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Los Angeles

Beyond blood: expanding immune studies to the human gut

A dissertation in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Cellular and Molecular Pathology

by

Jeffrey NelsFranklin Dock

2014



## ABSTRACT OF THE DISSERTATION

Beyond blood: expanding immune studies to the human gut

by

Jeffrey NelsFranklin Dock

Doctor of Philosophy in Cellular and Molecular Pathology

University of California, Los Angeles, 2014

Professor Rita B. Effros, Chair

A hallmark of age-related immunosenescence is the accumulation of late-differentiated memory CD8 T cells in peripheral blood with features of replicative senescence, such as inability to proliferate, absence of CD28 expression, shortened telomeres, loss of telomerase activity, enhanced activation, and increased secretion of inflammatory cytokines. Oligoclonal expansions of these late-differentiated memory CD8 T cells in peripheral blood are correlated with increased risk of morbidity and mortality in elderly humans. The studies described in this thesis focus on furthering our understanding of CD8 T cell replicative senescence in humans and its relationship to aging and HIV disease, with special emphasis on immune features of gut-associated lymphoid tissue (GALT), which contains 40-65% of lymphocytes (as opposed to peripheral blood, which houses only 2%) and is an area of high antigenic exposure.

We first review the data for CD8 T cell replicative senescence in peripheral blood. This includes a comprehensive discussion of experiments defining the phenotype and function of senescent cells *in vitro*. We then present evidence that CD8 T cell replicative senescence occurs *in vivo*, and discuss how senescent cells contribute to, and are affected by, dysregulated immunity in aging and HIV disease, ultimately contributing towards “inflammaging” and an immune risk phenotype (IRP) predictive of morbidity and mortality. We next move the focus of our studies to the GALT and compare blood and gut (specifically rectosigmoid colon) CD8 T cells, focusing on age-related phenotypic and functional alterations previously linked to senescence in peripheral blood. Overall, our results support the hypothesis that gut CD8 T cells have profiles suggestive of greater “aging” than those in peripheral blood. These results include a significant increase in RA-CD8<sup>+</sup> (memory), CD28-CD8<sup>+</sup> (differentiated), RA-CD28-CD8<sup>+</sup> (differentiated memory), DR<sup>+</sup>CD38<sup>+</sup>CD8<sup>+</sup> (activated), CD8  $\alpha/\alpha$ , RO+PD-1+CD8<sup>+</sup>, and a (non-significant) decrease in baseline telomerase activity. However, other criteria from this study (lower levels of CD57 expression, lack of inverted CD4:CD8 ratio, increase in KI-67 (proliferation) expression, and proliferative potential that is similar to blood) suggest that gut CD8 T cells may not be more senescent than peripheral blood. Our data suggest that additional work (such as looking at CD4 cells, other regions of the gut, and CD8 T cell gut senescence in aging and HIV disease) is needed to further elucidate this relationship. We then describe the development and optimization of a novel protocol to test our hypothesis of compartment-specific differences of *in vitro* proliferative dynamics of CD8 T cells. The protocol involves a 5 day culture of mononuclear leukocyte populations from blood and gut, respectively, labeled with CFSE and BrdU and stimulated with anti-CD2/3/28-linked microbeads. The specific experimental variables we have optimized include: mode of T cell stimulation, CFSE

concentration, inclusion of second proliferation marker BrdU, culture duration, initial cell concentration and inclusion of autologous irradiated feeder cells. Finally, we transition back to peripheral blood in order to examine a link between peripheral blood T cell senescence and a mild presentation of Kaposi's sarcoma in treated HIV-infected subjects with long-term suppression of HIV replication. This study indicates these subjects exhibit some features of immunosenescence relative to treated controls without sarcoma (increased frequency of CD57+ and CD28- CD4 and CD8 T cells, decreased proportion of naïve CD27+CD28+CD45RA+ CD4 and CD8 cells, increased expression of CCR5 in CD4 and CD8 T cells), indicating immunologic perturbations associated with immune senescence might be casually associated with development of this disease. Together, results from this research further our understanding of CD8 T cell senescence, especially in the rectosigmoid colon region of the GALT, and pave the way for further research elucidating the relationship of CD8 T cell immunosenescence and pathologies such as aging, HIV disease and Kaposi's sarcoma.

The dissertation of Jeffrey NelsFranklin Dock is approved.

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## PUBLICATIONS

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# **Chapter 1**

## **Introduction**

### **Role of CD8 T cell replicative senescence in human aging and in HIV-mediated immunosenescence**

## Review

## Role of CD8 T Cell Replicative Senescence in Human Aging and in HIV-mediated Immunosenescence

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**ABSTRACT:** As humans age, their immune systems undergo a process known as immunosenescence. This global aging of the immune system is associated with increased susceptibility to infectious diseases and cancer, reduced effectiveness of vaccination, increased autoimmune phenomena, and tissue damage due to dysregulated inflammation. One hallmark feature of immunosenescence is the accumulation of late-differentiated memory CD8 T cells with features of replicative senescence, such as inability to proliferate, absence of CD28 expression, shortened telomeres, loss of telomerase activity, and enhanced secretion of inflammatory cytokines. The proportion of senescent CD8 T cells increases progressively with age, and often consists of oligoclonal populations that are specific for cytomegalovirus (CMV) antigens. In addition, there is evidence that senescent memory CD8 T cells acquire suppressive functions and may also contribute to carcinogenesis. Chronic HIV disease, even when controlled through antiretroviral therapy (ART), is associated with accelerated immunosenescence, as evidenced by the higher numbers of senescent memory CD8 T cells and increased inflammatory milieu. Interestingly, even in HIV disease, a high proportion of late-differentiated, putatively senescent, memory CD8 T cells are specific for CMV antigens. As in age-related immunosenescence, these HIV-associated changes result in dysregulated immunity, chronic diseases linked to inflammatory damage, and increased morbidity and mortality. This review explores the evidence for CD8 T cell replicative senescence *in vitro* and *in vivo*, in the context of both chronological aging and HIV-mediated immunosenescence. We also highlight an important gap in our understanding of human immunosenescence, since all the studies to date have focused on peripheral blood, which contains a minority of the total body lymphocyte population.

**Key words:** CD8 T cells; HIV disease; Human Aging; immunosenescence; Replicative senescence

Immunosenescence is the global term used to describe the observed age-associated decline in immune competence characterized by functional and phenotypic alterations to the immune system as a whole [1]. Immunosenescence in the elderly human population is associated with increased susceptibility to infectious diseases and cancer, reduced effectiveness of vaccinations, increased autoimmunity, and damage to various organ systems through dysregulated inflammation [2-6]. Ultimately, these changes play a

significant role in increasing morbidity and mortality [7, 8]. Although immunosenescence is a ubiquitous process, as with all complex diseases its progression is highly variable from individual to individual, and is undoubtedly influenced by a number of genetic and environmental factors.

Human immunosenescence is believed to be driven via systematic remodeling of the immune system over a lifetime of antigenic exposures and responses, mainly due to inflammatory processes. An in-depth discussion

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of inflammation and its role in immunosenescence, and aging in general, has been covered in depth in other reviews [9-11] and is outside the scope of this discussion. Instead, this review will focus on how immunosenescence, in both aging and HIV disease, influences the adaptive immune system, especially the CD8 T cell compartment, and how such alterations contribute to morbidity and mortality.

Some of the major immune alterations associated with immunosenescence include reduced number and function of hematopoietic stem cells, thymic involution, reduced circulating naïve T cells, decreased CD4/CD8 ratio, and increased levels of proinflammatory cytokines such as IL-6 and TNF $\alpha$  [9]. Another alteration strongly associated with immunosenescence is the accumulation of late-differentiated memory CD8 T cells which have undergone significant phenotypic and functional changes, and show features reminiscent of cellular replicative senescence characterized in long-term *in vitro* cultures [12]. *In vitro*, replicative senescence refers to the process by which cells reach an irreversible stage of cell cycle arrest following extensive replicative activity; these end stage cells show consistent changes in function and gene expression [13]. *In vivo*, the presence of oligoclonally expanded populations of similar late-differentiated cells is increasingly being implicated in advanced immunosenescence and predictive of poor prognosis [14, 15]. Although it cannot be definitively proven the cells have reached irreversible cell-cycle arrest *in vivo*, and in some cases have been shown to retain limited proliferative capacity [16], for the purpose of this review such cells are referred to as having reached replicative senescence, due to their overall similarity in function and phenotype to their *in vitro* counterparts and their putative role in immunosenescence.

This review will examine the evidence for CD8 T cell replicative senescence, both *in vitro* and *in vivo*, in the context of chronological aging and HIV-mediated premature immunosenescence. Evidence that CMV-mediated replicative senescence is part of an immune risk profile (IRP) predictive of morbidity and mortality in the very old will be summarized. Finally, studies implicating 'inflammaging' as an important contributor to immunosenescence will be briefly discussed, together with the role of CD8 T replicative senescence in this process.

#### CD8 T cell replicative senescence *in vitro*

The phenomenon of replicative (aka, cellular) senescence of cultured cells was first described by

Hayflick 50 years ago [17], and has since been demonstrated in numerous cell types including fibroblasts, epithelial cells, hepatocytes, endothelial cells, and keratinocytes [12, 18]. Initially it was believed by immunologists that, with the addition of Interleukin 2 (IL-2), T cells in culture would be immortal and grow *in vitro* indefinitely [19]. However, it has since been repeatedly demonstrated that human T cells do, in fact, undergo replicative senescence *in vitro* [20, 21].

With proper activation via the T-cell receptor and constant exposure to IL-2, cultures of normal human T cells can undergo between 25 and 40 population doublings before reaching senescence and ceasing to proliferate [22], with an average lifespan of approximately 33 population doublings [23]. Although aging is correlated with the accumulation of cell types with senescence markers *in vivo*, there is no clear correlation between *in vitro* lifespan of T cell cultures and chronological age of the donor [24]. This holds true for both CD4 and CD8 T cells.

#### Characterization of CD8 T cell replicative senescence *in vitro*

As CD8 T cells progress toward replicative senescence *in vitro* they undergo predictable phenotypic and functional changes. In terms of cell surface markers, the most consistent and dramatic change is the loss of CD28 surface expression. CD28 is an essential T cell receptor (TCR) specific co-stimulatory molecule, and has been directly implicated in a number of critical T cell functions, such as lipid raft formation, IL-2 gene transcription, apoptosis, stabilization of cytokine mRNA, glucose metabolism and cell adhesion [25]. Thus, a T cell lacking CD28 is fundamentally different in numerous respects from a CD28+ T cell. It has been documented that during the progression of CD8 T cells to senescence in culture, the percentage of cells expressing CD28 decreases. Indeed, senescent cultures are > 95% CD28-, as compared to the starting population, which contains an average of 91% CD28+ T cells [26]; this initial value can vary, depending on such factors as chronological age and immune status of the blood donor. Although CD28 expression is regulated, at least in part, by protein turnover and transient transcriptional repression, there is evidence that by the time a cell reaches replicative senescence, CD28 transcription has been permanently silenced [16, 27].

Since CD28 is a critical co-stimulatory molecule and its transcription is repressed during cellular senescence, we hypothesized that sustained

transcription of CD28, via stable gene transduction, would affect the growth characteristics of primary CD8 T cells propagated in long term culture. Transduced cells did, in fact, demonstrate high initial CD28 transcription and cell surface expression, significantly increased proliferative potential, as well as a substantial delay in acquisition of certain features of replicative senescence, such as loss of telomerase activity and increase in inflammatory cytokine secretion [28]. Nevertheless, eventually CD28 cell surface expression was lost, CTLA-4 expression increased and replicative senescence was observed in the transduced cultures [28], indicating transcription of CD28 is important, but not in itself sufficient, to maintain replicative capacity indefinitely.

Another feature of senescent CD8 T cells in culture is resistance to apoptosis, consistent with observations on senescent fibroblasts [29]. In response to six different apoptotic stimuli, senescent and late passage cultures showed significantly lower levels of apoptosis as compared to quiescent early passage cultures derived from the same donor [30]. Senescent CD8 cells in culture also show a blunted response to stress, as measured by the ability to upregulate hsp70 [31], consistent with studies on other cell types such as fibroblasts [32]. Heat shock proteins are widely implicated in protecting cells from intrinsic and extrinsic damage, and their downregulation is believed to play a significant role in cellular aging [33].

Senescent CD8 T cells, although unable to replicate, still retain some functional capacity in response to antigenic stimulation. As CD8 T cell cultures progress towards senescence, they produce increasing amounts of inflammatory cytokines, such as IL-6 and TNF $\alpha$  [12]. Interestingly, addition of exogenous TNF $\alpha$  to long term T cell cultures has been shown to accelerate CD8 replicative senescence [34], suggesting a positive feedback role for senescent cells in promoting more rapid cellular senescence in pre-senescent cells within the same culture. Some senescent CD8 T cells (i.e., those driven to senescence by repeated alloantigen exposure) have been shown to retain antigen-specific cytotoxic function [24] in addition to acquiring major histocompatibility complex-unrestricted cytotoxicity [35]. By contrast, there is also evidence of compromised effector function. For example, senescent HIV-specific CD8 T cells show decreased antigen-specific production of IFN $\gamma$  [12] and the progressive decline in ability to perform antigen specific lysis and suppress HIV replication *in vitro* [36], as well as a reduction in perforin and Granzyme B expression [37].

### Telomere length and Telomerase

Telomeres are sequences of tandem (TTAGGG)<sub>n</sub> nucleotide repeats that protect the ends of chromosomes. An inevitable consequence of the end replication problem, originally hypothesized by Olovnikov [38], is that the free DNA ends of each chromosome are incompletely duplicated by DNA polymerase. The result is that the ends of human chromosomes can lose up to 200 base pairs per cell division [39]. When telomere length shortens to a critical minimum, a DNA damage signal leads to cell-cycle arrest and replicative senescence. It is believed this proliferative clock protects cells against malignant changes that can be brought about by chromosomal instability in dividing cells with shortened-telomeres. Consistent with this theory, a recent meta-analysis of 27 studies indicated a significant inverse correlation between telomere length of lymphocytes in surrogate tissues and cancer incidence [40], although it should be noted previous prospective studies have not shown a significant association. In addition to cell division, telomere length is also regulated by telomerase, an enzyme (consisting of the hTERT gene product, an RNA component TERC and other regulatory proteins) that can restore telomere ends during DNA replication, allowing cells to undergo proliferation with minimal telomere shortening. Most somatic cells do not have active telomerase, limiting proliferative capacity. However, cells of the immune system are able to upregulate telomerase in concert with early activation events, as will be discussed below.

At the beginning of culture, human CD8 T cells have mean telomere lengths of approximately 10-11 kb, but as the cells undergo multiple rounds of cell division and reach senescence, telomere length decreases to approximately 5-7 kb [41, 42], a value that is similar to reported telomere lengths in senescent fibroblast cultures [39]. Despite the overall decrease in telomere length during the progression to senescence *in vitro*, during initial activation, CD8 T cells (unlike most somatic cells) exhibit telomerase activity that is comparable with that of tumor cells [43], and which is associated with temporary maintenance of telomere length [44]. This initial high telomerase activity is consistent with the observed stable telomere length in virus-specific T cells during the early phase of adaptive immune responses to acute EBV infection *in vivo* [45]. As CD8 T cells are maintained in long-term cultures through repeated stimulation of the TCR, the telomerase activity upregulation associated with activation steadily decreases, until the 4<sup>th</sup> stimulation, at which time telomerase activity become undetectable

[44]. Interestingly, this trend is not seen in CD4 T cell cultures, which retain high telomerase activity through the 10<sup>th</sup> round of antigenic stimulation [44].

The loss of telomerase activity in senescing CD8 T cell cultures parallels the loss of CD28 cell surface expression [44, 46]. Blocking CD28 from binding to ligands on antigen-presenting cells (APC) during stimulation inhibits telomerase activity, indicating the key role in CD28 costimulation in upregulating telomerase activity during activation [44]. The importance of CD28 signaling in telomerase upregulation was further confirmed in the aforementioned experiments, in which the constitutive expression of CD28 via stable gene transduction in CD8 T cell cultures led to prolonged telomerase activity during multiple rounds of stimulation [28].

The importance of telomerase activity in regulating the process of replicative senescence has been documented via stable hTERT gene transduction in fibroblasts, endothelial cells and T cells [47-49]. Indeed, stable hTERT expression in HIV-specific CD8 T cells resulted in cultures that continued proliferating and maintained stable telomere lengths through many rounds of stimulation, well past the point at which the control vector transduced cultures had reached replicative senescence [49]. In addition, these transduced cultures showed enhanced inhibition of HIV replication and delayed loss of IFN $\gamma$  production in response to HIV peptides [49]. Other studies, in which telomerase activity in T cell cultures was upregulated by exposure to a small molecule chemical telomerase activator (TAT2) demonstrated modest retardation of telomere shortening, increased proliferative potential, and enhanced functional cytokine/chemokine production and antiviral activity [50]. All these effects were ablated in the presence of a specific telomerase inhibitor. Overall, these *in vitro* experiments strongly implicate loss of telomerase activity upon repeated stimulation as a central player in telomere-shortening associated replicative senescence, and further confirm the importance of CD28 expression and co-stimulation in the regulation of telomerase activity.

#### CD8 T cell replicative senescence *in vivo*

Murine models are somewhat limited in terms of studying CD8 T cell replicative senescence in the context of immunosenescence. Although mice do undergo immunosenescence and their T cells show age-related reduced proliferative activity *in vitro*, the dynamics of both processes do not correlate with those of humans, even if one takes relative lifespan into account [51]. Additionally, human immunological

aging is believed to be influenced by competition for space over several decades by lymphocytes that are specific for a variety of previously-encountered antigens [52], a situation that cannot be replicated in short lived animals that are subjected to minimal antigenic exposure, particularly with respect to persistent viral infections.

With current technology, there is no way to definitively prove that CD8 T cell replicative senescence occurs *in vivo*. Instead, researchers have conducted cross-sectional comparisons between young and old persons and short-term longitudinal studies with elderly cohorts to examine phenotypic and functional differences in selected cell populations and determine correlation to endpoints, such as morbidity and mortality. T cell replicative senescence probably occurs *in vivo* in an incremental fashion over the course of decades, and in a dynamic environment that molds cellular phenotype, making it difficult to pinpoint what exactly a senescent cell is, when senescence occurs, or the functionality of these cells. Indeed, whereas permanent *in vitro* loss of CD28 gene transcription is widely documented as a reliable indicator of senescence, *in vivo*, CD28- T cells represent a heterogeneous population of cells, some of which still exhibit modest proliferative potential [16]. As stated above, for the purpose of this review replicative senescence will be used to describe *in vivo* late-differentiated cells implicated in immunosenescence that share features with their *in vitro* counterparts, which have undergone irreversible cell-cycle arrest, with the caveat that *in vivo*, such cells may not have reached strict terminal differentiation or completely exhausted their proliferative potential.

Consistent with *in vitro* replicative senescence observations, perhaps the strongest indicator that CD8 T cell senescence occurs *in vivo* is the age-associated increase in T cells lacking CD28 expression. At birth, nearly 100% of human T cells express CD28 [53, 54]. As we age, the CD8+CD28- T cell population steadily increases, albeit at a variable rate, so that by age 80 these cells constitute up to 50%-60% of the peripheral blood CD8 T cell pool [55]. Cell culture and *ex vivo* work has clearly demonstrated that the CD28- population is derived from more early stage (CD28+) cells that have undergone multiple rounds of antigen-driven cellular division [56]. High proportions of CD8+CD28- T cells are correlated with reduced response to vaccinations in the elderly [57] and with autoimmune disease [58-60], providing evidence that their presence is an indicator, if not a cause, of dysregulated immunity. Moreover, despite the fact that CD28- T cells are not a homogeneous population,

overall telomere length of peripheral blood mononuclear cells (PBMC) has been shown to be significantly negatively correlated with the proportion of CD8+CD28- T cells [61]. Thus, the proportion of these late-differentiated (possibly senescent) T cells can serve as a surrogate marker for PBMC telomere length.

Cultures initiated from purified populations of CD8+CD28- T cells isolated *ex vivo* share multiple features with CD8+CD28- cells that arise in long-term repeatedly stimulated *in vitro* cultures. The signature feature of replicative senescence in cell culture is inability to enter the cell cycle, and *ex vivo* experiments on purified CD8+CD28- T cells isolated from peripheral blood are unable to proliferate *in vitro*, either in response to antigenic stimulation via the TCR or in response to mitogens, such as PMA and ionomycin [62]. Similar to CD8+CD28- cells that reach senescence *in vitro*, lymphocytes from elderly persons show attenuation in the molecular chaperone system hsp70, in steroid binding hsp90, and the chaperonin hsp60 [63], implicating reduced ability to respond to stress. *Ex-vivo* CD8+CD28- cell are also resistant to apoptosis, similar to their *in vitro* counterparts [56].

As stated earlier, CD8+CD28- T cells *in vivo* represent a very heterogeneous population, and it has been hypothesized that suppression of CD28 gene expression occurs prior to the ultimate state of differentiation/senescence [16]. Using analysis on *ex vivo* CD8 T cell populations, researchers have identified two other cell-surface markers, CD57 and CD27, which, when used in conjunction with loss of CD28, represent what is believed to be the most differentiated/senescent cell populations.

CD57 is an adhesion molecule, found on many cell types, that is believed to only be expressed on CD8 T cells that have undergone chronic proliferative activation, be it due to aging, persistent viral infection, autoimmune disease or cancer [64]. The CD8+CD57+ population increases with age, has been shown to have strong intracytoplasmic expression of cytotoxic granules [64], and has the shortest telomere lengths of any CD8 T cell subpopulation [65], indicative of an extensive proliferative history and a differentiative state close to replicative senescence. Short term culturing of CD8+CD57+ T cells indicates these cells respond to TCR stimulation with INF $\gamma$  production, but are unable to proliferate [65]. T-cell receptor excision circle analysis documents that these cells have undergone more proliferative generations than other cell subtypes [65], consistent with the telomere length data. The frequency of CD8+CD57+ cells has been

shown to increase in conditions associated with immune dysregulation, including HIV and CMV infection and autoimmune diseases [66]. These data constitute a compelling argument that CD8+CD57+ T cells have undergone proliferation-induced differentiation and replicative senescence.

CD8 T cells that have lost surface expression of the costimulatory molecule CD27 in conjunction with CD28 are also believed to be very late-differentiated/senescent cells. *Ex-vivo* experiments examining subpopulations of CD8+CD28+, CD8+CD28-CD27+ and CD8+CD28-CD27- T cells indicated that the CD28-CD27- subpopulation had the shortest telomeres, lowest telomerase activity, lowest IL-2 upregulation and highest INF $\gamma$  levels [67]. Despite being unable to proliferate or produce IL-2, primary CD28-CD27- CD8 T cells demonstrated high levels of cytotoxic molecules and enhanced toxicity *in vitro* [16, 68]. Initially it was believed such high cytotoxicity was a beneficial adaptation to fight infections, but recent studies have indicated the presence of CD28-/CD27- CD8 T cells is associated with poor disease control in persons infected with HIV and CMV [68, 69]. Interestingly, HIV-specific CD8 memory T cells have been shown to be predominately CD28-CD27+, whereas CMV-specific CD8 memory T cells are predominately CD28-CD27- [70]. This implies that different persistent viral infections drive memory T cells to different set-points, and that CMV may drive cells furthest along the path to replicative senescence, a notion to be addressed in depth later in this review.

#### HIV disease, CD8 replicative senescence and accelerated immunosenescence

With the success of antiretroviral therapy (ART) HIV infection has evolved into a chronic condition, allowing infected persons to survive into old age. Indeed, in the absence of ART, the median survival time following initial infection is 12 years [71], but with ART, patients are living up to several decades [72]. Coupling this increased survival with demographic trends showing an upward shift in the average age at initial infection, it is estimated that by 2015, more than 50% of all HIV-infected individuals in the U.S. will be older than 50 years of age [22].

Dramatically reducing CD4 T cell lymphopenia and progression towards AIDS in HIV-infected persons has increased our understanding of how the immune system responds to chronic infection over time, and the consequences of such a response propagated over several years. The results of such studies indicate that,

independent of peripheral blood CD4 lymphopenia, controlled HIV infection results in "premature immunosenescence," including accelerated CD8 T cell differentiation and replicative senescence.

HIV disease in relatively young cohorts is associated with changes in immune parameters that are remarkably similar to age-associated immunosenescence, including thymic involution, reduced circulating naïve T cells, decreased CD4/CD8 ratio, increased levels of proinflammatory cytokines, increased susceptibility to infectious disease and cancer, and reduced effectiveness of vaccines [9]. Moreover, new evidence, based on telomere and phenotypic studies, indicates that it is not only the CD8 T cell population that undergoes premature aging during HIV disease, but also specific naïve CD4 T cell subpopulations [73].

Additionally, there is strong evidence HIV disease results in accelerated increase in the number of CD8 T cell populations that have undergone replicative senescence. Compared to age-matched controls, HIV+ subjects have decreased average telomere length in the CD8 T cell compartment [74, 75], especially among CD28- T cells [62], indicating extensive cellular proliferation. Interestingly, these studies indicated no corresponding difference in CD4 T cell telomere length. Compared to age-matched controls, HIV-infected subjects also had a noticeable increase in CD28- and CD57+ CD8 T cell populations [70, 76, 77]. These late-differentiated populations tended to be highly oligoclonal and to exhibit senescent signatures, such as reduced capacity to proliferate in response to mitogens and high levels of perforin [78].

As age-associated CD8 T cell replicative senescence is associated with poor vaccine response and autoimmunity, it is not surprising that the accumulation of putatively senescent CD8 T cells in HIV disease is associated with poor prognosis. In one study, an increase in CD8+CD28-CD27- T cells was negatively correlated with CD4 T cell counts and positively correlated with disease progression [78]. In another study, an increased fraction of perforin expressing HIV-specific CD8 T cells, which were generally late differentiated, was indicative of disease progression [79]. Finally, a nested case-control study showed an increase in CD8+CD28- T cells early in disease was associated with more rapid progression to AIDS [80]. Interestingly, in that study, loss of CD28 proved to be a more accurate predictor of disease progression than proportion of CD57+ T cells.

Analysis of antigen specificity of late-differentiated oligoclonal CD8 T cell expansions in HIV disease indicates that many of the clones are specific for CMV

antigens. One study that examined the differentiation state of CD8 T cells binding to tetramers for immunodominant epitopes of HIV, CMV, EBV and influenza in HIV-infected subjects noted that the greatest number of tetramer-binding clones that were CD27- recognized CMV antigens [78]. Another study analyzed CD8 T cells specific for two immunodominant CMV epitopes in HIV patients on ART, noting that a late-differentiated (CD27-CD28-CCR7-) phenotype predominated [81]. These data are consistent with previous studies indicating chronic HIV infection drives HIV specific CD8 T cells towards a moderately differentiated phenotype (CD28-/CD27+), in contrast to CMV-specific cells, which show a more late differentiated (CD28-/CD27-) phenotype [70]. In sum, these observations suggest that chronic activation and accelerated immunosenescence in HIV infected persons might be responsible for driving CMV-specific CD8 T cells into replicative senescence.

