immune function because more dysfunctional T cells from both HCV infection and liver cirrhosis could have accumulated. The difference of 8% was not statistically significant and is most likely because of the small sample size that we collected in our preliminary data. Therefore, additional data are needed to obtain a more reasonable estimate of the ultimate differences.

In our experiments, we found that anti-CD3/anti-CD28 beads could efficiently induce the peripheral T cells from healthy controls. Whether the same mitogenic stimulation protocol will be effective for inducing T cell proliferation in other patients with potentially defective T cell function is not clear. In several clinical settings, such as malignancy, chronic viral and bacterial infections, T cells were reported to be impaired in their ability to proliferate and produce effector cytokines upon TCR-associated stimulation [46]. This T cell hypo-responsiveness was related to downregulation of CD3ζ±CD28 and possibly upregulation of inhibitory molecules [47]. We would expect patients with certain disease conditions, such as various types of cancer, immunodeficiency and untreated HIV infection, especially those with impaired T cell function, could be differentiated from other patients by this assay. However, some studies have previously claimed that T cells from patients with deficient immune

function, such as HIV-infected individuals, generally proliferate as well as from healthy individuals and retain functional capacity after stimulation by αCD3/αCD28 beads [48]. We were only able to test a small number of disease patients in our comparison of Ki67 expression in CD4+ T cells to healthy controls, and all were chronically infected with HCV. Most were found to have excellent proliferative responses in our assay, except for the two patients with hepatocellular carcinoma. More testing in different types of disease populations to see if there is a difference between disease and healthy controls are needed to come to a more convincing conclusion about this assay can be used. Finally, data from patients with primary immunodeficiency will be required to demonstrate that this Ki67 flow cytometric assay can replace the use of the standard tritiated thymidine assay in the evaluation of these disorders.

A potential drawback of the Ki67 flow cytometric assay on PBMC could be its cost. Indeed, in our hands, the cost remains slightly higher than the cost of the Cylex Immuknow assay, when taking into account cell culture, testing reagents, instruments, waste management, and technician handling time [49]. However, this drawback may be able to be diseased in the future, with advantages in PBMC processing techniques and increased volume of testing, which could decrease the individual test cost. It was disappointing that we could not to develop a more robust Ki67 assay in whole blood, as it would represent an easier and faster approach that would minimize the cost of handling time. Although future studies may be able to identify ways to improve results from whole blood, our present data indicate that the Ki67 assay results were optimal

using PBMC as the source material.

The advantages of the Ki67 flow cytometric assay we developed make it a promising tool for evaluating the immune status. First of all, unlike the Immuknow assay, which consists of CD4+ T cell selection first and then detection of ATP released from CD4+ T cells, we used fluorescent antibodies to label cell surface markers, which can be detected by a flow cytometer and help us to measure the Ki67 expression in different cell populations at the same time. Second, by using flow cytometer, we can add more cellular labels to measure the proliferation of different subtypes of CD4+ T cells, i.e. T_H1 or T_H2 cells, which can provide us with more specific information about the immune response. Third, Ki67 expression in CD4+ T cells can be stimulated with both non-specific mitogens and specific antigens. As a result, this assay provides us with a way to evaluate both non-specific and antigen-specific immune response of CD4+ T cells. Importantly, we showed that this nonradioactive method has excellent repeatability and reproducibility, which are important characteristics for a clinical assay.

In summary, even though more data are needed to further validate the Ki67 flow cytometric assay, our current observations provide new insights into a possible method of assessing the proliferation of CD4+ T cells by measuring the expression of Ki67 in cell nuclear after in vitro stimulation by using flow cytometer, and thereby a novel prognostic tool for evaluating the immune suppression.

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APPENDIX: PROCEDURES

PBMC Isolation Procedure

I. Reagents

1. Wash Diluent Reagents (WDR)

   Hanks' Balanced Salt Solution with Ca^{2+}/Mg^{2+} free (Gibco by Life Techbologies);

2. RPMI 1640 1x with L-Glutamine and 25mM Hepes

3. Ficoll-Hypaque (GE Healthcare)

4. Cell counting reagents

   0.4% trypan blue solution and 2% Acetic acid.

5. Cryopreservation reagents

   12.5% HSA in RPMI 1640 and 2x Cryoprotectant solution or 2x Freezing media.

II. PBMC isolation procedure

1. Recording collected blood volumes

   1) Document the type of blood received.

   2) Record the total number of tubes discarded or any other problems noted with the blood.

   3) Record the blood condition.

   4) Invert the blood collection tubes to mix. Pool the blood collection tubes from each PTID into 50mL conical tubes using a graduated pipette.

