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Research paper

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 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.

1. 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 (Dock and Effros, 2011). 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 (Banerjee et al., 2000; Mowat and Viney, 1997), 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

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.

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mucosal immune system earlier than in the peripheral immune compartment (Koga et al., 2000). 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 (Shacklett and Anton, 2010). 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 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; 70 cm^3 of peripheral blood for the proliferation and other assays were collected in seven 10 ml Heparin tubes. PBMC designated for the proliferation assay were immediately isolated by Ficoll gradient separation. Following Ficoll centrifugation, PBMC were washed with 1× PBS and resuspended in 10 ml culture media (1× RPMI 1640, 15% FBS, 10 mM HEPES, 2 mM glutamine, 50 IU/ml penicillin/streptomycin, 500 μg/ml Zosyn [piperacillin-tazobactam], 1.25 μg/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 (Shacklett et al., 2003). Briefly, rectosigmoid biopsies were endoscopically acquired by flexible sigmoidoscopy between 10 cm and 30 cm 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 50 ml tubes containing 20–25 ml of RPMI medium with 7.5% fetal calf serum (FCS) (R7.5), L-glutamine, amphotericin-B (1.25 μg/ml) and piperacillin-tazobactam (50 μg/ml). Samples were transported to the laboratory within 2 h of collection. Upon receipt, the transport media was aspirated and biopsies incubated in 20–25 ml 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, 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 h duration). The isolated mucosal mononuclear cells (MMC) were combined and resuspended in 5 ml of RPMI medium containing 10% FCS, amphoterin-B (1.25 μg/ml) and Zosyn (50 μg/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 (Parish et al., 2009), 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 2–3×10^6 mucosal CD3 T cells were separated into 15 ml polypropylene tubes, washed in 5 ml 1× PBS, centrifuged for 10 min, and pellets were resuspended in 1 ml 1× 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 min at 37 °C, washed twice (once in 10% pure FCS (9 ml PBS 1×, 1 ml FCS) and once in 1% pure FCS (9.9 ml PBS 1×, 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 1 ml 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.