#### **IRP, CMV and how CD8 senescent T cells may contribute towards immunosenescence**

The Swedish OCTO/NONA studies were short-term longitudinal analyses that followed free-living persons > 85 years of age, with the goal of finding immune biomarkers representing a risk profile for morbidity and mortality in the elderly [15]. These studies identified an immune risk profile (IRP) present in 15-20% of 85 year olds associated with 2, 4, and 6 year mortality at follow-up. The initial IRP included an inverted CD4:CD8 ratio, caused by accumulation of CD8 cells lacking CD28 expression, poor T cell proliferative response to mitogen, and low B cell numbers [82]. It was subsequently shown that seropositivity for human CMV (but not for other persistent viruses, such as EBV, HSV or VZV) was also predictive of the IRP. Analysis of CMV infection showed that 100% of 85 year olds with the IRP group were seropositive for CMV, versus only 85% who were not in the IRP [15].

Follow-up studies have provided valuable insights into why CMV-specific CD8 T cell differentiation towards exhaustion/replicative senescence is a defining feature of immunosenescence in aging and HIV disease, and how such late-differentiated/senescent cells might contribute to the IRP and increased morbidity and mortality. It was demonstrated the accumulation of CD8 T cells responsible for the inverted CD4:CD8 ratio tended to be late-differentiated oligoclonal expansions specific for CMV antigens [14, 83, 84], comprising up to 45% of the total CD8 T cell pool [85]. These late-differentiated CMV-specific

clones in the elderly were subsequently shown to have reduced functionality *in vitro* compared to CMV clones from younger subjects [14, 84]. Interestingly, it was shown that for persons in the IRP group, an inverted CD4:CD8 ratio correlated with a relatively low number of total CD8 T cell clonal expansions and increased mortality [14]. A possible explanation for this observation is that large populations of late-differentiated immunodominant clones, often replicatively senescent or otherwise displaying impaired functionality, occupy immunological space and restrict the development of new clones specific for other epitopes/antigens, compromising overall immunity. Consistent with this idea, it is believed the association between immunosenescence and a decrease in naïve T cells is due, in part, to late-differentiated CD8 T cells crowding out naïve T cells in the context of a T cell pool restricted to a specific size through homeostatic mechanisms [86].

A consideration of all the above evidence allows one to construct a model for how the IRP is created, and why it might both contribute to immunosenescence and also be a predictor of mortality. During the course of chronic CMV infection over many decades, CD8 T cells recognizing immunodominant CMV epitopes are periodically stimulated, and over time they proliferate, differentiate and develop features of senescence, including accumulation of cell-surface senescence markers (CD28-/27-, CD57+), downregulation of telomerase, telomere shortening, and loss of proliferative capacity. As these cells accumulate, due, at least in part, to apoptosis resistance, they displace more functional cells that are specific for other antigens, reducing the overall T cell repertoire and immune competence. To compound matters, when these late-differentiated cells reach senescence, they may lose efficacy. The general loss of immune control results in a higher pathogen load and correspondingly higher levels of systemic inflammation. Additionally, late-differentiated CD8 T cells themselves contribute to the inflammatory milieu through secretion of TNF $\alpha$  and IL-6. This high level of systemic inflammation, termed “inflammaging” and itself a feature of immunosenescence, is believed to further exacerbate age and HIV-mediated immunosenescence and contribute to mortality, as will be discussed below.

Thus far, CMV has primarily been viewed as a contributing factor — rather than an actual cause— of age-related immunosenescence. However, there is some evidence that CMV may be a primary cause of immunosenescence, independent of age. For example, one study showed a strong association between CMV seropositivity and increased number of CD28- CD4

and CD8 T cells, irrespective of age, although age and CMV status were important determinants of other immune parameters, such as total T cell number, the number of CD8 T cells, and the number of CD8 T cells expressing CD45RA or CD28 [87]. Another study showed CMV seropositivity to be correlated with a 60% increase in the size of CD8 T cell memory pool and with a reduction in naïve CD8 T cells across *all* age groups [88]. A third study indicated that, in addition to driving the loss of naïve CD8 T cells, CMV seropositivity is associated with a Th1 polarization of the immune system, possibly resulting in increased inflammation [89]. Supporting this notion, two epidemiological studies have noted a correlation between CMV seropositivity, increased inflammatory biomarkers, and morbidity in the elderly [90, 91].

In future studies of immunosenescence, it will be important to design experiments to test the effects of CMV seropositivity across various age groups, to further our understanding how CMV infection contributes towards age related immunosenescence and may influence immunosenescence independent of age. One variable that is impossible to determine is the length of time persons have been infected with CMV. It should also be noted that the rare CMV-negative elderly persons do, in fact, show some characteristics of immunosenescence [82] and there is evidence that, in the absence of CMV infection, immunosenescence may be driven by other persistent viruses, such as EBV [92], indicating CMV seropositivity is not absolutely necessary for immunosenescence (although all persons in the IRP are CMV seropositive).

Similarly, as stated above, there is some evidence that HIV infection may accelerate immunosenescence by driving differentiation and replicative senescence of not only HIV-specific, but also CMV-specific CD8 T cells. To test this idea it would be necessary to compare the progression of immunosenescence between HIV+CMV+ and HIV+CMV- cohorts, an extremely challenging endeavor, due to the paucity of HIV+CMV- individuals. Only through careful design of future experiments testing CMV seropositive versus seronegative cohorts will we be able to accurately elucidate the precise influence of CMV seropositivity on age and HIV-mediated immunosenescence, and determine whether vaccinating against CMV infection, or aggressively treating CMV reactivation will affect immune status, morbidity and mortality.

### Highly differentiated CD8+CD28- T cells: role in suppression and carcinogenesis?

In addition to contributing to immunosenescence through inflammatory cytokine secretion, impaired functionality against antigens, and decreasing overall T cell repertoire, late-differentiated CD8+CD28- T cells may have suppressor functions *in vivo*. CD8+CD28- cells have been found to suppress immune reactivity by inducing APC's to become tolerant to helper T cells [93, 94]. In addition, transplantation studies have identified donor-reactive CD8+28- cells in patients with successful organ transplants, but not in patients who experienced rejection, implicating a positive role for immune suppression, at least in this particular clinical situation [93]. In HIV-infected persons, CD8+57+ T cells were shown to be positively correlated with lymphocytic alveolitis disease progression and to have suppressive influences on HIV-specific CTL activity [95], indicating a role for these cells in immune suppression *in vivo*.

The effect of chronic antigenic stimulation in driving CD8 T cells to senescence may be relevant to tumor antigens as well as persistent viral infections. Indeed, CD8+CD28- T cells can be purified from various types of human tumors, including lung [96], colorectal [97], endometrial [98], ovarian [99], lymphoma [100] and breast [101, 102], as well as from metastatic satellite lymph nodes and peripheral blood of cancer patients [96, 103]. Interestingly, in patients with large granular lymphocyte leukemia, the CD8+CD28- T cells have acquired the ability to directly lyse arterial endothelial cells [104]. Expanded clonal populations of cytotoxic T cells with other features of replicative senescence (e.g., CD57 expression) are also present in melanoma and multiple myeloma patients [105, 106], and the abundance of senescent CD8 T cells correlates with tumor mass in head and neck cancer [107]. Moreover, in renal cancer, the proportion of CD8 T cells with features of replicative senescence is actually predictive of patient survival [108]. Tumor-reactive, memory CD8 T cells are also present in prostate cancer patients [109]. Importantly, immune evasion in these patients is associated with a population of quiescent CD8 T cells lacking perforin and interferon- $\gamma$  expression [110]—features that are identical to characteristics of *in vitro* senescent HIV specific CD8 T cell cultures [37]. At this time, it is not known whether late-differentiated CD8+28- actively repress anti-cancer immunity or passively fail to confer it.

Senescent cells have also been documented to contribute to cancer by creating a favorable tumor

microenvironment. For example, senescent fibroblasts have been shown to enhance pre-malignant epithelial cell growth both *in vitro* and *in vivo* [111]. Interestingly, molecules released by CD8+CD28- T cells from HIV-1-infected patients promote endothelial-cell (EC) growth and induce ECs to acquire spindle cell morphology and upregulation of surface receptors that mimic the phenotype of Kaposi's Sarcoma, an AIDS-defining cancer [112]. This intriguing connection between T cell replicative senescence, cancer immunosurveillance, and tumor cell biology is an area that merits further investigation. Finally, there is clinical evidence that adoptive immunotherapy using CD8 T cells may ultimately be affected by the process of replicative senescence. Indeed, the degree of immune impairment of adoptively transferred tumor-specific CD8 T cells is directly related to the number of rounds of *in vitro* cell division prior to cell transfer [113]. Also, the persistence and expansion capacity of tumor-infiltrating CD8 T cells correlates with two key parameters of replicative senescence, namely, telomere length and CD28 expression [114-116].

### “Inflammaging” and CD8 T cell replicative senescence

“Inflammaging,” a term promoted by Franceschi [117], describes how systemic low grade inflammation, a defining feature in age- and HIV-mediated immunosenescence, contributes to morbidity and mortality, often through damage to non-immune organs, resulting in chronic diseases. A common hallmark of aging and age-related immunosenescence is increased low-grade systemic inflammation, as evidenced by elevated serum CRP, IL-6, Amyloid A and TNF $\alpha$  [6, 118, 119], among other markers. Along with genetic interactions, this pro-inflammatory status is implicated as causative in a number of age-related conditions, including cardiovascular disease, atherosclerosis, metabolic syndrome, type 2 diabetes, obesity, neurodegeneration, arthrosis and arthritis, osteoporosis and osteoarthritis, sarcopenia, depression and frailty [6]. For example, CRP levels have been widely implicated as a moderate predictor of risk of coronary heart disease [120] and high levels of CRP, IL-6 and TNF $\alpha$  have been found to correlate with increased all-cause mortality in elderly men [121, 122].

Similar to aging, HIV disease is associated with relatively high levels of immune activation and systemic inflammation. Common biomarkers of this process include elevated levels of activated CD4 and CD8 T cells, high CD8 T cell counts, increased levels

of inflammatory cytokines such as IL-1 $\beta$ , IL-6 and TNF $\alpha$ , and activation of the coagulation pathway [9, 123]. An important factor believed to be driving HIV-mediated inflammation is the immune response directly against HIV antigens, both infectious and noninfectious particles. This is supported by observations that inflammatory markers decline with initiation of anti-retroviral therapy (ART) and suppression of HIV viral levels [9]. However, even in ART-suppressed subjects, inflammatory markers are elevated compared to non-infected controls [124, 125]. Possible causes for this sustained elevated inflammation include loss of inflammatory repressing regulatory CD4T cells, thymic atrophy, increased copathogen load, and loss of gut mucosal integrity and the resultant microbial translocation [9].

Recent evidence indicates this last point may be especially important in maintaining high systemic levels of inflammation, even when ART therapy successfully suppresses HIV replication. It is known the GALT (gut-associated lymphoid tissue) is an important site of acute viral replication, with severe local CD4 depletion [126], and that during ART therapy CD4 levels in the GALT do not recover nearly as well as they do in peripheral blood, presumably due to incomplete viral suppression and inflammatory effects [127]. Additionally, SIV models using rhesus macaques indicate Th17 CD4 cells in the GALT, which are particularly important in controlling microbial pathogens, are especially susceptible to SIV infection, leading towards a high rate of loss of these cells and increased microbial translocation from the gut into peripheral blood [128]. Supporting this evidence, additional studies on HIV-infected patients and SIV models indicate both conditions cause elevated levels of highly antigenic circulating microbial products, such as lipopolysaccharide [129]. As a whole, these data provide a compelling picture of how Th17 CD4 depletion, microbial translocation, and inflammatory responses against microbial products create an environment of chronic immune activation observed in HIV infection, even under conditions of ART therapy and peripheral blood viral suppression.

HIV disease is known to cause a 3-fold higher risk of death from all-cause mortality [130]. As expected, approximately 50% of the deaths in HIV patients on ART were attributed to AIDS-defining conditions, such as opportunistic infections [131, 132]. However, the remaining deaths are due to non-AIDS defining age-related illnesses associated with inflammation, including cardiovascular disease, kidney disease, liver disease, osteoporosis, non-AIDS cancers, neurologic disease and frailty [132-139]. Corroborating these

data, studies indicate an increase in inflammatory markers, such as CRP, in HIV infected patients is independently correlated with accelerated progression to AIDS and an increase all cause mortality [140-142]. For this reason HIV infection is generally believed to be causing "accelerated immunosenescence," due in large part to effects from chronic immune activation and inflammation [143].

Late-differentiated, putatively senescent CD8 T cells are both affected by, and contribute to, inflammaging. Viral antigens (e.g., HIV and CMV) and the inflammatory mediators they elicit are implicated in driving CD8 T cell proliferation and differentiation, ultimately leading to replicative senescence. For example, there is evidence linking high levels of cellular activation with greater CD8 T cell differentiation towards a senescent phenotype [78]. Senescent CD8 T cells, in turn, secrete inflammatory cytokines, such as TNF $\alpha$ , which promote further CD8 T cell differentiation and loss of CD28 [34], among other effects. Linking the presence of senescent CD8 T cells with disease, *in vitro* culture experiments and studies in mice both document that activated T cells exposed to inflammatory mediators show increased mRNA and protein expression of RANKL, an osteoclastic mediator implicated in osteoporosis [144]. Interestingly, accumulation of CD28- T cells is associated with several autoimmune diseases such as rheumatoid, multiple sclerosis, and ankylosing spondylitis [16], in which premature bone loss is observed, suggesting a role for these cells in dysregulated inflammatory conditions.

### Concluding Remarks

Antigen-driven differentiation towards the end stage of replicative senescence in CD8 T cells is an important component of both age- and HIV-mediated immunosenescence and the IRP. However, one important caveat regarding these observations is that most of our knowledge on immunosenescence in humans has been derived from studies on peripheral blood, which contains only 2% of total body lymphocytes. At this time, little is known about the dynamics of CD8 T cell aging in other tissues, especially the GI tract, which houses 60% of total body lymphocytes and is a major reservoir for HIV infection. As we move forward in defining the contribution of CD8 T cell replicative senescence to human immunosenescence, it will be critical to elucidate aging dynamics of lymphocytes in the GI tract. Our own preliminary studies suggest that T cells from the GI tract may be more antigen-experienced and further



differentiated than those in peripheral blood (J. Dock, unpublished observations). For example, as compared to the peripheral blood, the GI tract has a higher proportion of memory and effector memory CD8 T cells, increased expression of activation markers, and higher percentages of T cells lacking CD28 expression (J. Dock, unpublished observations). Thus, it is only via a systematic investigation of the process of lymphocyte aging dynamics in tissues outside the peripheral blood that we will be able to develop more effective therapies to combat both age- and HIV-mediated immunosenescence. Current research in our own laboratory is addressing this challenge.

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## **Chapter 2**

**Human CD8 T cell replicative senescence:  
comparison of blood and gut parameters**



## ABSTRACT

A hallmark of age-related immunosenescence is the accumulation of late-differentiated memory CD8 T cells in peripheral blood with features of replicative senescence, such as inability to proliferate, absence of CD28 expression, shortened telomeres, loss of telomerase activity, enhanced activation, and increased secretion of inflammatory cytokines. Oligoclonal expansions of these late differentiated memory CD8 T cells are correlated with increased risk of morbidity and mortality in elderly humans. Whereas blood contains only 2% of total body lymphocytes, the gut-associated lymphoid tissue (GALT) contains 40-65% of lymphocytes and is an area of high antigenic exposure, underscoring the need to study CD8 T cells within the GALT, and to compare them to peripheral blood CD8 T cells. In the current study we analyze CD8 T cells from peripheral blood and the GALT (specifically rectosigmoid colon) in young, healthy donors, focusing on age-related phenotypic and functional alterations previously linked to senescence in peripheral blood. Overall, our results support the hypothesis that gut CD8 T cells show profiles suggestive of greater “aging” than those in peripheral blood. Indeed, compared to blood from the same individuals, there is a significant increase within the GALT in RA-CD8<sup>+</sup> (memory), CD28-CD8<sup>+</sup> (differentiated), RA-CD28-CD8<sup>+</sup> (differentiated memory), DR<sup>+</sup>CD38<sup>+</sup>CD8<sup>+</sup> (activated), CD8  $\alpha/\alpha$ , and RO+PD-1+CD8<sup>+</sup> CD3 T cells. There is also a (non-significant) decrease in baseline telomerase activity. However, based on other criteria, CD8 gut cells may not necessarily be more senescent than blood. Specifically, gut CD8 T cells show lower levels of CD57 expression, an increase in KI-67 (proliferation) expression, and proliferative potential that is similar to those in the blood. In addition, the inverted CD4:8 ratio observed in older persons

with high proportions of senescent CD8 T cells is not observed in the gut, at least in this relatively young study population. Ongoing research in our lab is addressing the effects of aging and HIV disease, including the CD4 subset, on gut immunology. In addition, our studies will incorporate other regions of the GALT, in order to provide a far more comprehensive picture of the human immune system and immunosenescence.

## INTRODUCTION

Immunosenescence is defined as the age-associated decline in immune competence, characterized by functional and phenotypic alterations to the entire immune system [1, 2]. This constellation of features is associated with increased susceptibility to infectious diseases and cancer, reduced effectiveness of vaccination, increased autoimmune phenomena, tissue damage due to dysregulated inflammation, and ultimately higher mortality [3-6]. One hallmark feature of immunosenescence is the accumulation of late-differentiated memory CD8 T cells with features of replicative senescence, such as inability to proliferate, absence of CD28 expression, shortened telomeres, loss of telomerase activity, enhanced activation, and increased secretion of inflammatory cytokines [7, 8]. The presence of oligoclonal expansions of these late differentiated memory CD8 T cells in the elderly has been implicated in morbidity and mortality [9-11].

An important caveat in our understanding of immunosenescence, and the contribution of late-differentiated memory CD8 T cells to this process, is that most studies have been done on peripheral blood, which contains only 2% of total body lymphocytes. In the gut-associated lymphoid tissue (GALT), on the other hand, which contains 40-65% of lymphocytes and is an area of high antigenic exposure [12, 13], little is known concerning the senescence profile of resident CD8 T cells. Moreover, there is minimal information on the relationship of CD8 T cells within the GALT and peripheral blood, and how the composite of these two populations contribute to immunosenescence.

In the few studies that have compared GALT and peripheral blood cells, several phenotypic differences have been noted in the T cell compartment. Most notably, studies

comparing peripheral blood and intestinal lamina T cells indicated that whereas most peripheral blood T cells were naïve (CD45RO-) and non-activated, *ex-vivo* mucosal T cells are generally in a more activated state and were mainly (>98%) memory (CD45RO+) [14]. Also, HIV studies indicated gut CD4 T cells are more activated than their peripheral blood counterparts, and show greater susceptibility to HIV infection due to expression of the HIV CCR5 and CXCR4 co-receptors [15]. Other studies indicate that, compared to peripheral blood, breast milk CD8 T cells are more prevalent, activated and show increased expression of senescence related markers [16]. These observations of phenotypic and possibly functional differences across anatomical regions underscore the need for a more comprehensive analysis of the senescence profile of gut CD8 T cells, in order to further our understanding of immunosenescence.

In the current study, we compare CD8 T cells from peripheral blood and GALT (taken from rectosigmoid colon biopsies) of 39 healthy donors (range: 24-67 year old; mean age: 40.8), focusing on age-related phenotypic and functional alterations that had been previously linked to senescence in peripheral blood. First, we examined cell surface markers that are associated with senescence (and in many cases morbidity and mortality). These markers include the CD4/CD8 ratio, and surface expression of CD28, CD57, PD-1, and CD8  $\alpha/\alpha$ . Second, we analyzed markers of activation and homeostatic proliferation, namely, CD25, HLA-DR, CD38 and intracellular Ki-67, since these biomarkers are believed to be linked to cell turnover, differentiation and, ultimately, replicative senescence [2]. Third, we measured telomerase activity of *ex vivo* CD3 T cells, since CD28- T cells have been shown to have lower telomerase activity than their less senescent (CD28+) counterparts [17] and also show a blunted ability to upregulate telomerase when stimulated *in vitro* [18]. Finally, we performed a CFSE dilution assay to evaluate the proliferative capacity of CD8 T cells. Peripheral blood T cells that are driven to replicative

senescence in cell culture are unable to proliferate in response to both mitogenic and antigenic stimulation [19], and reduced *in vitro* T cell proliferative responses are correlated with increased morbidity and mortality in the elderly [20]. Our results indicate that in healthy adult donors, gut CD8 T cells *generally* have a more senescent profile than their peripheral blood counterparts, with notable exceptions. These findings indicate a need to further study immune phenotype and function in this immunologically important tissue, to further elucidate our understanding of immunosenescence, especially in the context of aging and HIV infection.

## **MATERIALS AND METHODS**

### ***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 blood and gastrointestinal (colorectal) mucosal biopsy (gut) samples collected from a total of 39 self-reported healthy participants. All participants are relatively 'young' (range: 24-67 years old; mean age: 40.8; male (n=35), female (n=3), MTF (n=1)). The data on these participants are part of a larger study aimed at examining the effects of aging and chronic antigenic stimulation on the mucosal immune system using both young and old subjects who are either HIV seronegative or seropositive. (AG032422 PI: Effros).

### ***Collection of Peripheral Blood Mononuclear Cells (PBMC)***

Human peripheral blood samples were acquired by standard venipuncture immediately prior to endoscopy; 70cc of peripheral blood to be used 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. An additional 8cc were collected in two 4ml EDTA tubes; one was transferred to the flow cytometry research laboratory for T cell immunophenotyping and the other was transferred to the CLIA certified Clinical Immunology Research laboratory for a White Blood Cell (WBC) with absolute lymphocyte count, which was

performed using the SYSMEX XT 1800i hematology analyzer. Samples (10ml of stored serum) were collected for future antibody and cytokine testing.

Following Ficoll centrifugation, PBMC were washed with 1X PBS and resuspended in 10 ml culture media (1X RPMI 1640, 15% FBS, 10mM HEPES, 2 mM glutamine, 50 IU/ml penicillin/streptomycin, 500 µg/ml piperacillin-tazobactam, 1.25ug/ml amphotericin B). Viable PBMC concentration was calculated via trypan blue exclusion. Five million PBMC were removed and irradiated at 5k rads 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  $10 \times 10^6$  CD3 T cells were collected from PBMC for CFSE staining.

### ***Collection of Mucosal Mononuclear Cells (MMC)***

Mucosal biopsy samples were collected as previously described [21]. 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 20-25mls of RPMI medium with 7.5% fetal calf serum (FCS) (R7.5), L-glutamine, amphoterin-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 20–25 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<sup>3</sup> disposable syringe attached to a blunt-ended 16-gauge needle (Stem Cell Technologies, Vancouver, BC). The entire suspension was then passed through a 70µm 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 pooled isolated mucosal mononuclear cells (MMC) were combined and resuspended in 5ml of RPMI medium containing 10% FCS, amphotericin-b (1.25µg/ml) and Zosyn (50µg/ml). The MMC were used for flow cytometry and functional studies. Absolute CD3 T cell numbers were approximated using TRUcount™ beads. In a total of 135 MMC donors to date, encompassing this study and additional studies addressing gut immunosenescence, the average recovery following 30 biopsies was  $6.2 \times 10^6$  CD3 T cells, with a standard deviation of  $3.5 \times 10^6$  and range of  $1.6 \times 10^6$ - $25.0 \times 10^6$  CD3 T cells.

### ***CFSE Staining***

CFSE staining was performed as previously described in [22], with assay-specific modifications to determine the final CFSE concentration. Briefly, for each donor, aliquots of whole PBMC or MMC including  $10 \times 10^6$  peripheral blood CD3 T cells,  $5 \times 10^6$  irradiated PBMC feeders, and  $2-3 \times 10^6$  mucosal CD3 T cells were separated into 15 ml polypropylene tubes, washed in 5 ml 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  $\mu$ M); (ii) Irradiated PBMC feeders (20.0  $\mu$ M); (iii) Mucosal T cells (5.0  $\mu$ M). 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 extracts were then resuspended in culture media (same as PBMC culture media) at  $1.0 \times 10^6$  CD3 T cells/ml and irradiated PBMC feeders were resuspended at  $0.5 \times 10^6$  cells/ml.

### ***Proliferation Assay***

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  $1 \times 10^6$  CD3 T cells/ml. For each participant's samples, both stimulated and unstimulated control wells were included in the assays. All wells also included  $0.5 \times 10^6$  irradiated (5k rads) feeder PBMC. Two additional wells, containing  $0.5 \times 10^6$  stimulated and unstimulated irradiated feeder PBMC, respectively, were established as further controls to verify no feeder PBMC were being included in the live CD3 T cell gate.

For all stimulated wells, 5  $\mu$ l T cell activation microbeads (anti-CD2/3/28; Miltenyi Biotec; Auburn, CA) were added to each culture ( $0.5 \times 10^6$  microbeads/ml culture). For all conditions 0.1 mg 5-bromo-2'-deoxyuridine (BrdU) (Becton Dickinson Immunocytometry Systems (BDIS); San Jose, CA), 0.1  $\mu$ g 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.

Cultures were incubated for 5 days at 37° C, 5% CO<sub>2</sub>. 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 an effort to optimize the cellular proliferation assays of blood and gut samples. These include stimulation method, initial cell concentration, culture kinetics, culture size, and whether or not to include irradiated autologous feeders.

### ***TRUCount™ and Absolute T-Cell Counting***

#### **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 ul of BD Multitest antibody cocktail (BD Biosciences, San Jose, CA) containing CD3-fluorescein (FITC), CD8-phycoerythrin (PE) , CD45-Peridinin Chlorophyll protein (PerCP) and 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 1X 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 instrument settings 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 [21]. 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. 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 vs SSC gate [23, 24]. The PBMC did not contain excess debris and gating was performed according to the manufacturer's procedures for whole blood analysis.

#### **Day 5 TRUCount™**

The 5 day cultured cells were harvested, washed and resuspended in 200ul of PBS staining buffer (1X phosphate buffered saline with 2 % heat inactivated newborn calf serum). A 100ul 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 500ul of staining buffer. The cells were transferred to TRUCount tubes just prior to acquisition on a FACSCanto cytometer (BD).

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 and because a forward scatter threshold can exclude small trucount beads and lead to erroneous counts. The feeder cells were off scale, (a result of high CFSE labeling) and thus were excluded.