   5) Record the blood volume.

2. Plasma removal and replacement

   1) Centrifuge the whole blood at 300 x g for 10 minutes.

   2) Transfer the plasma supernatant to a 15 or 50mL centrifuge tube for second centrifugation to remove any cellular debris.

   3) Add sufficient quantity of WDR to bring blood back to its original whole blood volume, mix gently and continue PBMC processing.

   4) Complete the plasma processing by centrifuging the collected plasma at 1500 x g for 10 minutes.

   5) Aliquot spun plasma into labeled aliquot tubes and discard any cellular debris in spun plasma tube.

3. Blood dilution and manual density gradient cell separation

   1) Pool all blood from same PTID into a sterile container large enough to fit 2x total blood volume.

   2) Add sufficient volume of WDR to dilute the blood. Maximum ratio of blood to WDR should be 2:1. Mix gently.

   3) Density gradient cell separation.

      This method uses 2 parts diluted blood to 1 part Ficoll.

      Ficoll-Hypaque overlay method: Aseptically add the required Ficoll to the sterile centrifuge tubes. Carefully and slowly pipet diluted blood on top of gradient medium.

   4) Carefully cap the tubes.

4. Lymphocyte density centrifugation and collection

1) Keep the tubes in an upright position and gently transfer them to the centrifuge.
2) Centrifuge 400 x g for 40 minutes at 15 to 30°C with the Brake OFF.
3) Prepare one sterile 50mL conical tubes for up to 2 Ficoll separation tubes used in the separation step.
4) Remove the tubes from the centrifuge.
5) Using a new sterile pipet for each PTID, remove the upper, yellowish, plasma-WDR fraction down to within approximately 2 cm of the cloudy white PBMC band located at the interface between the plasma-WDR fraction and the clear separation medium solution.
6) Using a sterile serological or transfer pipet, collect all cells at the cloudy white interface.
7) Combine collected cells from up to two centrifuge tubes to a single corresponding, pre-labeled, sterile 50mL conical tube.

5. Wash 1:
   1) QS the fraction of PBMC to 45mL by adding WDR.
   2) Re-cap all of the harvested cell tubes and mix by inverting gently 2 or 3 times.
   3) Centrifuge the tubes at 300 x g for 10 minutes at 15 to 30°C (brake).
   4) Remove the tubes from the centrifuge and check for cell pellets.
   5) Carefully remove and discard the supernatant by pouring or pipetting into bleach solution without disturbing the cell pellet.

6. Wash 2:
    1) Re-suspend each pellet in the remaining small volume of WDR by flicking the tube into a homogenous cell suspension.
    2) For 50mL conical tubes, combine up to four pellet suspensions from the same donor. Bring the total volume of PBMC fraction to approximately 45mL by adding WDR. Mix gently.
    3) Use a small volume of WDR to rinse the tubes from which the pellets were transferred.
    4) Re-cap the tubes and place the tubes in the centrifuge.
    5) Centrifuge diluted cells at 300 x g for 10 minutes at 15 to 30°C (brake).
    6) Remove and discard the supernatant without disturbing the cell pellet.
7. PBMC cell count
    1) Record the counting method used on the PBMC processing Worksheet.
    2) Calculate and record on the processing worksheet, the WDR counting resuspension volume (V). This is the volume on which the cell count is based.
    3) Mix cell gently, but thoroughly, before sampling for the cell count. All freshly isolated PBMC from whole blood should be manually counted using hemacytometer.
    4) Transfer 20uL of the cell suspension to a 96 well plate for counting.
    5) Add 180uL of 0.4% Trypan Blue solution or 90uL of 0.4% Trypan Blue and 90uL of 2% acetic acid solution to the 20uL of cell solution. The cells are

now diluted 1:10.

6) Load 10uL of cells in Trypan Blue solution to both sides of the hemacytometer and count each side.

7) To calculate the total of live and dead cells, add the values for sum of both sides together.

8) Divide the sum of both sides of live cells by 8 since 8 fields were counted.

9) To determine the concentration of cells in solution, multiply the average live cells with the dilution factor (10) and 10^4.

10) To determine the total cell number of live cells, multiply the cell concentration by volume of WDR.

11) The % viability = Total live cells/ (Total live + Dead cells)*100.

8. Calculation of final freezing media volume

9. Final centrifugation

    1) QS cell suspension to 45mL with WDR.

    2) Place the harvested cell tube in the centrifuge.

    3) Centrifuge diluted cells at 300 x g for 10 minutes at 15 to 30°C (brake).

10. Cryopreservation

    1) Remove and discard the WDR supernatant. Keep the pellet.

    2) Pre-chill vials and/or work on wet ice.

    3) Remove the 2x freezing media and 12.5% HSA from 4°C.

    4) Calculate volume of cryopreservation media needed.

    5) Re-suspend the pellet with cryopreservation media.

6) Aliquot 1mL of re-suspended cells per cryovial.

7) Immediately transfer all cryovials to the controlled-rate freezing container.

Thawing frozen PBMC procedure

1. Equipment and materials

    1) L-glutamine (GIBCO BRL Life Technologies)

    2) Penicillin-Streptomycin (GIBCO BRL Life Technologies)

    3) Human Serum heat inactivated

    4) Benzonase (Novagen)

    5) RPMI 1640 media (GIBCO BRL Life Technologies)

    6) Biological safety cabinet

    7) 37°C water bath

    8) Cell counter for counting cells

    9) Incubators (37°C, 5% CO_2)

    10) Pipettors

    11) Centrifuges

2. Procedure

    1) Prepare media

        a) Prepare R10 media

        Add 50mL of heat inactivated HS, 5mL of L-glutamine and 5mL of Penicillin-Streptomycin to 500mL of RPMI 1640 with 25mM HEPES buffer and L-glutamine.

        b) Prepare thawing media (TMB)

        Add 4uL of Benzonase to 20mL of prewarmed R10 culture media, final Benzonase concentration is 50U/mL.

c) Pipet 19mL of TMB into a 50mL conical tube. Place media-filled tubes in the incubator to warm for one hour.

2) Remove cell vials from liquid nitrogen freezers

3) Thaw cells

i) Take 1 cryovial and partially submerge each vial in the water bath, swirling the tubes.

ii) When a small bit of ice remains in the cryovial, transfer to the biosafety cabinet.

iii) Using a pipet, remove 1mL of TMB from the pre-warmed 50mL conical tube for every vial thawed.

iv) Add TMB dropwise to each cryovial.

v) Transfer cells from the vial into the remaining TMB in the 50mL conical tube.

vi) Centrifuge the conical tubes at 300 x g for 10 minutes.

vii) Following centrifugation of the thawed, diluted cells, decant the supernatant. Resuspend the cells in the small volume of media that remains after decanting by flicking the tube.

viii) Add 10mL of pre-warmed R10 to each tube.

ix) Centrifuge the conical tubes at 300 x g for 10 minutes.

x) Decant the supernatant.

xi) Resuspend the cells in the media that remains after decanting by flicking.

xii) Resuspend the cells in 2-5mLs of media (~4M/mL depending on the initial

cell count).

xiii) Remove 20uL for counting.

xiv) Bring the media volume to the desired concentration.

PBMC Stimulation & Culture Procedure

I. Reagents

1. Cell culture media

   1) RPMI 1640 500mL,

   2) 10% Fetal Bovine Serum,

   3) L-Glutamine 5mL,

   4) Penicillin-Streptomycin 5mL.

2. Cell stimuli

   1) $1x10^8$ anti-CD3/anti-CD28 coated magnetic beads 20uL,

   2) 1mg/mL Phytohaemagglutinin (PHA) 100uL,

   3) 200uL 10ug/mL anti-CD3 antibody and 10uL 1mg/mL anti-CD28 antibody.

3. DPBS (Dulbecco's Phosphate buffered saline, Gibco by Life Technologies).

II. Fresh PBMC preparation

1. PBMC isolation, referred to PBMC isolation procedure.

2. Freshly isolated PBMC was expanded with cell culture media at $2x10^6$ concentration.

3. 1mL well prepared PBMC mixture was added into 24-well plate.

III. Cell stimuli preparation

1. Anti-CD3/anti-CD28 coated magnetic beads preparation

   1) Resuspend anti-CD3/anti-CD28 beads in the vial (i.e. vortex for > 30 sec).

   2) Transfer a desired amount of anti-CD3/anti-CD28 coated magnetic beads into a new vial.

3) Wash beads with 1mL DPBS (vortex for 5 sec).

4) Place the vial on a magnet for one minute to separate magnetic beads from DPBS and discard supernatant.

5) Remove the vial from magnet and re-suspend anti-CD3/anti-CD28 beads with cell culture media back to the original volume.

6) Ready for cell stimulation.

2. Plate-bound anti-CD3/anti-CD28 antibody preparation

1) Dilute 1mg/mL anti-CD3 soluble antibody into 10ug/mL concentration at desired volume.

2) Coat each well with 200uL 10ug/mL anti-CD3 antibody overnight.