### **Absolute T-cell counting:**

Gating sequence: List mode data were first gated for viability using a forward versus side scatter gate. Next, the cells were gated using a CFSE-FITC versus CD3-PE display. 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 used to enumerate the CD4 and CD8 T-cells. Finally, a TRUcount™ beads gate was set on beads with high fluorescence using a plot of APC-CY7 versus PE-Cy7. 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*) X (*Total beads/ 100 μl cells*) = *Absolute T-cell count*].

Note: Please see Supplementary Figure 1 of Chapter 3 (**Optimization of protocol for comparing proliferative potential of human blood and gut T cells**) for a more detailed description of TRUCount™ gating sequence

### ***Measurement of T-cell proliferation cycles using BrdU and CFSE***

A 100 ul aliquot (the leftover volume of the original 200 ul after 100 ul had been taken for Day 5 TruCount™ staining—See Section 2.6) 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 exception that CD3-PE and CD8-PECy7 were added at the same time as the anti-BrdU-APC. When acquiring the data, the cytometer stop counters were set to routinely collect a minimum of 10,000 lymphocytes with the light scatter qualities of viable cells.

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, 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. The scatter gate excluded smaller cells with high side scatter, and the CD3 gate excluded CD3+ feeder cells that were off scale in CFSE fluorescence. The quadrant markers of the bivariate plot were used to differentiate proliferating (CFSE<sup>lo</sup>BrdU<sup>+</sup>) cells from non-proliferating (CFSE<sup>hi</sup>BrdU<sup>-</sup>) T-cells. The addition of BrdU allowed exclusion of contaminating debris and events that sometimes stained as CFSE<sup>lo</sup>BrdU<sup>-</sup>. We also included a 1D histogram of CFSE fluorescence, which allowed us to compare CFSE staining in non-proliferating populations in unstimulated and stimulated cultures, to verify the CFSE<sup>hi</sup> population is non-proliferating. The 1D CFSE histogram could also be used in data analysis of proliferative dynamics, such as determining number of proliferation cycles and frequency of cells in each cycle.

Note: Please see Figure 1 of Chapter 3 (**Optimization of protocol for comparing proliferative potential of human blood and gut T cells**) for a more detailed description of measurement of T-cell proliferation cycles using CFSE and BrdU.

### ***Cell Surface Phenotyping***

The staining panel consisted of 7 tubes with CD3 PerCP, CD4 (PE-CY7 or APC-H7)/ CD8 APC-H7. T-cells were investigated for expression of CD57, HLA-DR or CD45RO in the FITC parameter, CD28, CD38, CD31, CD8beta, or CD25 in the PE parameter, CD45RA PE-CY7, and PD-1, CD27, CD28, CD56 in the APC parameter. All monoclonal antibodies (mAbs) were purchased from BD Biosciences (San Jose, CA.) except; CD45RO was purchased from DAKO (Carpenteria, CA.), PD-1 and CD27 were purchased from eBioscience (San Diego, CA.). Appropriate gamma controls were run as a guide for cursor placement.

Cell surface staining was performed on 100ul aliquots of EDTA treated whole blood (WB) and freshly isolated MMC. WB samples were stained for 30' at room temperature followed by treatment with ammonium chloride lysing solution to remove erythrocytes. MMC were stained for 30' at 4° and washed twice with 1 ml PBS staining buffer(1X phosphate buffered saline with 2 % heat inactivated newborn calf serum and 0.1 % sodium azide), each wash was followed by centrifugation at 300xg for 5' followed by careful aspiration of the supernatant. Samples were resuspended in 500ul PBS staining buffer for acquisition by flow cytometry.

### ***Intracellular Ki-67 Staining***

MMC and PBMC staining included 2 tubes containing CD45RA FITC, CD3 Per-CP, CD4 PE-CY7, CD28 APC and CD8 APC-H7. Additionally MMC and PBMC were stained with the Ki-67 PE Antibody Set (*BD Pharmingen*<sup>TM</sup>) containing mouse anti-human Ki-67 antibody and matching IgG1 isotype control.

0.5x10<sup>6</sup> CD3+ MMC and 1.0x10<sup>6</sup> PBMC, as determined by BD TruCount™, were stained per tube. Cell surface staining was performed on MMC and PBMC as described above for MMC staining followed by intracellular staining using e-Bioscience Foxp3 Staining Buffer Set. After cell surface staining, samples were resuspended in 1 mL of Fixation/Permeabilization working solution and incubated for 30' at 4° followed by one wash in 1 ml of PBS staining buffer. Cells were then subjected to 2 washes in 2mL of 1X Permeabilization Buffer and centrifuged at 300xg for 7' followed by careful aspiration of the supernatant. Cells were incubated for 10' in 2% normal mouse serum after which the IgG1 isotype control was added to Tube 1 and Ki67 added to Tube 2. Samples were incubated for an additional 30' at 4° and washed 2 times with permeabilization buffer before being resuspended in 500ul PBS staining buffer for acquisition by flow cytometry.

#### ***Flow cytometry analysis of phenotypic markers***

Samples were acquired on a 2 laser BD FACSCanto™ flow cytometer using FACSDiva™ software. Cytometer performance was optimized each day using BD™ Cytometer Setup and Tracking (CS&T) Beads. In addition, glutaraldehyde fixed chicken red blood cells (Biosure, Grass Valley, CA.) were targeted daily to standardize for fluorescence. Single stained WB tubes were run to establish compensation settings using the manufacturer's automated program for compensation set-up, and optimized as necessary. The cytometer stop counter was set to count 50,000 CD3+ events. Although the number of MMC counted did not always reach that threshold, the average across all donors was 40,000 for cell surface staining; Ki67 staining averaged

>18,000 for both CD4<sup>+</sup> and CD8<sup>+</sup> events. Further sub setting of Ki67<sup>+</sup> CD4<sup>+</sup> or CD8<sup>+</sup> was performed on a minimum of 100 Ki67<sup>+</sup> events.

For sample analysis; a primary gate was set on CD3 positive events using a CD3 vs. SSC dot plot. CD3 positive events were then passed through a forward scatter (FSC) vs. SSC plot to omit low FSC events representing dead and dying cells that stained non-specifically for all markers, this step was critical for MMC but not relevant for WB. CD3 positive events were displayed as either CD3 vs. CD4 or CD8 or alternatively; CD8 vs. CD4 to select CD4/CD8 subsets for further interrogation.

#### ***Fluorescence-activated cell sorting (FACS) of blood and gut CD3<sup>+</sup> cells***

Purified T-cells (CD3<sup>+</sup>CD19<sup>-</sup>) were sorted from PBMC and MMC preparations stained with CD45 PerCP, CD3 FITC, CD19 PE, CD4 APC and CD8 APC-H7 (BD Biosciences, San Jose, CA.). Sorting was performed on a BD FACSAria™ flow cytometer. Instrument performance was validated with CS&T and BD™Accudrop beads. Lymphocyte sort gates were set using a CD45 vs. SSC gate and a FCS vs. SSC gate. Total T-cells were selected as CD3<sup>+</sup> and CD19<sup>-</sup>. Aliquots of 320,000 cells per vial were sorted for the telomerase assay. Post sort analysis indicated T-cell purities of 97-100%. To reduce the degree of cellular clumping during the sort, MMC samples were resuspended in PBS staining buffer at a dilute concentration of 1-2 million cells per ml and passed through 35um strainer capped tubes (BD, Franklin Lakes, NJ ). PBMC were processed similarly except the concentration was 5-10x10<sup>6</sup>/ml. Presort and sort collection vials were kept chilled (4°C). The sorted cells were washed in 1X PBS and pelleted into 1 ml



Eppendorf tubes as dry cell pellets for storage at -80°C until batch processed for telomerase activity measurement.

### ***Telomerase activity measurements***

Telomerase activity was determined using a modified version of the Telomere-repeat amplification protocol (TRAP), as previously described [25]. Briefly, for each sample,  $0.32 \times 10^6$  sorted CD3+ cells were pelleted and washed twice with PBS and cell pellets were lysed in 40  $\mu$ l of M-PER Protein Extraction Reagent lysis buffer (Pierce, Rockford, IL). To control for intersample cell number variance, activity was normalized by nucleic acid concentration, which was determined using a Nanodrop 1000 (ThermoScientific, Wilmington, DE). The endogenous telomerase present in the cell extract adds telomeric repeats to the telomerase substrate (TS), a nontelomeric oligonucleotide. The extension products are then amplified several-fold by the polymerase chain reaction (PCR) carried out by *Taq polymerase* using a Cy-5-labeled forward primer (known as TS: 5'-/5Cy5/AATCCGTCGACGCAGAGTT) as a substrate for telomerase-mediated addition of TTAGGG repeats, and an anchored reverse primer (ACX5'-*GCGCCGCTTACCCTTACCCTTACCCTAACC-3'*). Following amplification, each sample was mixed with 25  $\mu$ l of Bromothenol Blue loading dye and 25  $\mu$ l of sample and loading dye were loaded and run using 10% nondenaturing PAGE in 1X TBE buffer. Gels were run at approximately 300 V for 80 min. Two replicates of each sample were evaluated, with the average fluorescence value used. All values were normalized to a standardized cell number of a telomerase-positive control cell line (Jurkat). Gels were scanned on a STORM 865 (GE

Healthcare, Piscataway, New Jersey, USA) and quantified using the software ImageQuant 5.2, which integrates signal intensity over the telomere length distribution on the gel as a function of molecular weight (GE Healthcare).

## RESULTS

### *Comparison of senescence related markers in blood and gut*

We first evaluated the CD4:CD8 ratio of CD3<sup>+</sup> T cells. In healthy HIV seronegative individuals this ratio is approximately 2:1 in peripheral blood. Inversion of this ratio, due mainly to oligoclonal expansions of late-differentiated (CD28<sup>-</sup>) CD8 memory cells [26], is believed to be a hallmark of immunosenescence and part of a so-called “immune risk phenotype” that is predictive of early mortality in the very old [27]. As expected, the mean blood CD4:8 ratio from our specific study population was close to 2:1, at 2.25 (FIG. 1A); the gut CD4:8 ratio from these same donors was 2.07, indicating no significant difference between blood and gut ( $p=0.40$ ) (FIG. 1A). In addition, there was also no significant difference in the absolute percentage of CD8 expressing CD3 T cells from the same donors between blood and gut (blood 32.7%, gut 31.6%,  $p=0.52$ ) (FIG. 1B), suggesting that, according to these parameters, there was no evidence of increased senescence in gut relative to blood.

We next analyzed the percentage of CD8 T cells with a memory phenotype (CD45RA<sup>-</sup>) in the blood and gut compartments. Consistent with earlier work looking at intestinal lamina propria lymphocytes [14] and our own unpublished observations, gut CD8 T cells were almost completely memory cells, as opposed to blood (blood 29.2%, gut 88.5%,  $p<0.005$ ), (FIG. 1C). Consistent with these data, within the subset of naïve (CD45RA<sup>+</sup>) CD8 T cells, the percentages were inverted (figure not shown). We also evaluated the percentage of CD28<sup>-</sup> cells in blood versus gut. CD28 is a T cell-receptor (TCR) specific co-stimulatory molecule implicated in a number of critical T cell functions [9]. In long-term *In vitro* cultures of chronically stimulated

blood CD8 T cells, the percentage of CD28<sup>+</sup> cells steadily decreases as the cells proliferate and differentiate, starting from a population that is >90% CD28<sup>+</sup> to the point at which the culture reaches senescence, with >95% of cells being CD28<sup>-</sup>. *In vivo*, a low percentage of blood CD8 T cells expressing CD28, presumably due to differentiation caused by chronic antigenic stimulation, is correlated with old age [28] and increased risk of morbidity and mortality [26, 29-32]. In our study population, the percentage of CD8 T cells that were CD28<sup>-</sup> was significantly higher in gut compared to blood (blood 30.1%, gut 46.8%,  $p < 0.005$ ) (FIG. 1D). Additionally, the percentage “differentiated memory” CD8 T cells that were RA-CD28<sup>-</sup> was significantly higher in gut compared to blood (blood 4.8%, gut 43.1%,  $p < 0.005$ ) (FIG. 1E). The significant increase in memory (CD45RA<sup>-</sup>), differentiated (CD28<sup>-</sup>), and especially differentiated memory cells (RA-CD28<sup>-</sup>) in gut relative to blood strongly suggests a higher degree of CD8 T cell replicative senescence and immunosenescence in this compartment, presumably due to greater antigenic exposure and cell turnover.

CD57 is an adhesion molecule found on many cell types, and its expression is increased on peripheral blood CD8 T cells that have undergone chronic persistent activation [33]. *Ex-vivo*, blood CD8<sup>+</sup>CD57<sup>+</sup> cells have the shortest telomere length, and T-cell receptor excision circle analysis confirms they have undergone more proliferative generations than any other CD8 T cell subtype [34]. Additionally, CD8<sup>+</sup>CD57<sup>+</sup> cells respond to TCR stimulation in cell culture with IFN $\gamma$  production, but are unable to proliferate [34] and their frequency *in vivo* is correlated with conditions associated with immune dysregulation, such as CMV and HIV infection, and autoimmunity [35]. Evaluation of this marker shows that within the CD8 T cell population in the gut there is a significantly **lower** expression of CD57 (blood 31.0%, gut 11.2%,  $p < 0.005$ ) (FIG. 1F). Moreover, the population of CD28<sup>-</sup>CD57<sup>+</sup> (2 markers that are highly correlated in blood)

cells in the gut was almost nonexistent (blood 25.6%, gut 2.7%,  $p < 0.005$ ) (FIG. 1G). The significantly lower percentage of CD57-expressing CD8 T cells in gut relative to blood might suggest that the gut cells may not be more senescent than those in the blood. However, since CD57 expression is merely associated with senescence in blood CD8 T cells, and has no known function in this process, it is also possible this is just an artifact of variable protein expression between blood and gut and may not actually be involved in the senescence phenotype *per se*.

To further address the question of senescence, we analyzed expression of two additional markers, PD-1 (the inhibitory receptor of programmed death) and CD8  $\alpha/\alpha$ , which have been associated with peripheral blood CD8<sup>+</sup> T cell senescence. PD-1 is a negative regulator of activated T cells whose expression is significantly increased on the surface of exhausted virus-specific CD8 T cells in mice, and is also predictive of disease progression in HIV infected persons [36]. Our results indicate within the gut, there is a small, but significant, increase in PD-1 expression on RO<sup>+</sup> memory cells (blood 15.8%, gut 20.4%,  $p = 0.05$ ) (FIG. 1H), but not on the total population of CD8 T cells. Similarly, the homodimeric form of CD8 ( $\alpha/\alpha$ , instead of the usual  $\alpha/\beta$ ), which is more highly expressed on clonally expanded T lymphocytes in blood samples of elderly persons[37], is also significantly increased within the gut compartment (blood 3.2%, gut 4.2%,  $p = 0.05$ ) (FIG. 1I). These data support our hypothesis that the CD8 T cell compartment in the gut is more senescent than in peripheral blood.

### ***Comparison of markers of activation and homeostatic proliferation in blood and gut***

Chronic activation and homeostatic proliferation contribute to increased cell turnover and differentiation, which can ultimately lead to replicative senescence. We therefore looked at

markers associated with activation and proliferation in blood and gut CD8 T cells. In blood, HLA-DR is upregulated in concert with T cell activation, and expression of the multifunctional enzyme CD38 on CD8 T cells is associated with chronic activation and aging [38]. Consistent with established conventions [39], we evaluated CD8 T cells that were positive for HLA-DR and CD38 (HLA-DR+CD38+) to examine T cell activation in blood versus gut. Our results indicate that gut has significantly higher expression of HLA-DR+CD38+ CD8 T cells than blood (blood 3.8%, gut 7.1%,  $p=0.014$ ) (FIG. 2A). Interestingly, another marker associated with activation in blood, CD25 (alpha chain of IL-2 receptor), was barely expressed in gut CD8 cells (blood 5.8%, gut 0.9%,  $p<0.005$ ) (FIG 2B). The extremely low surface CD25 expression on gut T cells suggests that this particular activation marker may not be detectable due to possible differences in protein expression between blood and gut CD8 T cells, or to absence of IL-2 in the gut microenvironment, and not necessarily reflective of activation status *per se*.

Homeostatic proliferation of blood and gut CD8 T cells was assessed by intracellular staining of KI-67, a nuclear marker that is expressed in proliferating cells. Our results indicate gut have significantly higher percentage of KI-67+ proliferating cells (blood 2.6%, gut 4.3%,  $p=0.006$ ) (FIG 2C). Interestingly, when we gate on KI-67+ CD8 T cells, there is a higher percentage of “early memory” (RA-CD28+) proliferating cells in blood vs. gut (blood 42.7%, gut 31.5%,  $p=0.005$ ) (FIG 2D), but there is a greater than 6-fold increase in “differentiated memory” (RA-CD28-) proliferating cells in gut versus blood (blood 9.5%, gut 62.0%,  $p<0.005$ ) (FIG 2E). These results indicate that gut CD8 T cells have a significantly elevated activation profile relative to blood, including a much higher percentage of “differentiated memory” cells, supporting the argument gut cells have undergone more rounds of antigen driven cellular

division, and are more late-differentiated. However, an increase in KI-67+ CD8 T cells indicates these cells still have proliferative potential, and may not be senescent per se.

### ***Comparison of baseline telomerase activity of ex-vivo CD3 T cells in blood and gut***

The holoenzyme, telomerase, is critical for extended cellular proliferation, since it restores telomere termini during DNA replication [40]. Most human somatic cells show no telomerase activity ; however, both T and B lymphocytes express detectable, but low, baseline telomerase activity [41] that increases dramatically during initial activation [42]. *In vitro* telomerase activity from repeatedly stimulated T cells decreases as cells senesce [18], and low *ex-vivo* telomerase activity is correlated with disease in human [43] and animal [44] studies. Using the TRAP assay on 20 donor samples, we observed that overall blood T cell activity was greater than overall gut activity (blood 4.0, gut 2.9,  $p=0.14$ ) (FIG. 3A). However, since telomerase activity shows relatively high inter-individual variation[17], it is actually more appropriate to compare telomerase activity between blood and gut T cells within a single individual. Looking at the relative blood:gut ratio of telomerase activity on a per cell basis, we observed that the average blood activity was 1.5 times greater than gut activity, with a median ratio of 1.3 (FIG. 3B) . These results indicate baseline telomerase activity may be lower in gut than blood, possibly due to relatively accelerated senescence, although a higher sample size may be required to reach significance.

### ***Proliferative capacity of stimulated CD8 T cells in 5 day cultures***

We next looked at the proliferative capacity of CD3/CD28/CD2-stimulated blood and gut derived mononuclear cells in 5-day cultures. For each donor (n=13), both stimulated and unstimulated cultures were evaluated, using our recently developed double label CFSE/BrdU system (Dock et al. manuscript in progress, and Chapter 3 of this thesis). Briefly, proliferating cells registered as CFSE<sup>lo</sup>BrdU<sup>+</sup> and non-proliferating cells CFSE<sup>hi</sup>BrdU<sup>-</sup> (Chapter 3). On day 5, gut cultures contained significantly fewer viable CD3 T cells in both the unstimulated and stimulated cultures as compared to their blood counterparts ( $p < 0.005$ ) (FIG. 4A). For both blood and gut, stimulated fractions had significantly more CD3 T cells than unstimulated fractions ( $p < 0.005$ ) (FIG. 4A). Comparing the average ratio of CD3 T cells on D5 in stimulated:unstimulated cultures, there was no significant difference between blood and gut (blood 1.63, gut 1.84,  $p = 0.49$ ) (FIG. 4B).

The percentage of total CD8 T cells proliferating in the stimulated samples was similar in blood and gut cultures (FIG. 4C). However, in unstimulated cultures from the gut, CD8 T cells had a significantly greater stimulation profile (blood 1.4%, gut 15.1%,  $p < 0.005$ ) (FIG. 4C), consistent with our data showing that gut CD8 T cells are in a more activated state immediately *ex vivo* (FIG. 2A). At the day 5 time point there was no difference between blood and gut in terms of average number of proliferations per CD8 T cell in stimulated cultures using CFSE dilution (blood 2.3, gut 2.2,  $p = 0.50$ ) (FIG. 4D), nor was there any significant difference in the percentage of proliferating cells in stimulated cultures that have undergone 4 or more divisions (blood 5.8%, gut 4.5%,  $p = 0.56$ ) (FIG. 4E). Results from the proliferation assay suggest that gut CD3 cells have lower survival at day 5 than blood CD3 cells in both unstimulated and stimulated



cultures, but that the actual proliferative dynamics do not differ significantly, suggesting that those gut cells that are able to survive and proliferate are not more senescent than their blood counterparts.

## **DISCUSSION**

This study provides new details on several important differences between human T cells in two distinct immune compartments, namely peripheral blood and gut mucosa. We show that in healthy ‘young’ adults, CD8 T cells within at least one region of the gut have profiles suggestive of greater “aging” than in peripheral blood. Specifically, compared to blood from the same individuals, there is a significant increase within the GALT in RA-CD8<sup>+</sup> (memory), CD28-CD8<sup>+</sup> (differentiated), RA-CD28-CD8<sup>+</sup> (differentiated memory), DR<sup>+</sup>CD38<sup>+</sup>CD8<sup>+</sup> (activated), CD8  $\alpha/\alpha$ , and RO+PD-1+CD8<sup>+</sup> CD3 T cells.

Our analysis also showed that gut CD3 T cells have somewhat lower baseline telomerase activity than blood T cells, also suggestive of a greater degree of senescence. Importantly, whereas we and others have evaluated T cell telomerase activity using *in vitro* stimulation, which would have increased the available cell yield for the TRAP assay [18, 42], recent improvements in detection sensitivity [45] now enables a more biologically relevant assay, namely on *ex-vivo* B cells and various T cell subsets [17]. The current *ex vivo* analysis was performed on total T cells, rather than on specific subsets of CD8 T cells, the populations that would be predicted to show a greater degree of senescence. Due to limitations in T cell yield from mucosal biopsies (Chapter

3.), and the fact most blood and gut CD3 T cells in our young, healthy study population donor pool were CD4+8-, it was not possible to isolate sufficient numbers of the target CD8 T cell populations. Nevertheless, baseline telomerase activity among different lymphocyte subpopulations in individual donors has been shown to be highly correlated [17], and furthermore low telomerase in total leukocyte populations is correlated with increased morbidity [43, 46], indicating that total CD3 T cell telomerase activity is a useful surrogate. We suspect that the observed reduction in gut T cell telomerase activity would reach statistical significance with analysis of additional samples, which were not available due to low cell yields from many of the gut samples.

Because the gut is a primary line of defense that routinely faces antigenic challenges, as opposed to the blood, which is primarily a conduit for trafficking lymphocytes, we hypothesized that gut CD8 T cells would have undergone a greater degree of antigenic driven proliferation and differentiation, and be more senescent than their blood counterparts. This hypothesis is consistent with early work looking at intestinal lamina propria T cells [14] and previous unpublished observations from our laboratory indicating gut CD8 T cells were predominately RA- and more activated than their blood counterparts. This hypothesis is also consistent with mouse studies indicating age-associated alterations in the mouse GALT similar to peripheral blood immune senescence in humans; including changes in CD8 T cell distribution [47], decrease in naïve CD4 T cells and dendritic cells in Peyers patches [48], defects in mucosal IgA and IgG secretion [49, 50], and defects in T cell function and vaccine immune responses [51-53].

Whereas most of our data supports the hypothesis that gut CD8 T cells are more senescent than their peripheral blood counterparts, there were some exceptions. First, we found that CD57 is only expressed on 11.2% of gut CD8 T cells versus 31.0% of blood (FIG. 1F), and CD28-CD57+ T cells constitute only 2.7% of CD8 T cells, as opposed to 25.6% in blood (FIG. 1G), indicating most blood CD57+ cells are also CD28-, but only a small fraction of gut CD57+ cells are CD28-. *Ex-vivo* analysis on peripheral blood CD57+ CD8 T cells indicates they are the most senescent T cell subpopulation, based on their short telomere length, extensive proliferative history, and inability to proliferate [34]. It is possible the underrepresentation of CD57 in gut CD8 T cells suggests RA-CD28- gut cells in young donors may not be as functionally senescent as their peripheral blood counterparts in elderly subjects. It is also possible lower expression of CD57 expression has no impact on gut CD8 T cell senescence, as CD57 has no known functional role in peripheral blood senescence. Additionally, the gut has a significantly higher percentage of KI-67+ proliferating CD8 T cells than blood (blood 2.6%, gut 4.3%,  $p=0.006$ ) (FIG. 2C), and 62.0% of KI-67+ CD8 T cells in gut are RA-28-, as opposed to only 9.5% in blood (FIG. 2E), indicating that although gut cells are more differentiated they still have proliferative potential and are not senescent per se.

Our data on proliferation, most of which showed no significant difference between gut and blood on Day 5, also conflicts with the notion that greater aging occurs in the gut vs. blood. We considered that these findings may have been affected by the overall reduced survival of gut cells vs. blood cells during the 5 day culture period, based on the reduced cell yield in the former. This reduced cell yield might have reflected a lower initial input of gut cells into the culture, if more gut cells than blood cells had been lost during the extensive washes following CSFE labeling. However, using precise TRUCount™ cell enumeration, we verified that

equivalent numbers of gut and blood cells were lost during the washing. The fact that the CD4:8 T cell ratio in the gut does not show the inversion observed in blood of older individuals is also in conflict with the notion of accelerated aging within that compartment. During aging, the peripheral blood CD4:8 ratio becomes skewed, due to oligoclonal expansion of highly differentiated memory CD8 cells [10, 54], which may occupy immunological space and restrict the overall antigenic repertoire, compromising immunity. A lack of ratio inversion indicates a large percentage of gut CD8 T cells being memory may be a function of healthy gut immune homeostasis, and not indicative of dysfunction or senescence.

An interesting note is that previous *in-vitro* culturing systems of primary blood demonstrating compromised proliferation of senescent cells were comprised of pure isolation of either CD28- [19] or CD57+ [34] CD8 T cells. Although gut has a higher percentage of CD28- cells than blood (blood 30.1%, gut 46.8%,  $p < 0.005$ ) (FIG. 1D), over 50% of gut CD8 T cells in starting culture are still CD28+. Additionally, gut has a significantly lower percentage of CD57+ cells than blood (blood 31.0%, gut 11.2%,  $p < 0.005$ ) (FIG. 1F). A relatively high number of CD8 T cells that are CD28+ (and the absence of CD57+ CD8 cells) in gut may indicate proliferative potential is not compromised in the gut of healthy donors, even if other markers suggest these cells are more activated, differentiated and putatively senescent.