3) In the following day, take anti-CD3 antibody out of plate well and rinse each well with 1mL DPBS one time.

4) Ready for cell stimulation.

Ⅳ. Cell culture

1. Cells are separated into four different groups:

1) Unstimulated cell group,

2) 20uL $1x10^8$ anti-CD3/anti-CD28 coated magnetic beads

3) 100uL 1mg/mL PHA.

4) 10ug anti-CD3 antibody and 10ug anti-CD28 antibody.

2. Cell mixture is incubated at 37°C with 5% CO_2 for 43 hours.

PBMC staining procedure

1. Reagents

    1) 2% FACS buffer

        Add 2mL FBS into every 100mL DPBS to make 2% FACS buffer.

    2) DPBS (Dulbecco's Phosphate buffered saline, Gibco by Life Technologies)

    3) Fluorochrome conjugated antibodies

        Fixable Viability Dye eFluor 700 (BD Horizon), CD3-FITC (SK7, BD Bioscience), CD3-APC (UCHT1, Beckman Coulter), CD4-PE (RPA-T4, Biolegend), CD8-PerCP-Cy5.5 (SK1, BD Bioscience), CD14-PerCP-Cy5.5 (MϕP9, Biolegend), Ki67-APC (Ki67, Biolegend).

    4) 2% Paraformaldehyde (PFA)

2. Extracellular cell staining

    1) Transfer 500uL PBMC into each labeled 5mL FACS tube.

    2) Wash cells with 1mL FACS buffer one time. Spin tubes at 1200rpm for 5minuts.

    3) Flick the liquid off into sink.

    4) No stain tube: add 5uL FACS buffer.

    5) Single stain tubes: add 5uL CD3-FITC, 2uL CD4-PE, 5μL CD8-PerCP/Cy5.5 into each corresponding tube.

    6) Cultured cell tubes: 5uL CD14-PerCP/Cy5.5, 5uL CD3-FITC, 2uL CD4-PE into each tube.

    7) Incubate stained tubes at room temperature in dark for 30 minutes.

    8) After incubation, wash cells with 1mL FACS buffer one time. Spin at 1200rpm

for 5 minutes. Discard supernatant.

3. Cell viability staining
    1) After extracellular cell staining, wash cells with 1mL DPBS twice. Spin at 1200 rpm for 5 minutes. Discard supernatant.
    2) Dilute 5uL fixable viability dye eFluor 700 into 50uL with DPBS.
    3) Add 2uL 1:10 diluted fixable viability dye eFluor 700 into corresponding single stain cell tube and cultured cell tubes.
    4) Mix cells well. Incubate tubes at 4°C in dark for 30 minutes.
    5) Wash cells with 1mL FACS buffer twice. Spin at 1200rpm for 5 minutes. Discard supernatant.
4. Cell fixation/permeabilization
    1) Dilute Foxp3 solution with diluent at 1:4 ratio (1mL Foxp3 solution + 3mL diluent).
    2) Add 1mL diluted Foxp3 solution into each tube.
    3) Incubate cell tubes at room temperature in dark for 45 minutes.
    4) Dilute Foxp3 solution buffer with distilled water at 1:10 ratio (1mL 10x buffer and 9mL distilled water).
    5) Add 2mL 1x buffer into each tube. Spin at 1350rpm for 5 minutes. Discard supernatant. Repeat one more time.
5. Intracellular cell staining
    1) Add 5uL CD3-APC into APC single stain tube.
    2) Add 5uL Ki67-APC into cultured cells.

3) Incubate tubes at room temperature in dark for 30 minutes.

4) Wash cells with 1mL FACS buffer twice. Spin at 1350rpm for 5 minutes.

5) Add 200uL 2% Paraformaldehyde (PFA) into each tube.

6) Store tubes at 4°C in dark and ready to acquire.

Whole blood Stimulation & Culture Procedure

I. Reagents

a) Cell culture media

Serum-free culture media

b) Cell stimuli

i. $1x10^8$ anti-CD3/anti-CD28 coated magnetic beads at different beads/cells ratio,

ii. Different concentrations of Phytohaemagglutinin (PHA),

iii. Different ratio of 10ug/mL anti-CD3 antibody and 1mg/mL anti-CD28 antibody.

c) DPBS (Dulbecco's Phosphate buffered saline, Gibco by Life Technologies).