Thus, most, but not all, of our results accord with the notion that the gut shows a greater degree of immune aging than the blood. However, an important caveat to this conclusion is that our data were derived exclusively from the rectosigmoid colon, which may not necessarily be representative of other regions of the GALT. Indeed, unpublished pilot studies performed by our group indicate the small intestine has a lower CD4:CD8 ratio and a more 'activated' CD8 T cell

phenotype than the colon. Ultimately, it will be necessary to explore other parts of the GALT to get a more complete picture of senescence effects through the entire GI tract.

In conclusion, the current study established that gut CD8 T cells from the rectosigmoid colon of young, healthy donors have many features indicating they are more senescent than their peripheral blood counterparts, with some notable exceptions. This initial analysis was specifically directed at cytotoxic CD8 T lymphocytes, since oligoclonal expansion and differentiation of peripheral blood memory CD8 T cells towards senescence in old age and HIV disease is correlated with immunosenescence and morbidity and mortality [2, 5]. In fact, it has been proposed that HIV disease causes accelerated blood CD8 T cell replicative senescence and immune senescence, presumably due to high CD8 T cell turnover in response to exposure to viral antigens [19, 55-57]. The next stage of our research is, in fact, addressing the effects of aging and HIV on the senescence profile in the gut. In addition, based on certain published age-related CD4 T cell changes in blood [58-61], and our own preliminary observations on their early senescence within the gut, our future research will focus on both T cell subsets, and will cover additional regions of the GALT. Overall, this area of research aims to provide a far more comprehensive picture of the human immune system and the process of immunosenescence.

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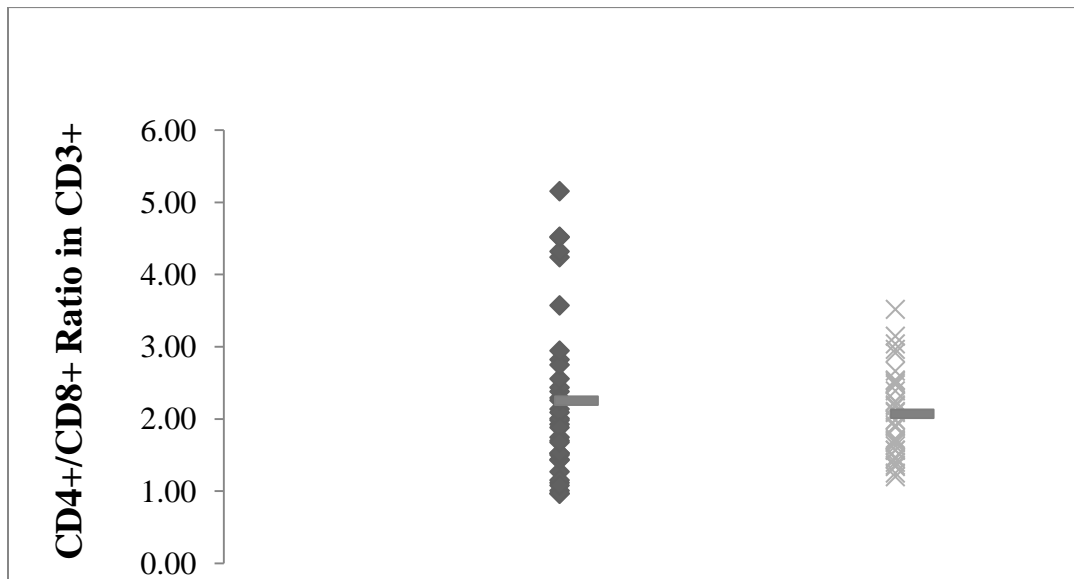
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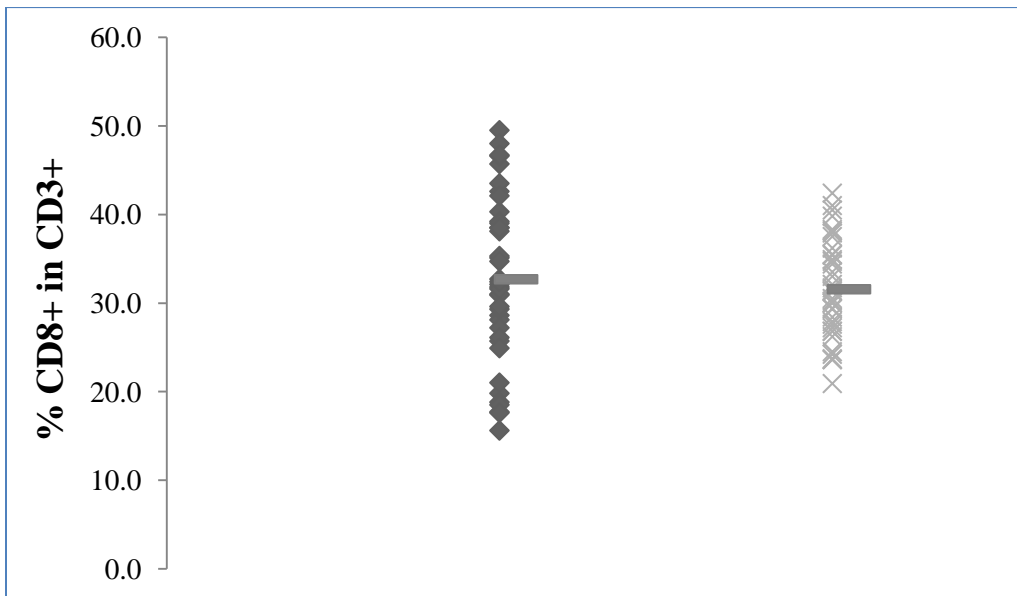
**Figure 1. Comparison of senescence-related phenotypic markers in blood and gut.** 100ul aliquots of EDTA treated whole blood (WB, blood) and freshly isolated MMC (gut) from 39 young, healthy donors (range: 24-67 years; mean age: 40.8) were resuspended in 500ul PBS staining buffer for acquisition by flow cytometry. WB samples were stained for 30' at room temperature followed by treatment with ammonium chloride lysing solution to remove erythrocytes. MMC were stained for 30' at 4° C and washed twice with 1 mL PBS staining buffer (1X phosphate buffered saline with 2 % heat inactivated newborn calf serum and 0.1 % sodium azide); each wash was followed by centrifugation at 300xg for 5' followed by aspiration of the supernatant. WB and MMC samples were gated on CD3+ cells and evaluated for the following markers: **A)** CD4+ and CD8+ to determine CD4/8 ratio (n=39, p=0.40) and, **B)** %CD8+ (n=39, p=0.50). **C)** %RA- in CD8+ (n=39, p<0.005). **D)** %CD28- in CD8+ (n=39, p<0.005). **E)** RA-CD28- in CD8+ (n=39, p<0.005). **F)** CD57+ in CD8+ (n=39, p<0.005). **G)** CD57+CD28- in CD8+ (n=39, p<0.005). **H)** RO+PD-1+ in CD8+ (n=34, p=0.05). **I)** CD8 $\alpha$ +CD8 $\beta$ - (n=33, p=0.05). For some markers fewer than 39 donor samples were evaluated due to low yield of freshly isolated MMC CD3+ cells from some donors. Asterisks signify the following value, \*\*\*, p $\leq$ 0.005; \*\*, p $\leq$ 0.05, and horizontal bars indicate mean values.

(♦) blood; (X) gut

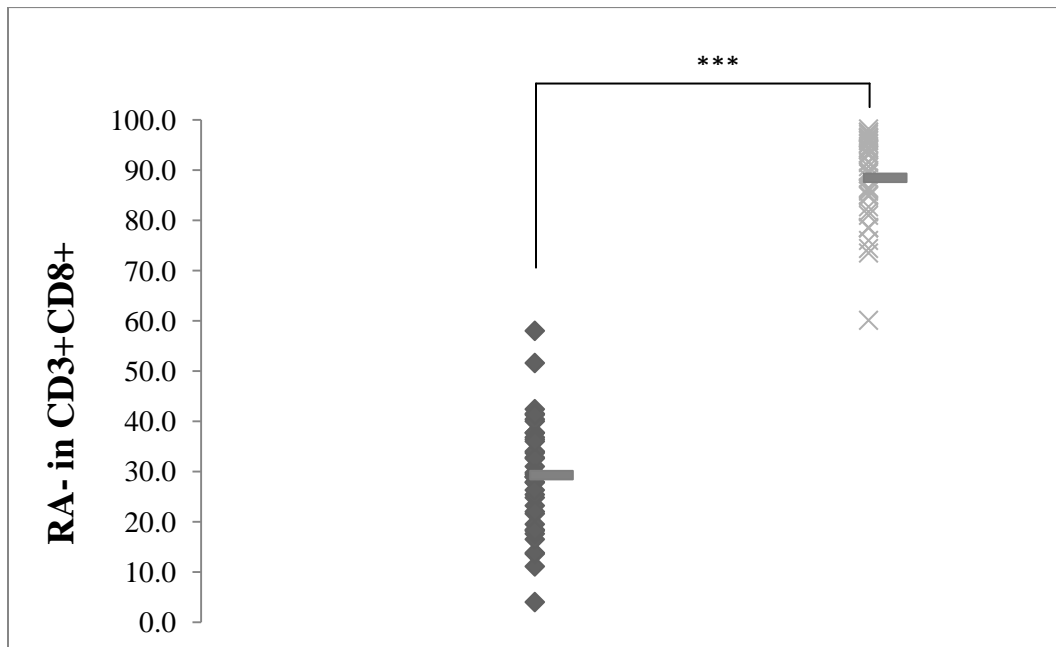
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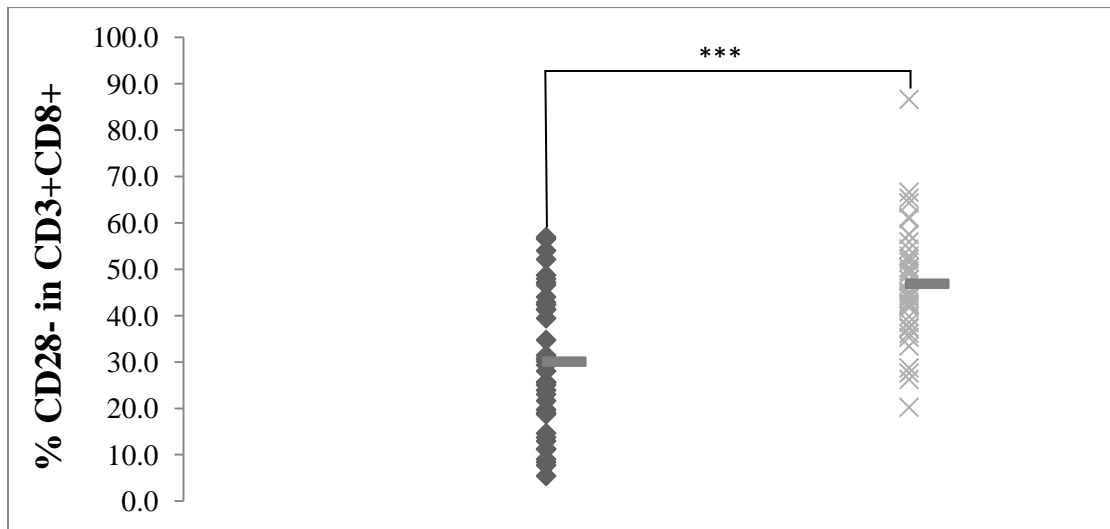
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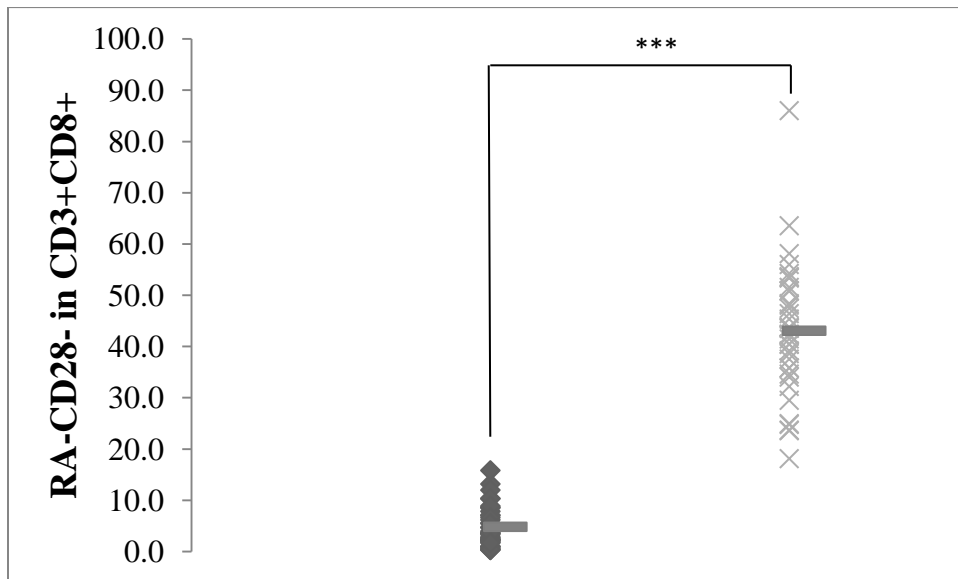
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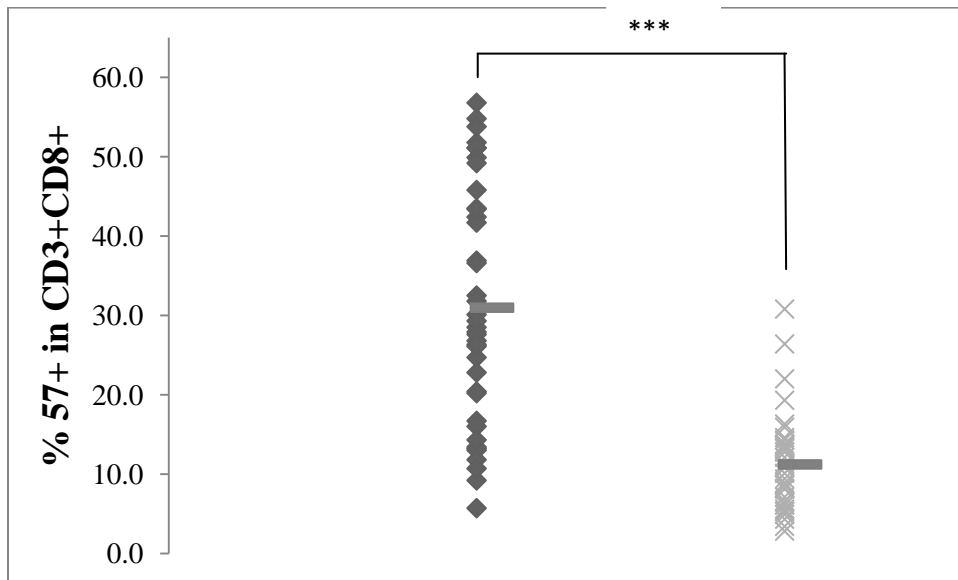
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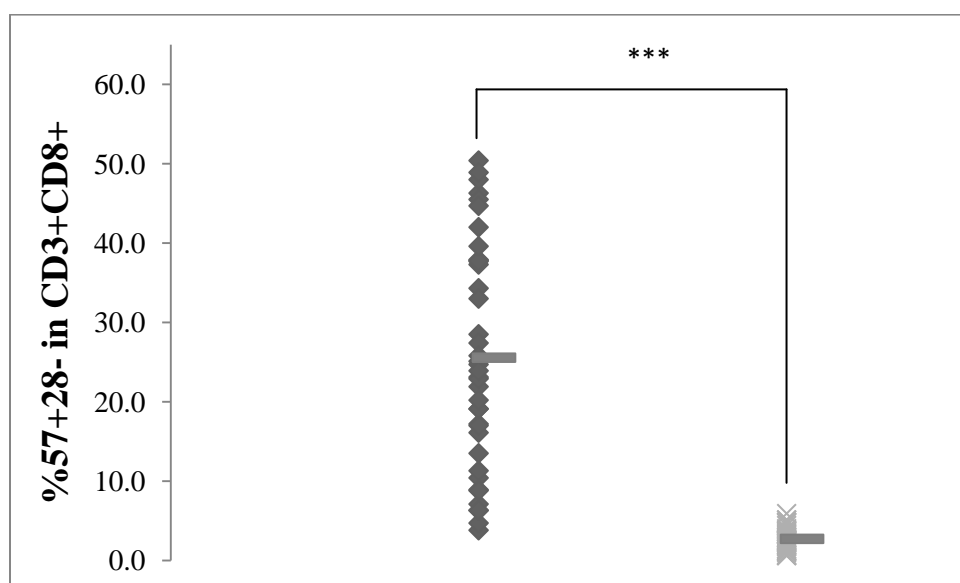
**E.**



**F.**

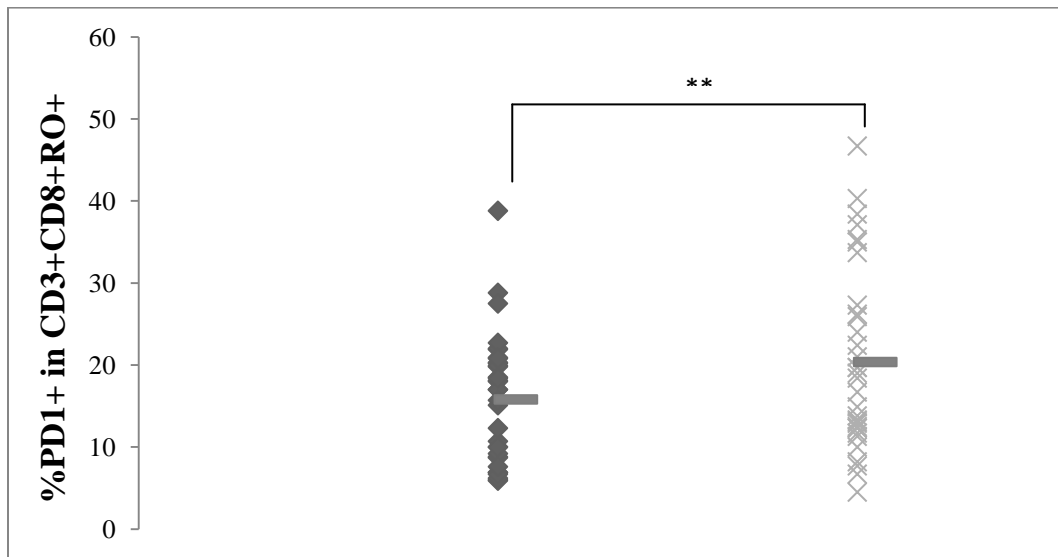


G.

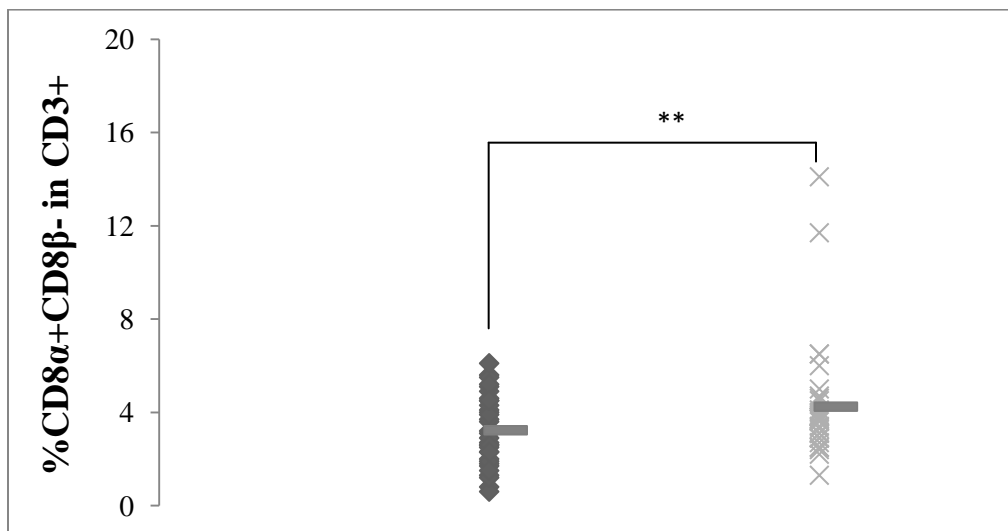




# H.



**I.**

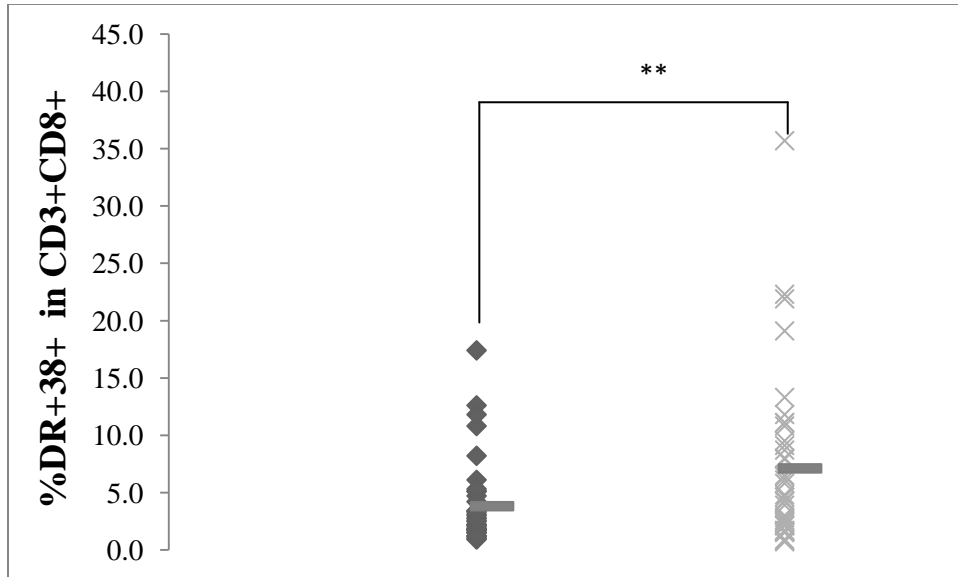


**Figure 2. Comparison of activation markers and homeostatic proliferation in blood and gut.** For Fig 2A-B, WB (blood) and MMC (gut) samples were collected, treated and prepared as in Fig. 1. Samples were gated on CD3<sup>+</sup> and evaluated for the following markers: **A)** HLA-DR+CD38<sup>+</sup> in CD8<sup>+</sup> (n=39, p=0.014). **B)** CD25<sup>+</sup> in CD8<sup>+</sup> (n=39, p<0.005). For Fig 2C-D, 0.5x10<sup>6</sup> CD3<sup>+</sup> freshly isolated MMC and 1.0x10<sup>6</sup> ficoll PBMC, as determined by BD TRUCount™, were stained per tube. Cell surface staining was performed on MMC and PBMC as described in Fig. 1 followed by intracellular staining using e-Bioscience Foxp3 Staining Buffer Set. Samples were then resuspended in 1 mL of Fixation/Permeabilization working solution and incubated for 30' at 4° followed by one wash in 1 mL of PBS staining buffer. Cells were then subjected to 2 washes in 2mL of 1X Permeabilization Buffer and centrifuged at 300xg for 7' followed by aspiration of the supernatant. Cells were incubated for 10' in 2% normal mouse serum, after which the IgG1 isotype control was added to Tube 1 and Ki67 added to Tube 2. Samples were incubated for an additional 30' at 4° and washed 2 times with permeabilization buffer before being resuspended in 500ul PBS staining buffer for acquisition by flow cytometry. PBMC (blood) and MMC (gut) samples were gated on CD3<sup>+</sup> and evaluated for the following markers: **C)** Ki-67<sup>+</sup> in CD8<sup>+</sup> (n=38, p<0.005). **D)** RA-CD28<sup>+</sup> in Ki-67<sup>+</sup>CD8<sup>+</sup> (n=38, p=0.005). **E)** RA-CD28<sup>-</sup> in Ki-67<sup>+</sup>CD8<sup>+</sup> (n=38, p<0.005).

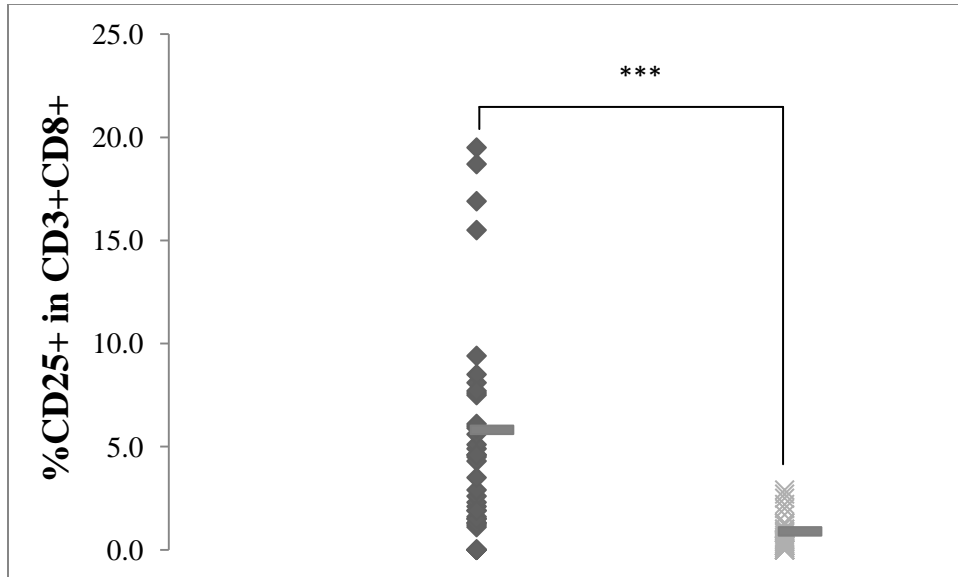
Asterisks signify the following value, \*\*\*, p≤0.005; \*\*, p≤0.05, and horizontal bars indicate mean values.

(♦) blood; (X) gut

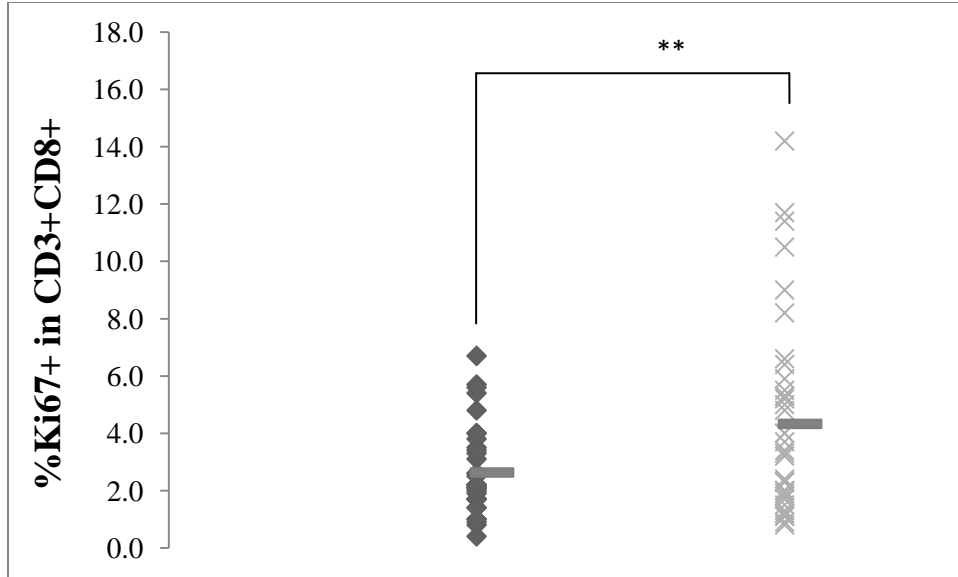
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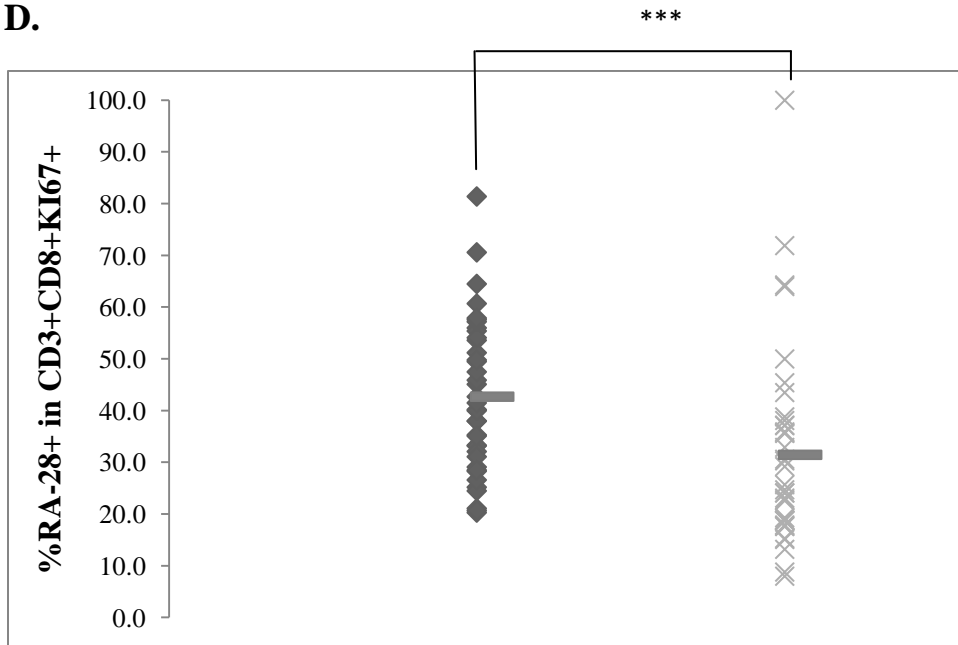
**B.**



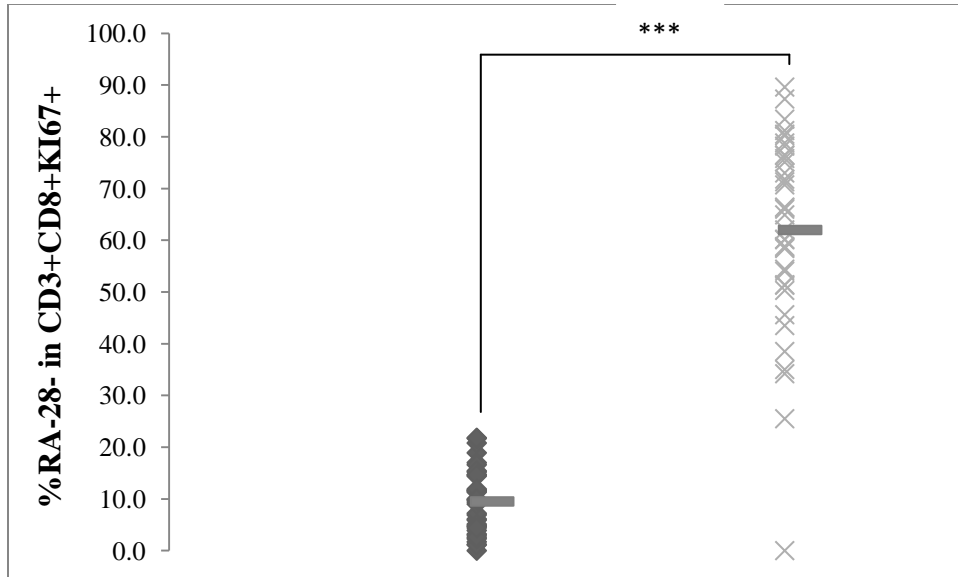
C.



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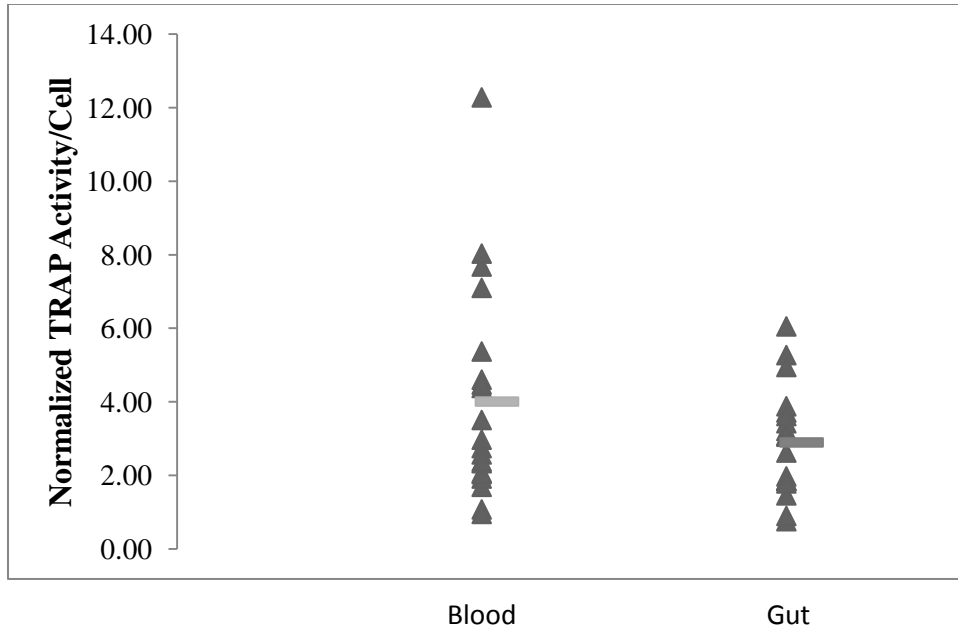
**E.**



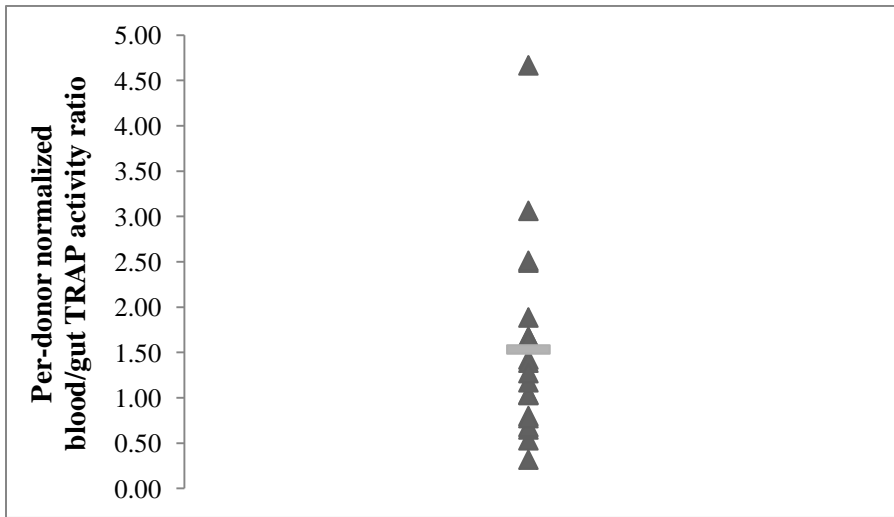


**Figure 3. Comparison of baseline telomerase activity of ex-vivo CD3 T cells.**  $0.32 \times 10^6$  sorted CD3+ cells from PBMC (blood) and MMC (gut) were lysed in 40  $\mu$ l M-PER buffer and relative telomerase activity/cell was determined using a modified version of the PCR-based telomeric repeat amplification protocol (TRAP) assay. Following the PCR, samples were run using 10% denaturing PAGE in 1X TBE buffer. Two replicates (lanes) were recorded for each sample and mean fluorescence value determined on a STORM 865. Each sample was normalized by nucleic acid amount (Nanodrop) and compared to a standardized number of telomerase-positive cells (Jurkat). **A)** Normalized TRAP activity/cell for blood and gut CD3+ T cells. (n=20, p=0.14) **B)** Per-donor normalized blood TRAP activity/gut TRAP activity ratio (n=20, mean 1.5, median 1.3).

A.



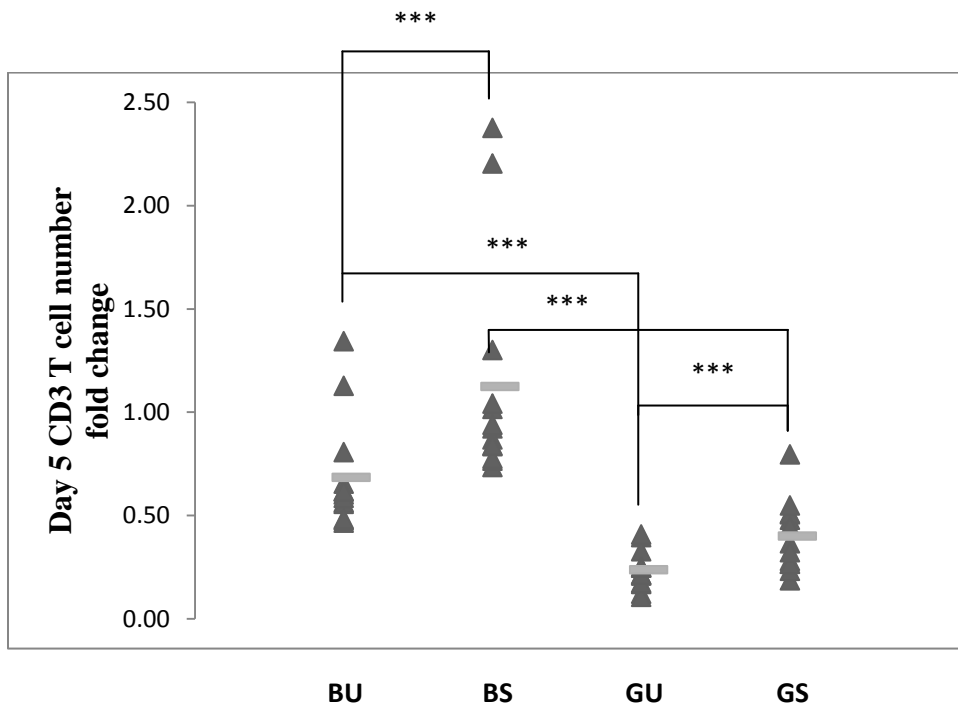
**B.**



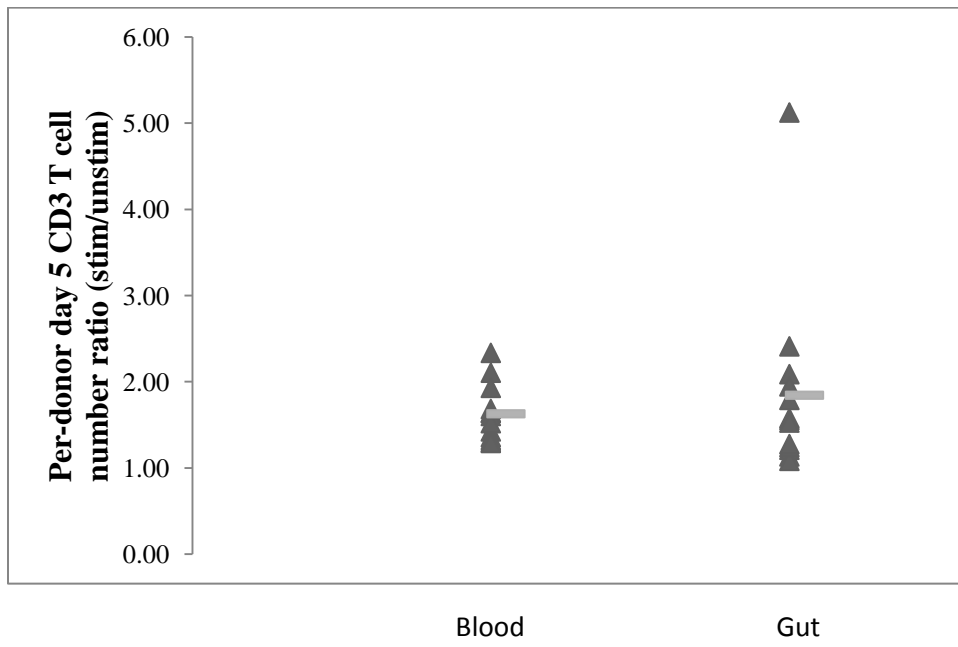
**Figure 4. Proliferative capacity of stimulated CD8 T cells in 5 day cultures.** Cultures of CFSE- and BrdU- labeled CD3/CD28/CD2-stimulated blood and gut cells (initial cells /well= $1.0 \times 10^6$ ) were analyzed at Day 5 (n=13). For each donor, 4 cultures were established, namely a stimulated and unstimulated control sample for blood and gut, respectively. Each well contained  $0.5 \times 10^6$  autologous irradiated feeder PBMC. On Day 5 each sample was divided into 2 fractions and analyzed following protocols for TRUCount™ staining and CFSE/BrdU dual-staining (See Sections 2.6 and 2.7 for detailed description of protocols). **A)** Day 5 CD3 T cell number fold-change for unstimulated and stimulated blood and gut cells was compared using TRUCount™ beads (n=13). **B)** Per-donor Day 5 CD3 T cell number ratio (stimulated/unstimulated) for blood and gut cells was compared using TRUCount™ beads (n=13, p=0.49). **C)** The percentage of unstimulated and stimulated blood and gut proliferating CD8+ T cells (CFSE<sup>lo</sup>BrdU<sup>+</sup>) on day 5 was compared using CFSE/BrdU dual-staining (n=13, unstim blood vs gut p<0.005; stim blood vs gut p=0.30). **D)** The mean number of proliferation cycles/cell (calculated by CFSE dilution) was compared for proliferating (CFSE<sup>hi</sup>BrdU<sup>+</sup>) CD8+ T cells in stimulated blood and gut cultures using CFSE/BrdU dual staining (n=13, p=0.50). **E)** The percentage of highly proliferative (operationally defines as 4 or more divisions) cells was compared for blood and gut proliferating (CFSE<sup>lo</sup>BrdU<sup>+</sup>) CD8+ T cells in stimulated cultures using CFSE/BrdU dual staining (n=13, p=0.56). Asterisks signify the following value, \*\*\*, p≤0.005

(BU) blood unstimulated; (BS) blood stimulated; (GU) gut unstimulated; (GS) gut stimulated

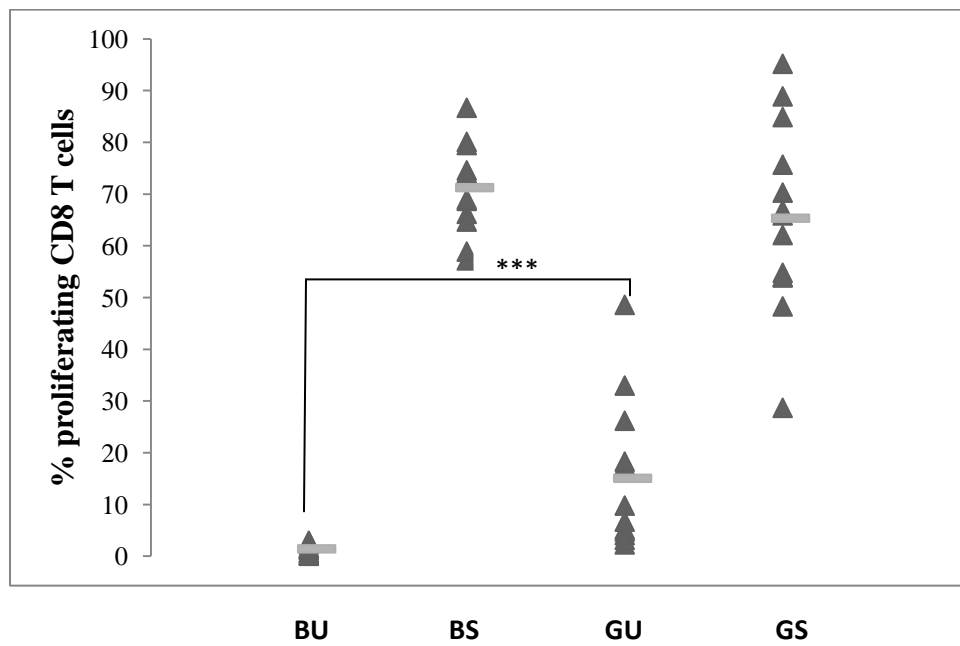
A.



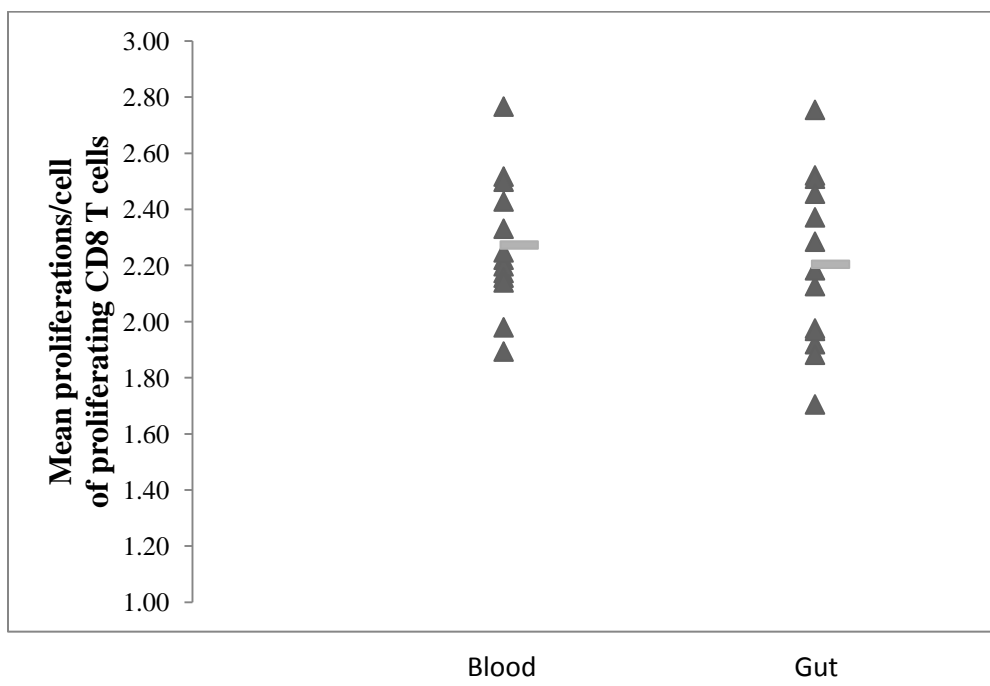
**B.**



C.

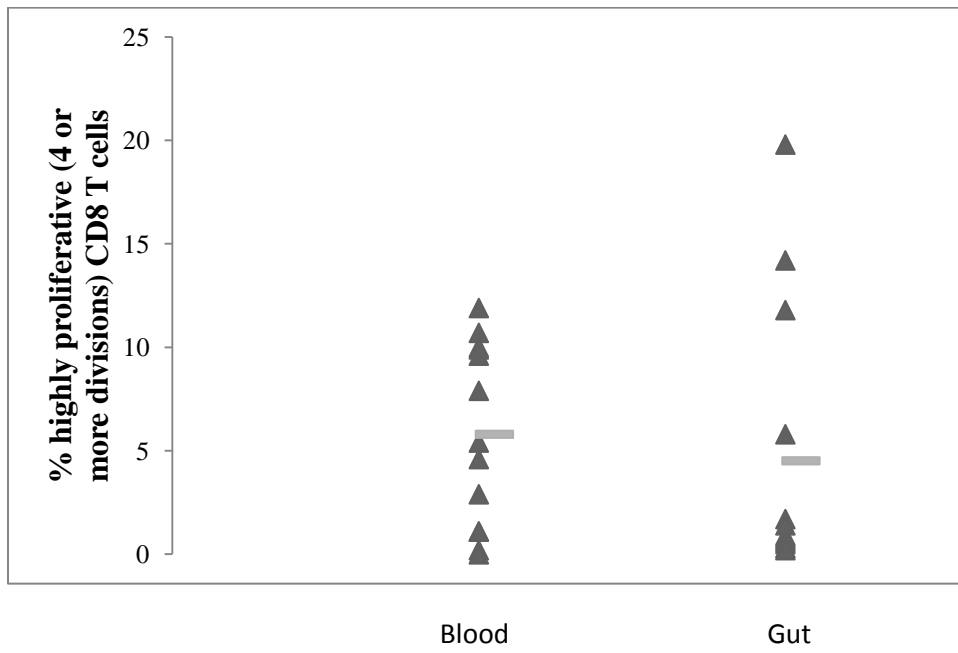


**D.**





**E.**



## **Chapter 3**

### **Optimization of protocol for comparing proliferative potential of human blood and gut T cells**

## ABSTRACT

The accumulation of peripheral blood late-differentiated memory CD8 T cells with features of replicative senescence, including inability to proliferate *in vitro*, has been extensively studied and is associated with increased morbidity and mortality in elderly humans. Of note, peripheral blood contains only 2% of the total body lymphocyte population. By contrast, the Gut Associated Lymphoid Tissue (GALT), the most extensive lymphoid organ, housing up to 60% of total body lymphocytes, has never been assessed with respect to senescence profiles. We report here the development and details of a novel, reproducible method for measuring and comparing proliferative dynamics of peripheral blood-and-gut-derived CD8 T cells. The protocol involves a 5 day culture of mononuclear leukocyte populations, from blood and gut 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 protocol development include: mode of T cell stimulation, CFSE concentration, inclusion of second proliferation marker BrdU, culture duration, initial culture concentration and inclusion of autologous irradiated feeder cells. This novel protocol has enabled us to test our hypothesis of compartment-specific differences of *in vitro* proliferative dynamics of CD8 T cells, as an indicator of replicative senescence and immunological aging.

## INTRODUCTION

The accumulation of late-differentiated memory CD8 T cells with features of replicative senescence has been associated with increased morbidity and mortality in elderly humans [1]. Senescent T cells are unable to enter cell cycle, lack CD28 expression, have shortened telomeres, and show enhanced inflammatory cytokine secretion. Of note, 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 senescence profiles.

Interestingly, our preliminary studies indicate that T cells from the gastrointestinal (GI) tract are more differentiated and antigen-experienced than their peripheral blood counterparts, suggesting that these two immune compartments may differ in their senescence trajectories. This agrees with rodent studies showing that age-associated alterations arise in the GI mucosal immune system earlier than in the peripheral immune compartment [4]. Similarly, in HIV infection, 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.

Here, we provide a detailed description of a novel reproducible protocol for measuring and comparing proliferative dynamics of peripheral blood-and gut-derived T cells. These

methods enabled us to test our hypotheses of compartment-specific differences in replicative senescence and then compare compartment results between study groups in an ongoing research project.

## **MATERIALS AND METHODS**

### ***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 blood and gastrointestinal (colorectal) mucosal biopsy (gut) samples collected from a total of 43 participants: 21 HIV seropositives (HIV-SP) (aged 23-57, median age 41.0, 19 male and 2 female) and 22 HIV seronegatives (HIV-SN) (aged 25-60, median age 42.9, 20 male and 2 female). The data on these participants are part of a larger study aimed at examining the effects of aging and chronic antigenic stimulation on the mucosal immune system using both young and old subjects who are either HIV seronegative or seropositive. (AG032422 PI: Effros).

### ***Collection of Peripheral Blood Mononuclear Cells (PBMC)***

Human peripheral blood samples were acquired by standard venipuncture immediately prior to endoscopy; 70cc of peripheral blood to be used 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. An additional 8cc were collected in two 4ml EDTA tubes; one was transferred to the flow cytometry research laboratory for T-cell immunophenotyping and the other was transferred to the CLIA certified Clinical Immunology Research laboratory for a White Blood Cell (WBC) with absolute lymphocyte count, which was

performed using the SYSMEX XT 1800i hematology analyzer. Samples (10ml of stored serum) were collected for future antibody and cytokine testing.

Following Ficoll centrifugation, PBMC were washed with 1X PBS and resuspended in 10 ml culture media (1X RPMI 1640, 15% FBS, 10mM HEPES, 2 mM glutamine, 50IU/ml penicillin/streptomycin, 500 µg/ml piperacillin-tazobactam, 1.25ug/ml amphotericin B). Viable PBMC concentration was calculated via trypan blue exclusion.  $5.0 \times 10^6$  PBMC were removed and irradiated at 5k rads 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  $10 \times 10^6$  CD3 T cells were collected from PBMC for CFSE staining.

### ***Collection of Mucosal Mononuclear Cells (MMC)***

Mucosal biopsy samples were collected as previously described [6]. 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 20-25mls of RPMI medium with 7.5% fetal calf serum (FCS) (R7.5), L-glutamine, amphoterin-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 20–25 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<sup>3</sup> 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 hours duration). The pooled isolated mucosal mononuclear cells (MMC) were combined and resuspended in 5ml of RPMI medium containing 10% FCS, amphotericin-b (1.25ug/ml) and Zosyn (50ug/ml). The MMC were used for flow cytometry and functional studies. Absolute CD3 T cell numbers were approximated using TRUcount™ beads. In a total of 135 MMC donors to date, encompassing this study and additional studies addressing gut immunosenescence, the average recovery following 30 biopsies was  $6.2 \times 10^6$  CD3 T cells, with a standard deviation of  $3.5 \times 10^6$  and range of  $1.6 \times 10^6$  -  $25.0 \times 10^6$  CD3 T cells.