II. Whole blood preparation

a) Dilute heparinized whole blood with serum-free culture media at a 1:10 ratio.

b) Add 500μL 1:10 diluted whole blood to 48-well flat bottom plate.

c) (Optional) Pre-lysing RBC assay: add 1:10 diluted RBC Lysis Buffer 10X to each well. (if don't perform pre-lysing RBC assay, skip to this step)

III. Cell stimuli preparation

a) Anti-CD3/anti-CD28 coated magnetic beads preparation

i. Resuspend anti-CD3/anti-CD28 beads in the vial (i.e. vortex for > 30 sec).

ii. Transfer a desired amount of anti-CD3/anti-CD28 coated magnetic beads into a new vial.

iii. Wash beads with 1mL DPBS (vortex for 5 sec).

iv. Place the vial on a magnet for one minute to separate magnetic beads from DPBS and discard supernatant.

v. Remove the vial from magnet and re-suspend anti-CD3/anti-CD28 beads with cell culture media back to the original volume.

vi. Ready for cell stimulation.

b) Plate-bound anti-CD3/anti-CD28 antibody preparation

i. Dilute 1mg/mL anti-CD3 soluble antibody into 10ug/mL concentration at desired volume.

ii. Coat each well with desired volume of 10ug/mL anti-CD3 antibody overnight.

iii. In the following day, take anti-CD3 antibody out of plate well and rinse each well with 1mL DPBS one time.

iv. Ready for cell stimulation.

IV. Cell culture

a) Cells are separated into four different groups:

i. Unstimulated cell group,

ii. $1x10^8$ anti-CD3/anti-CD28 coated magnetic beads with desired concentration.

iii. Desired volume of 1mg/mL PHA.

iv. Desired ratio of 10ug/mL anti-CD3 antibody and 1mg/mL anti-CD28 antibody.

V. Cell mixture is incubated at 37°C with 5% CO_2 for 43 hours.

Whole blood staining procedure

1. Reagents

   1) 2% FACS buffer

      Add 2mL FBS into every 100mL DPBS to make 2% FACS buffer.

   2) DPBS (Dulbecco's Phosphate buffered saline, Gibco by Life Technologies)

   3) Fluorochrome conjugated antibodies

      Fixable Viability Dye eFluor 700 (BD Horizon), CD45-Pacific Blue (Biolegend), CD3-APC (UCHT1, Beckman Coulter), CD4-PE (RPA-T4, Biolegend), CD8-PerCP-Cy5.5 (SK1, BD Bioscience), CD14-FITC (MϕP9, BD Pharmingen), Ki67-APC (Ki-67, Biolegend).

   4) 2% Paraformaldehyde (PFA)

2. Extracellular cell staining

   1) Transfer 500uL PBMC into each labeled 5mL FACS tube.

   2) No stain tube: add 5uL FACS buffer.

   3) Single stain tubes: add 5uL CD45-Pacific Blue, 15uL CD14-FITC, 2uL CD4-PE, 10uL CD8-PerCP/Cy5.5 into each corresponding tube.

   4) Cultured cell tubes: 5uL CD45-Pacific Blue, 15uL CD14-FITC, 10uL CD8-PerCP/Cy5.5, 2uL CD4-PE into each tube.

   5) Incubate stained tubes at room temperature in dark for 30 minutes.

3. Red blood cells lysing (Post-lysing RBC assay only)

   1) Dilute 10x BD PharmLyse solution with distilled water at 1:10 ratio into 1x PharmLyse solution.

2) Add 1mL 1x PharmLyse solution into each tube.

3) Incubate at room temperature for 20 minutes.

4) Add 2mL FACS buffer into each tube. Spin at 1200rpm for 5 minutes. Discard supernatant.

5) Repeat washing cells one more time. Discard supernatant.

4. Cell fixation/permeabilization

1) Dilute Foxp3 solution with diluent at 1:4 ratio (1mL Foxp3 solution + 3mL diluent).

2) Add 500uL diluted Foxp3 solution into each tube.

3) Incubate cell tubes at 4°C in dark for 45 minutes.

4) Dilute Foxp3 solution buffer with distilled water at 1:10 ratio (1mL 10x buffer and 9mL distilled water).

5) Add 1mL 1x buffer into each tube. Spin at 1350rpm for 5 minutes. Discard supernatant. Repeat two times more.

5. Intracellular cell staining

1) Add 5uL CD3-APC into APC single stain tube.

2) Add 5uL Ki67-APC into cultured cells.

3) Incubate tubes at room temperature in dark for 30 minutes.

4) Wash cells with 1mL FACS buffer twice. Spin at 1350rpm for 5 minutes.

5) Add 200uL 2% Paraformaldehyde (PFA) into each tube.

6) Store tubes at 4°C in dark and ready to acquire.