### ***CFSE Staining***

CFSE staining was performed as previously described in [7], with assay-specific modifications to determine the final CFSE concentration. Briefly, for each donor, aliquots of whole PBMC or MMC including  $10 \times 10^6$  peripheral blood CD3 T cells,  $5 \times 10^6$  irradiated PBMC feeders, and  $2-3 \times 10^6$  mucosal CD3 T cells were separated into 15 ml polypropylene tubes, washed in 5 ml 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  $\mu$ M); (ii) Irradiated PBMC feeders (20.0  $\mu$ M); (iii) Mucosal T cells (5.0  $\mu$ M). 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 extracts were then resuspended in culture media (same as PBMC culture media) at  $1 \times 10^6$  CD3 T cells/ml and irradiated PBMC feeders were resuspended at  $0.5 \times 10^6$  cells/ml.

### *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  $1 \times 10^6$  CD3 T cells/ml. For each participant's samples, both stimulated and unstimulated control wells were included in the assays. All wells also included  $0.5 \times 10^6$  irradiated (5k rads) feeder PBMC. Two additional wells, containing  $0.5 \times 10^6$  stimulated and unstimulated irradiated feeder PBMC, respectively, were established as further controls to verify no feeder PBMC were being included in the live CD3 T cell gate.

For all stimulated wells, 5  $\mu$ l T cell activation microbeads (anti-CD2/3/28; Miltenyi Biotec; Auburn, CA) were added to each culture ( $0.5 \times 10^6$  microbeads/ml culture). For all conditions 0.1 mg 5-bromo-2'-deoxyuridine (BrdU) (Becton Dickinson Immunocytometry Systems (BDIS); San Jose, CA), 0.1  $\mu$ g 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.

Cultures were incubated for 5 days at 37° C, 5% CO<sub>2</sub>. 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 an effort to optimize the cellular proliferation assays of blood and gut samples. These include stimulation method, initial cell concentration, culture kinetics, culture size, and whether or not to include irradiated autologous feeders.

### ***TRUCount™ and Absolute T-Cell Counting***

#### **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 ul of BD Multitest antibody cocktail (BD Biosciences, San Jose, CA) containing CD3-fluorescein (FITC), CD8-phycoerythrin (PE) , CD45-Peridinin Chlorophyll protein (PerCP) and 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 15 minute incubation at room temperature in the absence of light, 450 ul of 1X 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 instrument settings 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. 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 vs 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.

### **Day 5 TruCount**

The 5 day cultured cells were harvested, washed and resuspended in 200ul of PBS staining buffer (1X phosphate buffered saline with 2 % heat inactivated newborn calf serum). A 100ul 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 500ul of staining buffer. The cells were transferred to TRUCount tubes just prior to acquisition on a FACSCanto cytometer (BD).

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 and because a forward scatter threshold can exclude small trucount beads and lead to erroneous counts. The feeder cells were off scale, (a result of high CFSE labeling) and thus were excluded.

### **Absolute T-cell counting:**

Gating sequence: List mode data were first gated for viability using a forward versus side scatter gate (Supp. Fig. 1i). 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 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 [ $(T\text{-cell count}/Bead\ count) \times (Total\ beads/100\ \mu l\ cells) = Absolute\ T\text{-cell\ count}$ ].

## RESULTS

### *Measurement of T-cell proliferation cycles using BrdU and CFSE*

A 100 ul aliquot (the leftover volume of the original 200 ul after 100 ul had been taken for Day 5 TruCount™ staining—See Section 2.6) 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 exception that CD3-PE and CD8-PECy7 were added at the same time as the anti-BrdU-APC. When acquiring the data, the cytometer stop counters were set to routinely collect a minimum of 10,000 lymphocytes with the light scatter qualities of viable cells.

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 (Fig. 1Ai-iii). The scatter gate excluded smaller cells with high side scatter, and the CD3 gate excluded CD3+ feeder cells that were off scale in CFSE fluorescence. The quadrant markers of the bivariate plot were used to differentiate proliferating ( $CFSE^{lo}BrdU^{+}$ ) cells from non-proliferating  $CFSE^{hi}BrdU^{-}$  T-cells (Fig. 1Aiv). The addition of BrdU allowed exclusion of contaminating debris and events which sometimes stained as  $CFSE^{lo}BrdU^{-}$  (Fig 1Aiv). We also included a 1D histogram of CFSE fluorescence (Fig. 1B), which allowed us to compare CFSE staining in non-proliferating populations in unstimulated and

stimulated cultures, to verify the CFSE<sup>hi</sup> population is non-proliferating. The 1D CFSE histogram could also be used in data analysis of proliferative dynamics, such as determining number of proliferation cycles and frequency of cells in each cycle.

### ***Determining the optimal mode of T cell stimulation***

Three commonly used methods to stimulate peripheral blood T cells were compared on T cells present in the mucosal mononuclear cells (MMC): (1) Miltenyi anti-CD2,3,28 Ab beads (2) Immobilized OKT3 + CD28 Ab and (3) Bispecific CD3 + CD4 Ab (selectively stimulates CD8+ T cells). Miltenyi beads were tested on MMC from four donors; on day 5 after stimulation, the percent of live gated CD3+ cells that were activated (CFSE<sup>lo</sup>) was 84%, 83%, 60% and 72% (mean 75%), respectively (Fig. 2A). Immobilized OKT3 was tested on MMC from three donors. Only one donor sample successfully activated, with 17% activation of live gated cells (Fig. 2A). Bispecific Abs were tested on MMC from two donors, with 42% and 54% (mean 48%) of live gated CD3+ cells being activated (Fig. 2A). In a side-by-side comparison of Miltenyi beads and Bispecific Abs from the same 2 donors, bead stimulation resulted in a higher percentage of stimulated CD3+ cells (mean 66% vs. 48%, data not shown). Fig. 2B illustrates a representative 1D histogram of proliferating (CFSE<sup>lo</sup>) versus non-proliferating (CFSE<sup>hi</sup>) CD3+ cells for each stimulation method (bead method 72% proliferating, OKT3 17% proliferating, bispecific ab 54% proliferating). Because Miltenyi bead stimulation resulted in the greatest percentage of proliferating (CFSE<sup>lo</sup>) CD3+ cells, this stimulation method was selected for our protocol going forward. An additional advantage of the Miltenyi reagent over the bispecific (CD3/CD4) Abs is

that the former results in both CD4 and CD8 T cell stimulation, whereas the latter selectively activates only the CD8+ T cell subset.

### ***Evaluating the bead: cell ratio***

Miltenyi anti-CD2,3,28 Ab beads have been used extensively by us and others in studies of human peripheral blood CD3+ T cells, 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 bead:cell ratios of 1:2 and 1:4 were evaluated in gut-derived CD3+ T cells in one donor on days 3 and 7. Results of this experiment indicated that stimulation with 1:2 and 1:4 bead:cell ratios were comparable (Day 3 CFSE<sup>lo</sup> CD3+ cells: mean CFSE 9,818 vs. 10,423 Mean Fluorescence Intensity (MFI) ; Day 7: mean CFSE 643 vs. 567 MFI respectively) (Data not shown). After refining other parameters of the assay, a follow-up experiment on cells from a single donor was performed, testing three bead:cell ratios, 1:4, 1:2, and 1:1.. On day 5 post-stimulation, the percentage of non-dividing gut derived CD3+ T cells was 4%, 3.5% and 3.2% respectively, and the percentage of dividing CD3+ T cells was 75.6%, 77.4% and 79.3% respectively (Data not shown). As there was no substantial quantitative difference between the different concentrations (consistent with the earlier experiment looking at Days 3 and 7) we opted to stay with the manufacturer's recommendation of a 1:2 bead:cell ratio for PBMC and MMC.

### ***Optimizing the CFSE concentration***

CFSE is an intracellular fluorescent dye that has been shown to be particularly useful in measuring peripheral blood T lymphocyte proliferation in response to stimulation, both *in vitro* and *in vivo* [7, 10]. During the labeling phase, prior to T cell activation, CFSE is able to stably incorporate into cells via covalent coupling to intracellular proteins with a high fluorescence intensity, exceptionally low variance, and low toxicity [7]. Following activation, CFSE concentration is halved with each cellular division. Due to the low variance, proliferating populations form up to 7 or 8 distinct CFSE peaks corresponding to populations of cells that have undergone a distinct number of cellular divisions [11]. Thus, staining T cells with CFSE prior to culturing allows differentiation of non-divided (CFSE<sup>hi</sup>) CD3 T cells from divided (CFSE<sup>lo</sup>) CD3T cells, and calculation of the number of divisions undertaken by each cell, either visually or quantitatively by CFSE dilution relative to the non-dividing population.

In developing the proliferation assay, it was necessary to find an appropriate CFSE concentration with which to stain blood and gut derived cells prior to culturing, where all non-proliferating and proliferating cells can be detected and differentiated from background. Preliminary assay development using blood derived T cells confirmed our previously established efficacy of staining PBMC containing  $10 \times 10^6$  CD3 T cells with 2.5  $\mu$ M CFSE (Data not shown). However, due to limitations in cell number, only  $2-3 \times 10^6$  gut derived CD3 T cells from each subject were available for culture. In experiments using biopsies from two subjects, we evaluated the efficacy of exposing  $2-3 \times 10^6$  gut derived CD3 T cells prior to culture with 2.5  $\mu$ M CFSE. Results on Day 5 indicated CFSE<sup>hi</sup> (undivided) CD3 T cells were in the instrument's detection range, but CFSE<sup>low</sup> CD3 T cells with the highest number of cell divisions had CFSE



concentrations so low as to be in the background range as determined by a CFSE unstained control culture. In both experiments, doubling the initial CFSE concentration in gut derived cultures to 5.0  $\mu\text{M}$  increased CFSE levels to a point where all dividing and non-dividing cells CD3 T cells were in detectable range above background with no apparent toxicity issues. Fig. 3 demonstrates in one donor using a CFSE concentration of 2.5  $\mu\text{M}$  allows all divided peripheral blood T cells to remain out of background CFSE range, but with gut T cells this concentration is not sufficient and a concentration of 5.0  $\mu\text{M}$  is necessary to avoid dividing cells falling into background CFSE range. Based on these results, our final protocol involved staining of  $10 \times 10^6$  blood derived CD3 T cells with 2.5  $\mu\text{M}$  CFSE and  $2-3 \times 10^6$  gut derived CD3 T with 5.0  $\mu\text{M}$  CFSE for each 5 day culture.

#### ***Inclusion of a second proliferation marker***

Although CFSE staining is a highly effective method to measure proliferation of peripheral blood T cells *in vitro* [10], our early efforts studying proliferation of gut-derived T cells indicated the need for further refinement of this protocol. Indeed, 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. 4A). Additionally, the demarcation between proliferation and non-proliferating populations was difficult to determine (Fig. 4A). To confirm that CFSE dilution was, in fact, occurring via 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. Adding this second marker has proven to be an invaluable tool in

differentiating T cell populations with low CFSE that is due to cellular division (and are, therefore CFSE<sup>lo</sup>BrdU<sup>+</sup>), as opposed to poor staining (CFSE<sup>lo</sup>BrdU<sup>-</sup>) (Fig. 4B).

We performed 5 day proliferation assays on blood and gut samples from 29 subjects with dual CFSE and BrdU staining. We specifically focused on the proportion of CD8<sup>+</sup>CFSE<sup>lo</sup>BrdU<sup>-</sup> cells, which would be registered as proliferating if CFSE had been the sole marker being used, but could now be eliminated as non-proliferating due to lack of intracellular BrdU absorption. On Day 5 in stimulated cultures, CFSE<sup>lo</sup>BrdU<sup>-</sup> cells accounted for, on average, 3.3% of blood derived CD8 T cells and 9.8% of gut derived CD8 T cells (p<0.005) (Fig. 4C). These observations underscored the importance of including BrdU, particularly in gut cultures, as a second stain to help define true CFSE-related proliferation and eliminate CFSE<sup>lo</sup> false positive cells.

Further retrospective analysis of CFSE and BrdU levels on these 29 donors indicate relative CFSE equivalency at day 5 for dividing and non-dividing blood and gut derived cells (Proliferating T Cells: blood 7232.4 MFI, gut 6233.0 MFI; Non-proliferating T Cells: blood 33193.0 MFI, gut 31088.5 MFI), supporting the efficacy of chosen CFSE and BrdU dosing regimens (Data not shown).

### ***Kinetics of the proliferative response***

To determine optimal culture duration for analysis of T cell proliferation of blood- and gut-derived T cells, we performed a time course experiment of samples from three donors and evaluated proliferation parameters on Day 3, Day 5 and Day 7. For blood derived T cell

cultures, T cell numbers and proliferative generations steadily increased in all three donors from Day 3 to Day 7 (Data not shown), consistent with published data [12, 13].

The results for the gut-derived cultures were less uniform. We analyzed the CD3 T cell number fold-change in total T cell population size (ending number of live CD3 T cells/initial number of CD3 T cells) at Day 3, 5 and 7 in cultures from 3 subjects. The peak mean T cell 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). In terms of individual donor kinetics, from Day 5 to Day 7 two donors show a decrease in T cell population size, while one donor shows an increase (Fig. 5A).

Within the CD8 T cell population, on average, the percentage of proliferating (CFSE<sup>lo</sup>BrdU<sup>+</sup>) cells plateau at approximately Day 5 (Day 3, 37%; Day 5, 54%; Day 7, 56%) (Fig. 5B). It should be noted that there was high donor-to-donor variation in proliferative kinetics, especially from Day 5 to Day 7 (Fig. 5B). Based on the data from these initial experiments, day 5 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 represent a time frame of uniform proliferative growth.

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

Having established stimulation, labeling and culture duration parameters, we sought to determine the optimal number of cells for initiating the cultures. 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 create what we determined to be viable

proliferating and non-proliferating populations in 2 dimensional gating of CFSE vs. BrdU.

Based on visual determination of stimulated and unstimulated blood and gut samples from the 43 donors, we estimated this threshold to be approximately 1500 events. For blood derived cultures, cell input of  $0.5 \times 10^6$  and  $1.0 \times 10^6$  CD3 T cells in 48-well flat bottom plates resulted in 100% assay success as defined by this criterion (Data not shown). For gut, cultures starting with  $0.5 \times 10^6$  T cells (n=13) yielded a 54% success rate at Day 5 and cultures starting with  $1 \times 10^6$  T cells (n=30) yielded a 70% success rate (Data not shown). In unsuccessful cultures (i.e., <1500 events in BrdU tube) it appeared there was no viable CD3+ MMC population at Day 5, for unknown reasons. This finding led us to initiate cultures with  $1.0 \times 10^6$  cells for PBMC and MMC.

In short-term cultures of blood derived T cells, inclusion of irradiated autologous feeders facilitates T cell survival and proliferation [14, 15], however 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  $0.5 \times 10^6$  autologous irradiated feeder PBMC. Prior to culture, irradiated feeders were incubated for 10 minutes with a very high concentration of CFSE ( $20 \mu\text{M}$  CFSE per  $5.0 \times 10^6$  feeder PBMC), so they could be gated out during analysis on Day 5, due to off-scale high CFSE levels. For every culture we included two feeder cell control wells, one stimulated and one non-stimulated, to confirm that no feeders were present in the CFSE gate. 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. 2). Despite only modest

enhancements in cell viability, we chose to include  $0.5 \times 10^6$  autologous irradiated PBMC feeder cells stained with 20.0  $\mu\text{M}$  CFSE per  $5.0 \times 10^6$  feeder cells in our assay.

### ***Comparison of blood and gut CD8 T cell proliferation***

Our goal has been to develop a reproducible method to evaluate proliferative dynamics of human peripheral blood- and gut-derived lymphocyte populations. To illustrate the utility of the results described above, we now show a comparison of the Day 5 proliferation of CD3+8+ T cells from blood versus gut cells derived from cultures of 29 of our study subjects, whose cells yielded technically successful results. We first identified and gated non-proliferating (CFSE<sup>hi</sup>BrdU<sup>-</sup>) versus proliferating (CFSE<sup>lo</sup>BrdU<sup>+</sup>) CD3+8+ T cell compartments in bead stimulated cultures, as described in Fig. 1A. We next used the mean CFSE fluorescence values for non-proliferating and proliferating compartments and determined mean number of proliferative generations, using the formula:  $n = \log(\text{mean non-prolif CFSE} / \text{mean prolif CFSE}) / \log(2)$ . The mean number of CD3+8+ proliferative generations for bead stimulated cultures on Day 5 is 2.19 for PBMC and 2.28 for MMC (n=29, p=0.19) (Fig. 6).

## DISCUSSION

Our studies provide the first detailed protocol for evaluating the proliferative potential of T cells within the human gut, the largest lymphoid organ in the body. This assay will be critical in future comparisons between blood and gut, such as in studies to determine the effect of aging and certain chronic diseases on the human gut. Moreover, given emerging data on the importance of individual variations within the gut microbiome, evaluation of the immune system status within the gut will become increasingly important clinically [16, 17].

In developing the proliferation assay, it became immediately clear that mucosal T cells stained with CFSE did not routinely form the tight, divisional bands observed with peripheral blood T cells (Fig. 4A), and instead formed a broad slope, where divisions could not be qualitatively judged and the demarcation between proliferating (CFSE<sup>lo</sup>) and non-proliferating (CFSE<sup>hi</sup>) cells was sometimes difficult to determine (Fig. 4A). Although the reasons for this difference are not clear, one possible explanation is that protein carryover in the mucosal suspension after tissue digestion interferes with CFSE uptake, resulting in high variability of CFSE content per cell.

Interestingly, based on the Day 5 TRUCOUNT™, the number of live CD3 T cells recorded for stimulated cultures of PBMC and MMC was lower than one would expect. Based on all successful cultures (n=29), on Day 5 the average CD3 T cell fold-change for PBMC stimulated wells was 0.94 and for MMC was 0.36 (p<0.005) (Supp. Fig. 3), well below the starting cell number. It is known that following non-specific stimulation of blood-derived T cell subsets *in vitro* or *in vivo*, it takes 1-2 days for activated cells to begin proliferating and initially cell

numbers may decline as activation induced cell death (AICD) outpaces proliferation [11, 18, 19]. This is consistent with our own unpublished observations of *in vitro* T cell growth dynamics following stimulation with Miltenyi anti-CD2, 3, 28 Ab beads. However, in the aforementioned studies and our own unpublished observations, by Day 5 the stimulated T cell populations had recovered and were above starting cell population numbers.

A possible explanation for why the Day 5 cell numbers are so low for blood and gut derived cultures relates to our cell counting methodology. CD3 T cell concentration is initially determined using TruCount™. However, prior to culture, cells are stained with CFSE, which requires several washing steps, where cell loss may occur. To test this hypothesis, we performed an additional TRUCount™ on blood and gut samples on Day 0 following CFSE staining prior to culturing. The Day 0 post-CFSE staining TruCount™ results indicate our estimated cell numbers overestimate the number of T cells in beginning cultures; however, there was no bias in CD3 T cell loss during processing for blood or gut cells (blood  $0.42 \times 10^6$  mean starting CD3 T cells, gut  $0.44 \times 10^6$  mean starting CD3 T cells, n=2) (data not shown). These results help explain why the day 5 yields may be relatively low for blood and gut cultures.

Another potential explanation for low Day 5 CD3 T cell numbers is that CFSE from irradiated feeder PBMC (labeled with a very high dose of 20.0  $\mu$ M CFSE) is leaching into the culture medium and causing toxicity. To exclude this possible cause, for two of our donors we included an additional bead stimulated condition for blood and gut cells, using non-CFSE labeled autologous irradiated feeder PBMC. We also included a control well with only non-labeled autologous feeders for each donor. Our results indicate that the unlabeled irradiated feeder PBMC caused the D5 CD3T cell yield to only marginally increase (blood 14% increase, gut 17%

increase, n=2) (data not shown). Furthermore, in the control wells containing unlabeled irradiated PBMC, feeders were entering the live CD3 T cell gate and being recognized as proliferating (CFSE<sup>lo</sup>) CD3 T cells during D5 TruCount™. CFSE/BrdU analysis confirmed this, as percentage of CD8 T cells that were CFSE<sup>lo</sup>BrdU<sup>-</sup> increased (CFSE<sup>lo</sup>BrdU<sup>-</sup> CD8 population: blood- CFSE labeled feeders 1.7%, unlabeled feeders 4.5%; gut- labeled 7.3%, unlabeled 9.8%, n=2) (data not shown). Taken together, these data indicate that labeling feeder PBMC with 20.0 μM CFSE does not confer significant toxicity, and it is likely that most (if not all) the increase in D5 CD3 T cell yield can be attributed to inclusion of non-labeled feeder PBMC in the live CD3 gate.

Regardless of whether final cell numbers are reduced due to cell loss during processing, it is clear that by Day 5 gut derived cultures consistently had significantly fewer CD3 T cells than peripheral blood cultures from the same donor. In stimulated cultures, on average blood derived cultures had almost three times as many live CD3 T cells as gut-derived cultures on day 5 (average fold change 0.94 vs. 0.36) (Supp. Fig. 3). Possible factors contributing to this reduced viability include (a) physical trauma to cells during extraction and processing, (b) interference from impurities in mucosal cell suspensions, (c) biological differences inherent in mucosal T cells which would render them more sensitive to culturing and stimulation. For example, unpublished work in our laboratory indicates gut CD8 cells have higher expression of several markers associated with replicative senescence relative to blood CD8 cells, possibly affecting culture growth.

The use of irradiated feeders is a well established method to facilitate T cell survival and proliferation *in vitro* [20, 21]. In an effort to enhance cellular survival and proliferation,



especially in gut derived cultures, we included  $0.5 \times 10^6$  autologous irradiated PBMC feeders in every well. However, side by side comparisons of experiments conducted with and without feeders indicated very modest increases of 14% in blood derived cultures and 5% in gut derived cultures (n=7) (Supplementary Figure 2). The above data reflect experiments conducted starting with  $1.0 \times 10^6$  cells. Interestingly, we looked at a small number of preliminary cultures starting with  $0.5 \times 10^6$  cells, and feeders facilitated a 24% increase in blood derived cultures (n=4) and 55% increase in gut derived cultures (n=2) (Data not shown), indicating irradiated feeders may enhance cellular proliferation more dramatically under different assay conditions.

Because mucosal cultures have neither high cell yields nor consistently tight, reliable CFSE proliferation bands, it was necessary to establish parameters to determine whether or not a particular Day 5 stimulation was “technically successful” (i.e., reliable). Quantitatively, the proliferation stimulation was considered successful if there were greater than 1500 CD3+ events in the BrdU treated aliquot at Day 5 following BrdU treatment, staining and flow. We chose this threshold after analyzing several donors with borderline culture results and determining 1500 CD3+ events was a lowest threshold which we could definitively identify viable populations. The unstimulated control cultures were critical in terms of both quality control and for determining culture viability. First, the fluorescence distribution of the unstimulated cells was important to verify satisfactory loading of the cells. The unstimulated cells were also used as a guide for determining the CFSE fluorescence that corresponded to non-proliferating cells. We consistently observed in the **unstimulated** mucosal T cell cultures, a measurable cluster of proliferating T-cells which was only rarely observed in the blood derived T-cells (% CD3+8+ cells proliferating: PBMC 1.7%, MMC 15.3%,  $p < 0.005$  (n=29)) (Data not shown). This cluster was observed in the TRUcount™ assay and confirmed by BrdU expression in the BrdU assay.

In determining the initial cell number for the proliferation assay, we were limited to a maximum of  $1.0 \times 10^6$  cells/well, due to low number of available MMC T cells. We examined two different cell numbers, namely,  $0.5 \times 10^6$  and  $1.0 \times 10^6$  cells per well in 48 well flat bottom culture plates. Ultimately we determined that  $1.0 \times 10^6$  cells/well was optimal, because the assay success rate was significantly higher, 70% versus 54%, than with  $0.5 \times 10^6$  starting cells. It is believed the increase in assay success rate with  $1.0 \times 10^6$  cells was mainly because the higher starting cell number allowed for more CD3+ events to be gated on Day 5, especially in the BrdU treated cells, from which considerable numbers of cells were lost during treatment. As stated above, CD3+ population survival was significantly lower with MMC than PBMC, and in many donors starting with  $0.5 \times 10^6$  cells, there were not enough live CD3+ cells left at Day 5 to form viable populations for analysis. Based on visual analysis, we determined the minimum population threshold to be around 1500 viable CD3+ events for each condition.

One of the issues encountered in developing this protocol was that mucosal T cell proliferation cultures yielded wide CFSE fluorescence distributions, lacking the usual distinct, tight proliferative populations seen with PBMC cultures. Thus, there was no definitive method to reliably differentiate proliferating from non-proliferating populations using CFSE alone. We therefore elected to incorporate a 2<sup>nd</sup> marker, BrdU, and to separate proliferating (CFSE<sup>lo</sup>BrdU<sup>+</sup>) and non-proliferating (CFSE<sup>hi</sup>BrdU<sup>-</sup>) populations using 2 dimensional analysis (Fig. 1). This 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 (in the study total number of proliferating cells using BrdU was consistently 0.8 that of CFSE), and established both methods were non-toxic in the range being tested [11].

Using CFSE and BrdU dual staining, we were able to precisely demarcate proliferating and non-proliferating populations. In addition, we were able to exclude cells that were CFSE<sup>lo</sup> without incorporating BrdU and becoming BrdU<sup>+</sup>. Although this population was negligible in peripheral blood cultures (AVG: 3.3%) it was a significant component of mucosal cultures (AVG: 9.8%) (Fig. 4C), indicating potential issues with CFSE uptake or antibody labeling in mucosal suspension.

Both CFSE and BrdU were administered in dose ranges established as efficacious and non-toxic for peripheral blood T cells in previous work by our laboratory. Preliminary experiments on peripheral blood and mucosal T cell cultures confirmed negligible toxicity (data not shown) for both labels at doses utilized (CFSE, peripheral blood 2.5  $\mu$ M/10x10<sup>6</sup> T cells, gut 5.0  $\mu$ M/2-3x10<sup>6</sup> T cells; BrdU, 0.1mg/ml). We also sought to eliminate a potential confounder with BrdU administration, namely insufficient free synthetic nucleotide for incorporation into all dividing cells in gut cultures, so dividing cells would be mis-labeled as non-proliferating because they were CFSE<sup>lo</sup>BrdU<sup>-</sup>. This could possibly explain the relatively high CFSE<sup>lo</sup>BrdU<sup>-</sup> populations in many gut derived cultures. To test this, in one donor we compared identical gut derived cultures with one culture receiving the standard BrdU dose at Day 0 and another culture receiving this dose plus a second standard dose at Day 3. Comparing the two cultures, the culture receiving the second dose of BrdU actually had a slightly higher percentage CFSE<sup>lo</sup>BrdU<sup>-</sup> population (21.8% vs 18.4%) (Data not shown); indicating BrdU was not a limiting reagent. Interestingly, the culture receiving the extra BrdU had a significantly lower cell number at Day

5, (mean CD3 fold-change 0.23 vs 0.56) and lower % of proliferating cells (68.7% vs 75.0%) compared to the culture only receiving one BrdU dose (Data not shown), indicating some possible toxicity issues with a 2nd dose of BrdU.

In developing the proliferation assay for mucosal T cells, it was critical to determine the optimal activation method. Numerous studies by our own groups and others have validated activation via direct stimulation of the TCR and coreceptors [12, 13] as well as by mitogens such as PMA [22] and PHA [23]. 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 [24]. We therefore focused on activation methods that stimulate via the T cell receptor to evaluate the global proliferative potential of CD3<sup>+</sup> T cells. Accordingly, we compared the following three methods of TCR-mediated activation: (1) Miltenyi anti-CD23,28 Ab beads, (2) Immobilized OKT3 + CD28 Ab, and (3) Bispecific CD3 + CD4 Abs. Following direct comparison cultures of T cells from peripheral blood and gut (Fig. 2A); 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 bispecific antibodies, this stimulation method demonstrated no bias towards proliferation of CD4 or CD8 T cells, at least in a 5 day culture.

In determining an optimal CFSE concentration for blood and gut derived cultures, the main considerations were avoiding toxicity, and ensuring that on day 5 all dividing and non-dividing cells were in the flow cytometer's sensitivity range following antibody staining. For peripheral blood cultures, CFSE concentration and staining protocols fitting these criteria had

already been established, and were confirmed by comparing CFSE stained cultures to cultures without CFSE (Fig. 3). For MMC, because of the considerable amount of mucosal debris in these cultures, we hypothesized the CFSE staining concentration might have to be considerably higher. Through trial and error we determined a concentration of 5.0  $\mu\text{M}$  for mucosal samples containing  $2\text{-}3 \times 10^6$  T cells to be optimal. At this concentration both non-dividing and dividing cells are within instrument detection parameters and above background levels for CFSE, whereas at half the concentration (2.5  $\mu\text{M}$  CFSE) mucosal cells undergoing the most divisions (i.e., lowest CFSE) cannot be differentiated from background (Fig. 3).

As Miltenyi beads are known to stimulate both CD4 and CD8 T cells, the final issue we addressed was whether during a 5 day culture period the beads would differentially favor one T cell subset in gut vs. blood, a situation that has been observed in long term cultures ( $> 30$  days) with Miltenyi bead stimulation (Effros, unpublished observations). To test for possible selective proliferation/survival of CD4 or CD8 subsets in gut vs. blood, we compared the % CD8 T cells (of total T cells) on Day 1 versus Day 5 in the unstimulated and stimulated compartments. To eliminate possible bias due to effects of HIV infection, we included only SN donors in this analysis. The results indicate no significant change in % CD8 T cells from Day 1 to Day 5 in blood unstimulated (30.6 vs. 28.8,  $p=0.65$ ), blood stimulated (30.6 vs. 31.3,  $p=0.87$ ), gut unstimulated (31.9 vs. 31.9,  $p=0.99$ ), or gut stimulated (31.9 vs. 35.9,  $p=0.24$ ) cultures ( $n=13$ ); confirming that our newly established culture system did not exert any stimulation bias toward a particular T cell subset in a 5 day timeframe.

In conclusion, the assay described in this report outlines the first detailed protocol for evaluating proliferative dynamics of blood and rectal mucosal T cells *in vitro*. To date, this

experimental procedure has been successfully performed on samples from 29 research subjects, including HIV seronegative and seropositive donors, as part of a larger study to determine how mucosal T cells relate to their peripheral blood counterparts, and how age and HIV infection may differentially affect mucosal T cells. Ultimately, this assay, along with additional functional tests, will allow immunologists and clinicians to better understand how disease processes affect immune performance in this vital immune organ, and facilitate development of effective therapies specific for immune dysregulation in this system.

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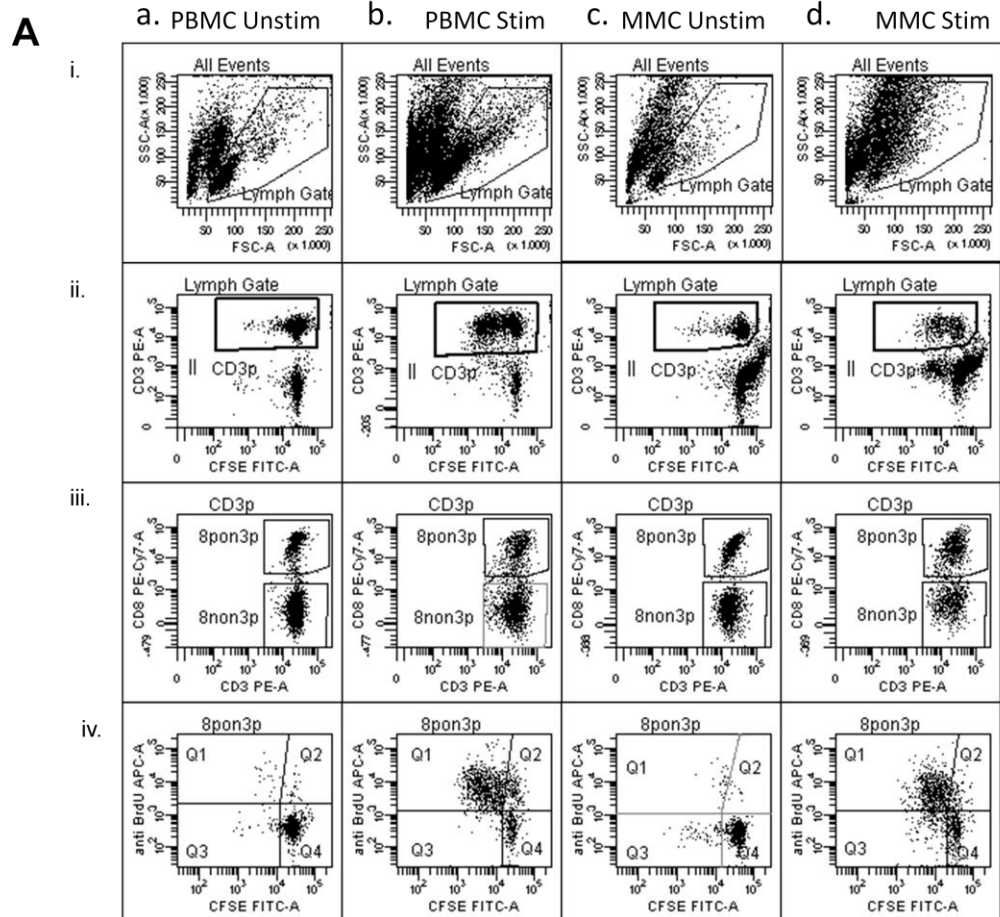
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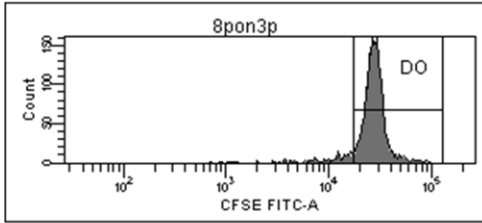
**Figure 1. Enumeration of proliferating (CFSE<sup>lo</sup>BrdU<sup>+</sup>) and non-proliferating (CFSE<sup>hi</sup>BrdU<sup>-</sup>) live CD3+8 T cell populations utilizing bivariate plots of CFSE-FITC (x-axis) versus BrdU-APC (Y-axis).** **A.** (i) Viable cells were first gated on forward versus side-scatter feed, (ii) followed by gating on CD3-PE versus CFSE-FITC), (iii) then CD8+ cells were isolated gating using CD8-PECy7 on viable T cells. (iv) The quadrant markers of the bivariate plot were used to enumerate proliferating (CFSE<sup>lo</sup>BrdU<sup>+</sup>) cells from non-proliferating (CFSE<sup>hi</sup>BrdU<sup>-</sup>) T-cells. **B.** Histogram of CFSE fluorescence is used to confirm demarcation between proliferating and non-proliferating populations and to evaluate proliferative dynamics. Columns a-d are representative plots for (a) PBMC unstimulated, (b) PBMC stimulated, (c) MMC unstimulated, and (d) MMC stimulated Day 5 cultures, respectively. (Cultures represented in histograms previously gated on CD3+ and CD8+ as illustrated in Fig 1A)

[Legend: CD3p =CD3+, PBMC = blood, MMC = gut]

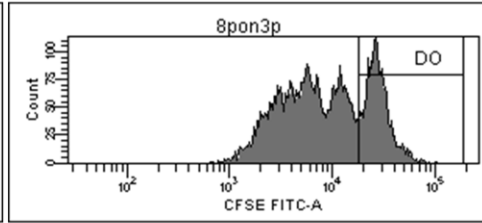


**B**

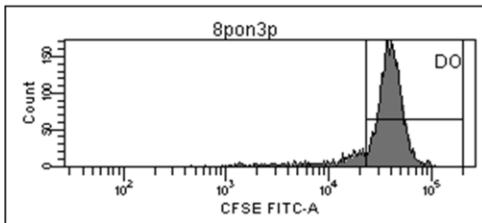
a. PBMC Unstimulated



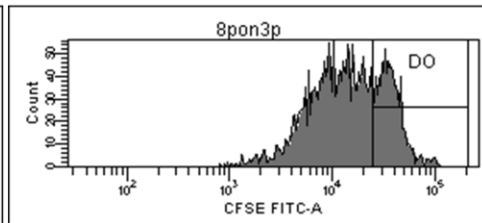
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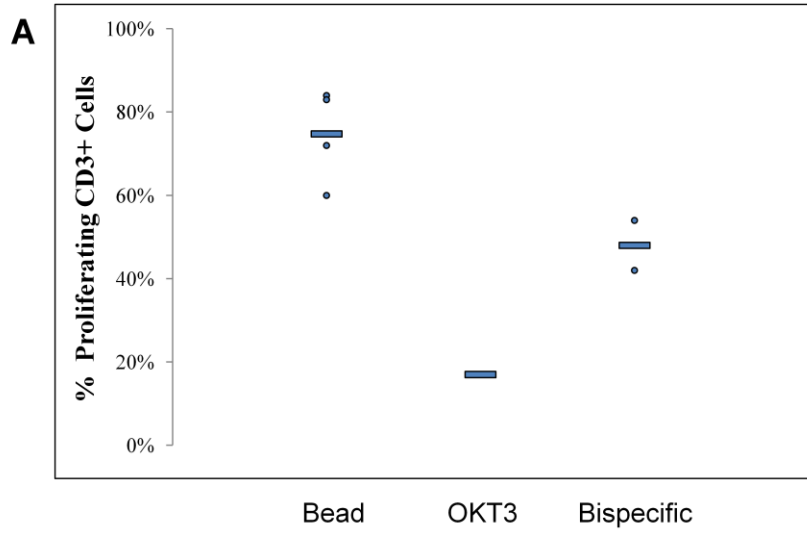
c. MMC Unstimulated



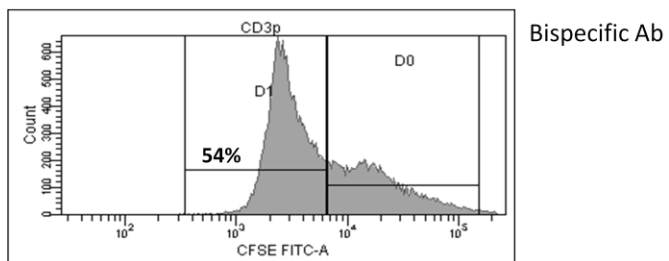
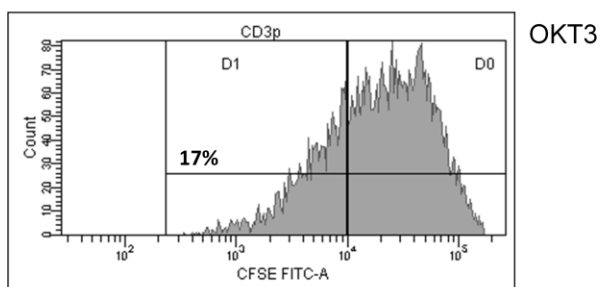
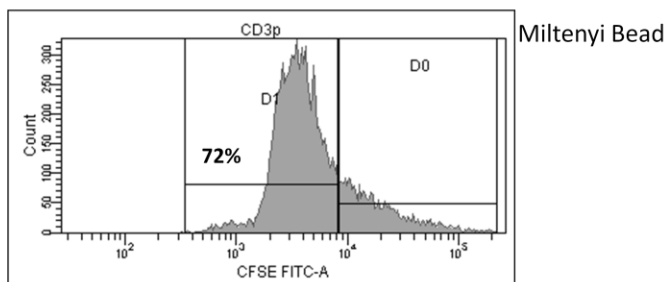
d. MMC Stimulated



**Figure 2. Comparison % proliferation of gut CD3+ cells using three TCR mediated stimulation methods:** MMC were stained with 5  $\mu$ M CFSE and cultured for 5 days following CD3 stimulation via three different methods, at recommended concentrations; (1) Miltenyi anti-CD2, 3, 28 Ab beads (5 $\mu$ l/ml) (n=4), (2) OKT3 (1 $\mu$ g/ml) + CD28 Ab (20 $\mu$ g/ml) (n=1), (3) Bispecific CD3/CD4 (blocking) Ab (1 $\mu$ l/ml) (n=2). At day 5, cells were stained with CD3-PE and %CD3+ cells that had proliferated (CFSE<sup>lo</sup>) was determined. (A) Comparison of % proliferating (CFSE<sup>lo</sup>) cells using Miltenyi beads (n=4), OKT3 (n=1) and Bispecific Ab (n=2). (B) Representative 1D flow histogram demonstrating proliferating (CFSE<sup>lo</sup>) versus non-proliferating (CFSE<sup>hi</sup>) CD3+ cell populations of gut derived cells for each stimulation method.

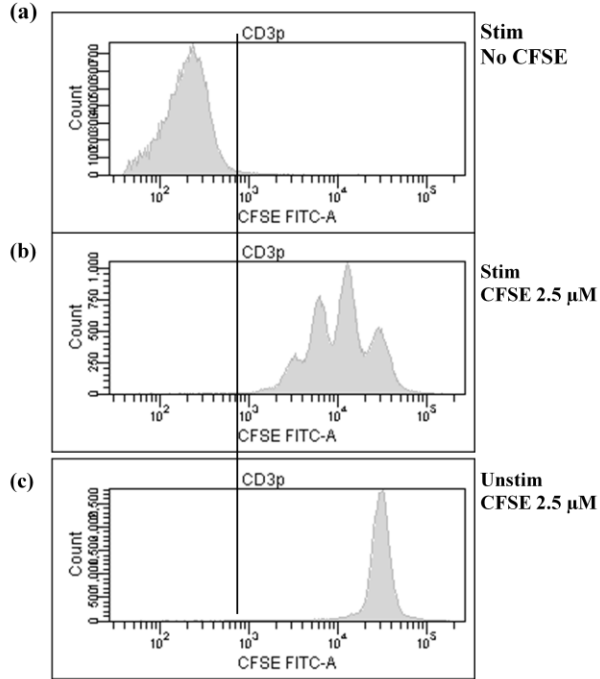


**B**

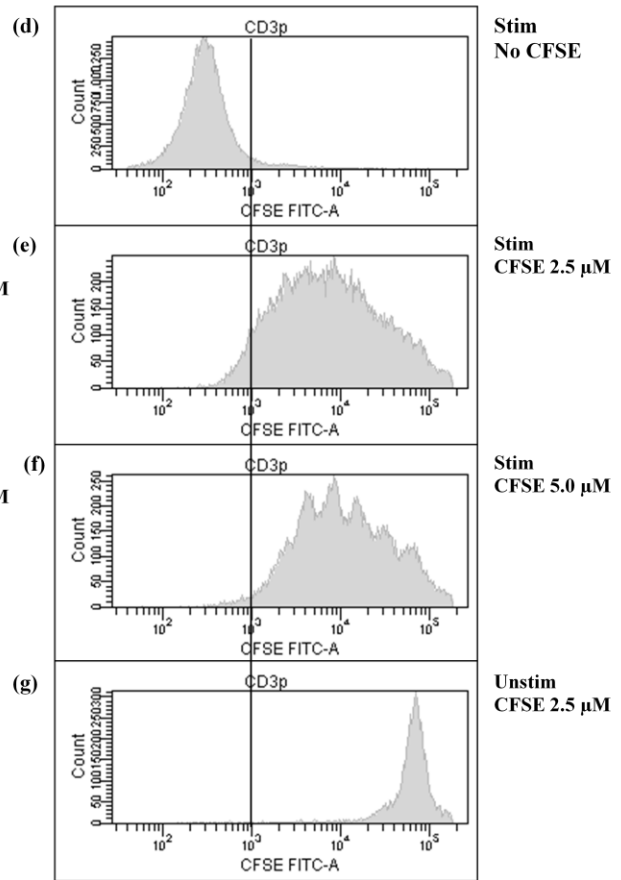


**Figure 3. Determination of optimal CFSE concentration:** Staining  $10.0 \times 10^6$  blood derived T cells with  $2.5 \mu\text{M}$  CFSE and  $2-3 \times 10^6$  gut derived T with  $5.0 \mu\text{M}$  CFSE provides an optimal CFSE range where on Day 5 (D5), all non-replicating and replicating cells are in the flow cytometer's detection range and not in background range (n=2). Panels a-g represent (a) PBMC stim (No CFSE), (b) PBMC stim (CFSE  $2.5 \mu\text{M}$ ), (c) PBMC unstim (CFSE  $2.5 \mu\text{M}$ ), (d) MMC stim (No CFSE), (e) MMC stim (CFSE  $2.5 \mu\text{M}$ ), (f) MMC stim (CFSE  $5.0 \mu\text{M}$ ) and (g) MMC unstim (CFSE  $2.5 \mu\text{M}$ ) compartments from a representative donor.

**PBMC**



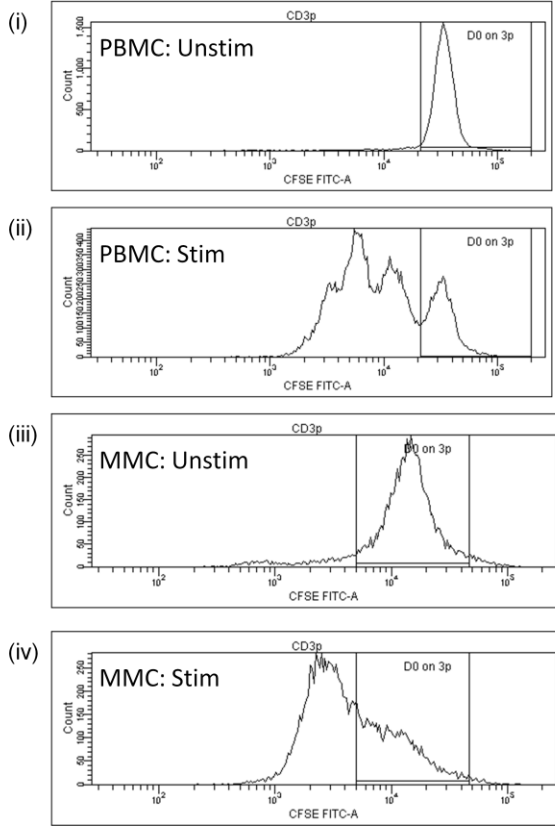
**MMC**

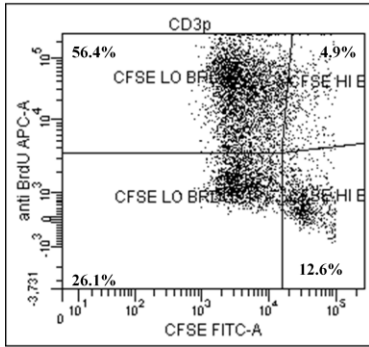
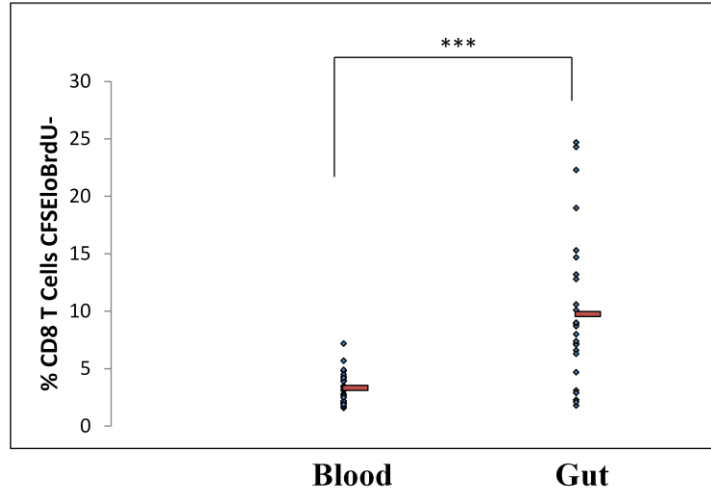




**Figure 4. Enhanced demarcation of proliferating vs. non-proliferating MMC using BrdU (BrdU also permits exclusion of any false positive CFSE<sup>lo</sup> cells that are BdrU<sup>-</sup>):** **A.** CFSE histogram of live CD3<sup>+</sup> cells following a representative 5 day culture of (i) PBMC unstim, (ii) PBMC stim, (iii) MMC unstim, (iv) MMC stim cultures. **B.** Representative bivariate plot from a gut derived culture of CFSE-FITC (x-axis) versus BrdU-APC (Y-axis) gating used to enumerate replicating (CFSE<sup>lo</sup>BrdU<sup>+</sup>) versus non-replicating (CFSE<sup>hi</sup>BrdU<sup>-</sup>) CD3<sup>+</sup> populations, and illustrating (CFSE<sup>lo</sup>BrdU<sup>-</sup>) non-replicating populations. **C.** Mean % of live blood and gut derived CD3<sup>+</sup>8<sup>+</sup> cells that are (CFSE<sup>lo</sup>BrdU<sup>-</sup>) following bead stimulation and 5 day culture (n=29); \*\*\*, p<0.005.

**A**

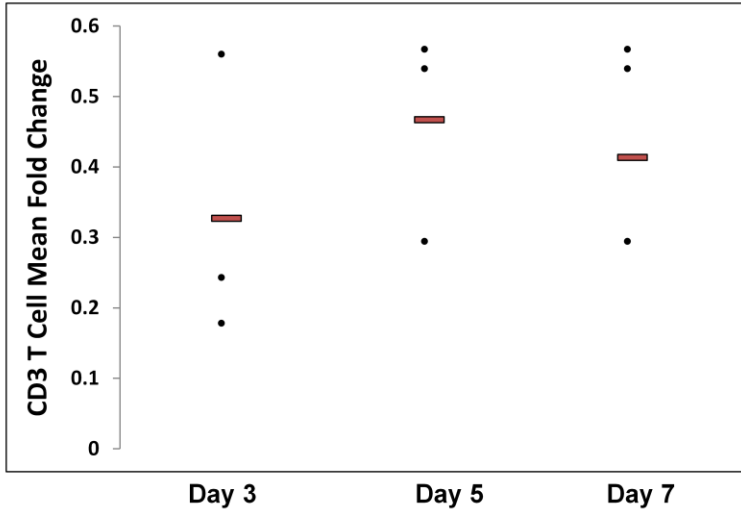


**B****C**

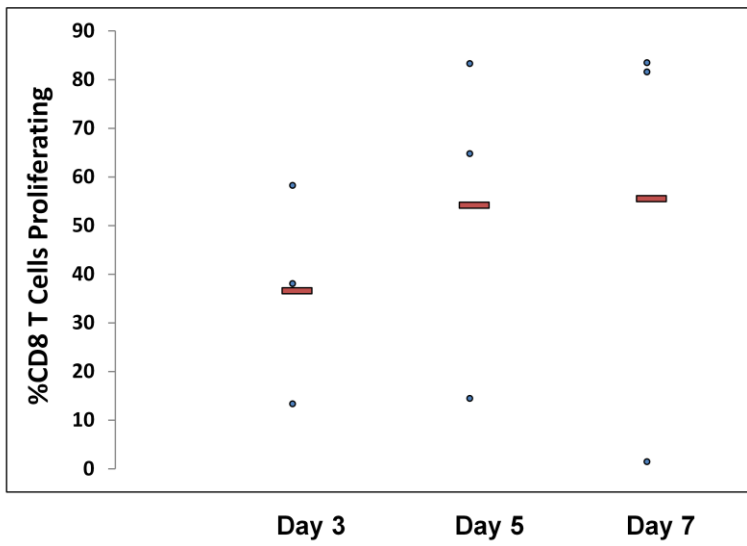
**Figure 5. Determination of optimal culture length for gut and blood CD3+ cells:**

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<sup>lo</sup>BrdU<sup>+</sup>) in gut-derived cultures at Day 3, 5, 7 (n=3).

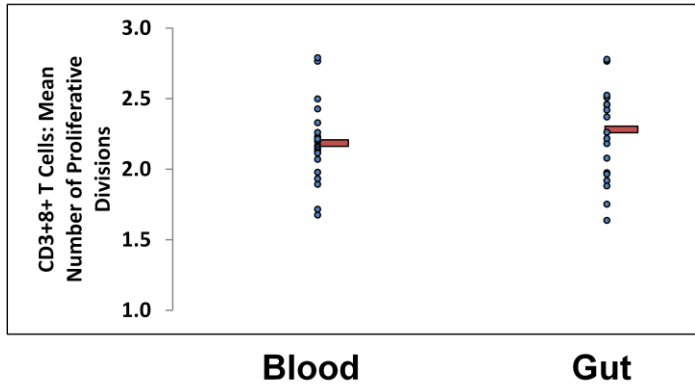
**A**



**B**

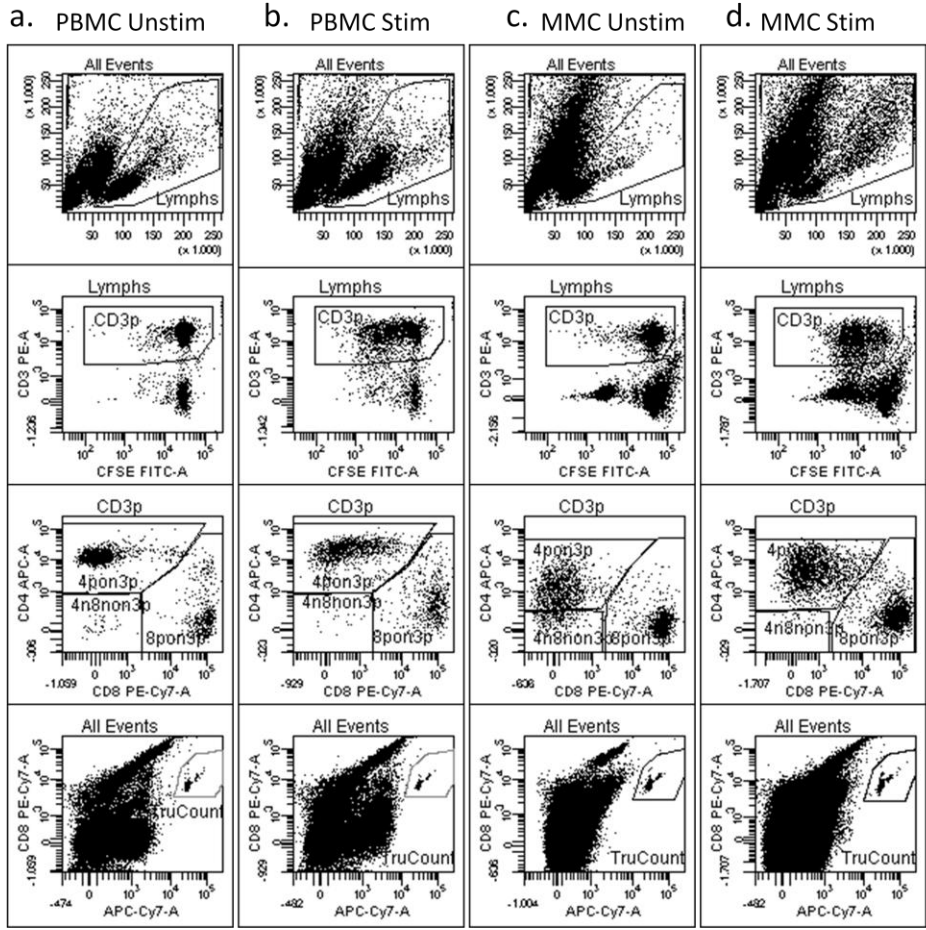


**Figure 6. Comparison of day 5 mean CD8+ blood and gut proliferation:** Proliferating (CFSE<sup>lo</sup>BrdU<sup>+</sup>) CD8+ T cells were evaluated for mean number of proliferations relative to non-proliferating (CFSE<sup>hi</sup>BrdU<sup>-</sup>) CD8+ cells on Day 5 bead stimulated blood and gut cultures (n=29). We first identified and gated non-proliferating (CFSE<sup>hi</sup>BrdU<sup>-</sup>) versus proliferating (CFSE<sup>lo</sup>BrdU<sup>+</sup>) CD3+8+ T cell compartments in bead stimulated cultures. We next used the mean CFSE fluorescence values for non-proliferating and proliferating compartments and determined mean number of proliferative generations, using the formula:  $n = \log(\text{mean non-prolif CFSE} / \text{mean prolifer CFSE}) / \log(2)$ .

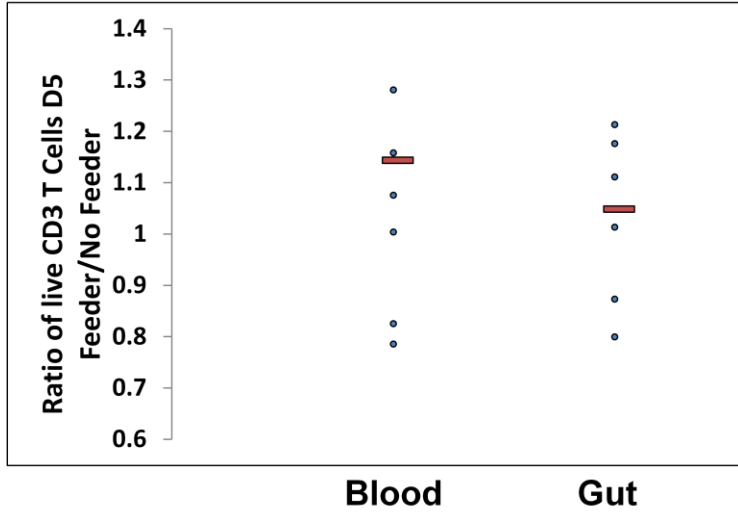




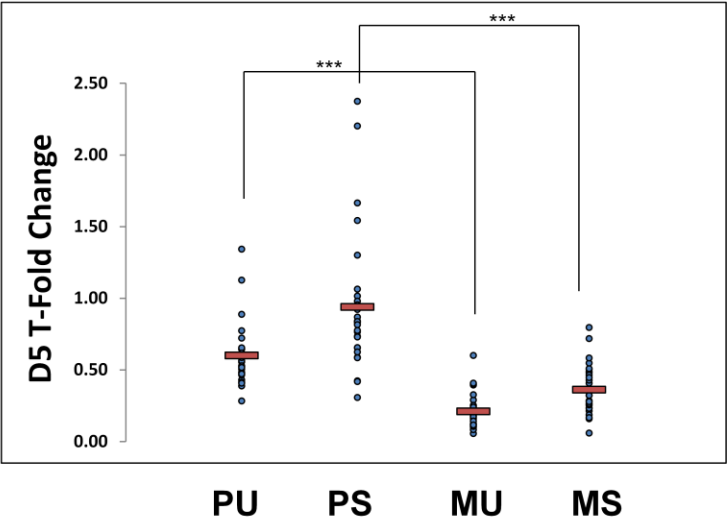
**Supplementary Figure 1. Determination of absolute CD3, CD4 and CD8 T cell counts for Day 5 cultures using TRUcount™ beads:** Cells were first gated for viability using a forward versus side scatter gate **(i)**. Next, the cells were gated using a CFSE-FITC versus CD3-PE display **(ii)**. A CD4-APC versus CD8-PE-Cy7 display gated on the viable T-cells was used to enumerate the CD4 and CD8 T-cells **(iii)**. Finally, a TRUcount™ beads gate was set on beads with high fluorescence using a plot of APC-CY7 versus PE-Cy7 **(iv)**. Columns a-d are representative plots for (a) PBMC unstimulated, (b) PBMC stimulated, (c) MMC unstimulated, and (d) MMC stimulated Day 5 cultures respectively.



**Supplementary Figure 2. Effect of irradiated feeders:** Inclusion of  $0.5 \times 10^6$  irradiated autologous PBMC feeders (stained with  $20 \mu\text{M}$  CFSE/  $5 \times 10^6$  feeders) imparts a very modest, non-significant, enhancement in CD3 T cell number on Day 5 in stimulated blood and gut derived cultures. Ratio of live CD3+ T cell culture size (with feeders/without feeders) as determined by TRUcount™ on day 5 stimulated blood and gut cultures (n=7).



**Supplementary Figure 3. Day 5 mean CD3 T cell number fold change (Day 5 culture size/Day 1 culture size) in stimulated and unstimulated blood and gut cultures following D5 TRUcount™ (n=29); \*\*\*, p<0.005. [Legend: PU=PBMC unstimulated, PS=PBMC stimulated, MU=MMC unstimulated, MS=MMC stimulated]**



## **Chapter 4**

**Immunosenescence is associated with presence of  
Kaposi's sarcoma in antiretroviral treated HIV  
Infection**

# Immunosenescence is associated with presence of Kaposi's sarcoma in antiretroviral treated HIV infection

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**Objective:** Some antiretroviral treated HIV-infected patients develop Kaposi's sarcoma despite long-term suppression of HIV replication. These Kaposi's sarcoma lesions are consistent with Kaposi's sarcoma observed in the elderly uninfected population ('classical Kaposi's sarcoma'). We investigated potential mechanisms for this phenomenon, focusing on measures of immune activation and T-cell senescence.

**Design:** We compared markers of immunosenescence, naive T cells, activation, and inflammation in CD4<sup>+</sup> and CD8<sup>+</sup> T cells from antiretroviral-treated participants with new-onset Kaposi's sarcoma (cases, *n* = 19) and from treated individuals without Kaposi's sarcoma (controls, *n* = 47).

**Results:** There was increased frequency of CD4<sup>+</sup> and CD8<sup>+</sup> T cells with an immunosenescence phenotype (CD57<sup>+</sup> and CD28<sup>-</sup>) in cases vs. controls (CD4<sup>+</sup> T cells: CD57<sup>+</sup> 7.4 vs. 3.7%, *P* = 0.025; CD28<sup>-</sup> 9.1 vs. 4.8%, *P* = 0.025; CD8<sup>+</sup> T cells: CD57<sup>+</sup> 41.5 vs. 27.7%, *P* = 0.003; CD28<sup>-</sup> 60.5 vs. 51.3%, *P* = 0.041). Cases had lower proportions of naive T cells (CD27<sup>+</sup>CD28<sup>+</sup>CD45RA<sup>+</sup>) in CD4<sup>+</sup> (23.0 vs. 32.2%, *P* = 0.023) and CD8<sup>+</sup> (11.3 vs. 20.7%, *P* < 0.001) T-cell compartments. CCR5 was more highly expressed in CD4<sup>+</sup> (16.3 vs. 11.0%, *P* = 0.025), and CD8<sup>+</sup> (43.1 vs. 28.3%, *P* < 0.001) T-cell compartments in cases vs. controls. There was no difference in telomere length or telomerase activity in peripheral blood mononuclear cells, or in T-cell expression of activation markers (HLADR<sup>+</sup>CD38<sup>+</sup>).

**Conclusion:** Among antiretroviral-treated patients, increased frequencies of T cells with an immunosenescence phenotype and lower frequencies of naive T cells were associated with presence of Kaposi's sarcoma among effectively treated patients. These data suggest that certain immunologic perturbations – including those associated with aging – might be causally associated with development of Kaposi's sarcoma.

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**Keywords:** aging, CCR5, CD28, CD57, immunosenescence, Kaposi's sarcoma, senescence

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## Introduction

Traditionally, Kaposi's sarcoma in the general population has been associated with advanced age, in which the lesions are often isolated, indolent, and rarely systemic (classical Kaposi's sarcoma). Kaposi's sarcoma is also found in patients with advanced immunodeficiency, in which the lesions can be far more aggressive [1–3]. Although the advent of antiretroviral therapy (ART) may have greatly decreased the incidence of Kaposi's sarcoma in HIV-infected adults, Kaposi's sarcoma continues to be the most prevalent AIDS-defining malignancy worldwide and carries with it significant morbidity and mortality [4–6].

In spite of the clinical relevance of Kaposi's sarcoma, mechanisms behind development of Kaposi's sarcoma in the elderly have yet to be fully elucidated. We previously reported an atypical cohort of patients who developed cutaneous Kaposi's sarcoma despite having undetectable plasma HIV RNA levels and relatively high CD4<sup>+</sup> T-cell counts [7]. The Kaposi's sarcoma lesions seen in these patients ran an indolent course, and were morphologically and clinically reminiscent of classical Kaposi's sarcoma, or the Kaposi's sarcoma seen in the HIV-negative elderly patients. Given emerging evidence suggesting that prolonged periods of untreated HIV infection result in accelerated and perhaps irreversible 'aging' of the immune system [8–12], we hypothesized that gradual loss of immunologic function during advancing age, or 'immunosenescence,' may account for the development of Kaposi's sarcoma in these individuals.

Given that immunologic aging may present in both populations which develop classical Kaposi's sarcoma – namely very old uninfected adults and long-term antiretroviral treated adults – we hypothesized this group of patients who developed Kaposi's sarcoma in spite of well controlled HIV would carry an immunosenescent phenotype, which would include increased proportions of CD28<sup>-</sup> and CD57<sup>+</sup> T cells, decreased telomere length, decreased proliferative capacity, increased T-cell activation, and/or lower levels of naïve T cells.

## Methods

### Design

This is a case–control study comparing global immunosenescence markers (CD28<sup>-</sup> and CD57<sup>+</sup>), naïve T-cell markers (CD27<sup>+</sup>CD28<sup>+</sup>CD45RA<sup>+</sup>), activation markers (HLADR<sup>+</sup>CD38<sup>+</sup> and CCR5<sup>+</sup>), proliferative response (carboxyfluorescein diacetate succinimidyl ester, CFSE), and telomere length and telomerase activity in antiretroviral treated HIV-positive individuals with active Kaposi's sarcoma (cases) vs. those without Kaposi's sarcoma (controls). In order to determine whether any

observed differences in immune response might be due to presence or absence of human herpesvirus 8 (HHV8), we also determined the HHV8 serology of the controls.

### Participants

We identified 19 individuals on ART who developed or had active biopsy proven, unremitting Kaposi's sarcoma after an interval of at least 24 months with viral loads less than 75 copies RNA/ml, and peripheral CD4 cell counts more than 300 cells/ $\mu$ l. We also recruited 47 HIV-positive and Kaposi's sarcoma-negative controls on ART with viral loads less than 75 copies RNA/ml and peripheral CD4 cell counts more than 300 cells/ $\mu$ l. For controls, absence of Kaposi's sarcoma in history was determined by self-report. Thus, cases and controls were matched in terms of viral load (all <75 copies RNA/ml), and in terms of CD4 cell threshold (>300 cells/ $\mu$ l). All cases and controls were HIV-positive, as determined by standard ELISA documented at any time in the past, or by documented viral load within 6 months of enrollment, and had no history of autoimmune disease, systemic fungal or mycobacterial infection, immunomodulatory or chemotherapy use within 1 year of enrollment, or current other active malignancy. Women were excluded because of potential gender effects on immune responses [13].

### Recruitment and follow-up

Cases and controls were recruited from Dermatology and HIV Primary Care Practices in San Francisco and adjacent counties, University of California, Los Angeles (UCLA) related HIV primary care clinics, and from the Study of the Consequences of the Protease Inhibitor Era (SCOPE), a clinic-based cohort of over 1500 HIV-infected individuals at the University of California, San Francisco (UCSF). This study was reviewed and approved by institutional review boards at UCSF and UCLA.

### T-cell immunophenotyping

Cryopreserved T cells were characterized by flow cytometry as previously described [14]. Briefly, cryopreserved peripheral blood mononuclear cells (PBMCs) were rapidly thawed washed and assessed for cell count and viability using the Viacount assay on a Guava Personal Cell Analysis system (Guava Technologies Inc., Hayward, California, USA). Cells were stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, Grand Island, New York, USA), and then stained with the following combination of fluorescently conjugated monoclonal antibodies: panel 1: CD3–Pacific Blue and CCR5–PE CY5 (BD Biosciences Pharmingen, San Diego, California, USA), CD4–PE CY7, HLA–DR–FITC and CD38–PE (BD Biosciences), CD57–Alexa Fluor 647 (BioLegend, San Diego, California, USA), and CD8–Q–Dot605 (custom conjugate; Invitrogen); Panel 2: CD3–Pacific Blue and CD28–APC (BD Biosciences Pharmingen), CD45RA–PECY7 (BD Biosciences), CD27–APC–ALEXA 750 and CD4 PE–TR (Invitrogen), and CD8–Q–Dot605 (custom conjugate; Invitrogen).



categorized as HHV8 antibody-negative. This serologic algorithm has an estimated specificity of 97.5%, as evidenced by testing blood donors [20], and a sensitivity of 96.3%, as evidenced by testing patients with Kaposi's sarcoma [21].

### Statistical methods

Mann-Whitney comparisons were used to compare variables between HIV-positive and Kaposi's sarcoma-positive cases vs. HIV-positive and Kaposi's sarcoma-negative controls. Linear regression and quartile analysis were used to further control for potential confounding by age.

## Results

### Age and HIV markers of infection

The cases and controls were not significantly different with regard to CD4<sup>+</sup> T-cell count, CD8<sup>+</sup> T-cell count, and viral load, although the cases were significantly older than the controls (medians of 54 and 43 years of age, respectively,  $P < 0.001$ , see Table 1).

### Global immunosenescence markers CD28 and CD57

The frequency of CD8<sup>+</sup> T cells expressing CD57 was higher in the cases than controls (median of 41.5 vs. 27.7%,  $P = 0.003$ , Table 2). This difference remains significant after adjusting for age and CD4 cell count (age-adjusted  $P = 0.005$ , age-adjusted and CD4 cell count-adjusted  $P = 0.009$ ). As compared with the controls, the cases also had a higher frequency of CD28<sup>-</sup>CD4<sup>+</sup> cells (median of 9.1 vs. 4.8%,  $P = 0.025$ , age-adjusted  $P = 0.030$ , age-adjusted and CD4 cell-adjusted  $P = 0.06$ , Table 2) and CD28<sup>-</sup>CD8<sup>+</sup> cells (median of 60.5 vs. 51.3%,  $P = 0.041$ , age-adjusted  $P = 0.044$ , age-adjusted and CD4 cell count-adjusted  $P = 0.05$ , Table 2) vs. controls. There was a trend suggesting that the cases had a higher frequency of CD57<sup>+</sup>CD4<sup>+</sup> T cells than controls (median of 7.4 vs. 3.7%,  $P = 0.045$ , age-adjusted  $P = 0.07$ , age-adjusted and CD4 cell count-adjusted  $P = 0.12$ , Table 2).

### T-cell maturation

The proportion of naïve (CD27<sup>+</sup>CD28<sup>+</sup>CD45RA<sup>+</sup>) CD8<sup>+</sup> T cells was lower in the cases than the controls (median of 11.3 vs. 20.7%,  $P < 0.001$ , age-adjusted  $P = 0.022$ , age-adjusted and CD4 cell count-adjusted  $P = 0.01$ , Table 2). In addition, the proportion of T-effector cells (CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>-</sup>) was greater in cases than the controls in both CD4<sup>+</sup> (median of 6.7 vs. 3.0%,  $P = 0.014$ , age-adjusted  $P = 0.022$ , age-adjusted and CD4 cell count-adjusted  $P = 0.05$ , Table 2) and CD8<sup>+</sup> (35.0 vs. 19.5%,  $P = 0.005$ , age-adjusted  $P = 0.05$ , age-adjusted and CD4 cell count-adjusted  $P = 0.01$ , Table 2) T-cell compartments. There was a trend suggesting cases had a lower frequency of naïve (CD27<sup>+</sup>CD28<sup>+</sup>CD45RA<sup>+</sup>) CD4<sup>+</sup> T cells than controls (median of 23.0 vs. 32.2%, age-adjusted  $P = 0.11$ , age-adjusted and CD4 cell count-adjusted  $P = 0.07$ , Table 2).

### CCR5 expression

The median percentage CD4<sup>+</sup> T cells expressing CCR5 was 16.3 for Kaposi's sarcoma-positive cases vs. 11.0 for Kaposi's sarcoma-negative controls, ( $P = 0.025$ , age-adjusted  $P = 0.05$ , age-adjusted and CD4 cell count-adjusted  $P = 0.04$ , Table 2) and the median percentage CD8<sup>+</sup> T cells expressing CCR5 was 43.1 for Kaposi's sarcoma-positive cases vs. 28.3 for Kaposi's sarcoma-negative controls (unadjusted, age-adjusted, and age-adjusted and CD4 cell count-adjusted  $P < 0.001$ , Table 2).

### Measures for activation, proliferation, telomere length, and telomerase activity

There were no differences between cases and controls for the HLA-DR and CD38 activation markers, proliferation as determined by CFSE assays, nor were telomerase activity and telomere lengths different between the groups. However, we observed a significant negative correlation between the overall PBMC telomere length and the proportion of more differentiated CD8<sup>+</sup>CD28<sup>-</sup> T cells ( $\rho = -0.41$ ,  $P = 0.001$ ), as has been observed in healthy individuals [22].

### Age adjustment via quartile analysis

As additional means of controlling for age as a potential confounding variable, we employed quartile adjustment. When quartile analysis was applied, differences between

**Table 1. Age and markers of HIV infection in HIV-positive and Kaposi's sarcoma-positive cases and HIV-positive and Kaposi's sarcoma-negative controls.**

	HIV-positive and Kaposi's sarcoma-positive cases: [median (range)]	HIV-positive and Kaposi's sarcoma-negative controls: [median, (range), $P$ value*]
Number of patients (N)	19	47
Age (years)	54 (39–74)	43 (29–60), <0.001
CD4 cell count (cells/ $\mu$ l)	701 (338–1205)	521 (311–1194), 0.08
CD8 cell count (cells/ $\mu$ l)	933 (429–2543)	1200 (425–3485), 0.41
Viral load (copies/ml)	<75 (<75 to <75)	<75 (<75 to <75), >0.5

Women were excluded due to potential gender effects on immune responses [13].

\* $P$  values involve comparison between given measure and Kaposi's sarcoma-positive cases.

**Table 2.** Selected immune markers for HIV-positive and Kaposi's sarcoma-positive cases vs. HIV-positive and Kaposi's sarcoma-negative controls.

	Kaposi's sarcoma-positive cases [median (range)] ( <i>n</i> = 19)	Kaposi's sarcoma-negative controls [median (range)] ( <i>n</i> = 47)	Age-adjusted <i>P</i> values (linear, quartile)
Percentage of CD8 <sup>+</sup> T cells			
CD57 <sup>+</sup>	41.5 (30.2–49.3)	27.7 (19.4–38.3)	0.005, 0.005
CD28 <sup>−</sup>	60.5 (46.5–72.9)	51.3 (38.7–60.7)	0.044, 0.07
CD27 <sup>+</sup> CD28 <sup>+</sup> CD45RA <sup>+</sup>	11.3 (6.8–14.8)	20.7 (14.1–30.1)	0.022, 0.025
CD27 <sup>−</sup> CD28 <sup>−</sup> CD45RA <sup>−</sup>	35.0 (19.9–37.8)	19.5 (12.8–28.1)	0.05, 0.05
CCR5 <sup>+</sup>	43.1 (36.6–60.2)	28.3 (23.5–36.3)	<0.001, <0.001
Percentage of CD4 <sup>+</sup> T cells			
CD57 <sup>+</sup>	7.4 (3.9–17.3)	3.7 (1.6–10.4)	0.07, 0.09
CD28 <sup>−</sup>	9.1 (5.0–20.9)	4.8 (2.0–11.9)	0.030, 0.05
CD27 <sup>+</sup> CD28 <sup>+</sup> CD45RA <sup>+</sup>	23 (14.3–34.0)	32.2 (20.0–43.1)	0.11, 0.05
CD27 <sup>−</sup> CD28 <sup>−</sup> CD45RA <sup>−</sup>	6.7 (3.9–20.4)	3.0 (1.2–9.0)	0.022, 0.04
CCR5 <sup>+</sup>	16.3 (9.4–28.9)	11.0 (7.7–16.1)	0.05, 0.008

Kaposi's sarcoma-positive cases and Kaposi's sarcoma-negative controls were preserved for measures of global immunosenescence, T-cell maturation, and CCR5 expression (Table 2).

### The impact of human herpesvirus 8 serology on immune responses

To further examine the effect of HHV8 itself, the Kaposi's sarcoma-negative controls were divided by HHV8 status (HHV8-positive and HHV8-negative). These two groups were compared to each other and finally, compared to Kaposi's sarcoma-positive cases.

Of the 47 Kaposi's sarcoma-negative controls, 26 had a positive serology. Notably, when comparing HHV8-positive vs. HHV8-negative subgroups with one another, there were no differences in age, markers of HIV infection, global markers of immunosenescence, T-cell maturation markers, CCR5 markers of expression, or measures of activation, telomere length, and telomerase activity.

### Human herpesvirus 8 subgroups: age and HIV markers of infection

When comparing Kaposi's sarcoma-positive cases vs. HHV8-positive and HHV8-negative subgroups, there were no differences in HIV markers of infection. Kaposi's sarcoma-positive cases were older than the HHV8-positive subgroup (median 54 and 45 years of age,  $P=0.003$ ), as well as the HHV8-negative subgroup (median 54 and 43 years of age,  $P<0.001$ ).

### Human herpesvirus 8 subgroups: global markers of immunosenescence

Kaposi's sarcoma-positive cases had a higher frequency of CD8<sup>+</sup>CD57<sup>+</sup> T cells than the HHV8-positive subgroup (median 41.5 and 30.4%, respectively,  $P=0.01$ , age-adjusted  $P=0.02$ ), and HHV8-negative subgroup (median 41.5 and 26.2%, respectively,  $P=0.04$ , age-adjusted  $P=0.05$ ). There was a trend suggesting Kaposi's sarcoma-positive cases had a higher frequency of

CD8<sup>+</sup>CD28<sup>−</sup> T cells than the HHV8-positive subgroup (median 60.5 and 52.1%, respectively,  $P=0.06$ , age-adjusted  $P=0.08$ ). As well, there was a trend suggesting that the Kaposi's sarcoma-positive cases had a higher frequency of CD4<sup>+</sup>CD28<sup>−</sup> T cells than the HHV8-positive subgroup (medians 9.1 and 4.8%, respectively,  $P=0.09$ , age-adjusted  $P=0.09$ ).

### Human herpesvirus 8 subgroups: T-cell maturation markers

Naïve CD4 and CD8 T cells were lower in the Kaposi's sarcoma-positive cases as compared with the Kaposi's sarcoma-negative controls, a comparison that was preserved when Kaposi's sarcoma-negative controls were further subdivided into HHV8-positive and HHV8-negative subgroups. The Kaposi's sarcoma-positive cases had a lower frequency of naïve (CD27<sup>+</sup>CD28<sup>+</sup>CD45RA<sup>+</sup>) CD8<sup>+</sup> T cells than the HHV8-positive subgroup (median 11.3 and 23.9%, respectively,  $P=0.04$ , age-adjusted  $P=0.05$ , Table 3) and the HHV8-negative subgroup (median 11.3 and 19.1%, respectively,  $P=0.01$ , age-adjusted  $P=0.02$ , Table 3). The Kaposi's sarcoma-positive cases had a lower frequency of naïve (CD27<sup>+</sup>CD28<sup>+</sup>CD45RA<sup>+</sup>) CD4<sup>+</sup> T cells than the HHV8-negative subgroup (median 23.0 and 28.0% respectively,  $P=0.06$ , age-adjusted  $P=0.05$ , Table 3).

### Human herpesvirus 8 subgroups: CCR5 expression

The Kaposi's sarcoma-positive cases had a higher frequency of CCR5<sup>+</sup> CD8<sup>+</sup>T cells than the HHV8-positive subgroup (median 43.1 and 29.0%, respectively,  $P<0.01$ , age-adjusted  $P<0.01$ , Table 3) and the HHV8-negative subgroup (median 43.1 and 27.5%, respectively,  $P<0.01$ , age-adjusted  $P=0.03$ , Table 3).

### Human herpesvirus 8 subgroups: measures of activation, telomere length, and telomerase activity

There were no significant differences between Kaposi's sarcoma-positive cases and HHV8 control subgroups

**Table 3. Selected immune markers for HIV-positive and Kaposi's sarcoma-positive cases, HIV-positive, Kaposi's sarcoma-negative, and human herpesvirus 8-positive controls, and HIV-positive, Kaposi's sarcoma-negative and human herpesvirus 8-negative controls.**

	Kaposi's sarcoma-positive cases: [median (range)] (n = 19) Reference	Kaposi's sarcoma-negative and HHV8-positive controls [median, (range), age-adjusted P value] (n = 26)	Kaposi's sarcoma-negative and HHV8-negative controls: [median, (range), age-adjusted P value] (n = 21)
Percentage of CD8 <sup>+</sup> T cells			
CD57 <sup>+</sup>	41.5 (30.2–49.3)	30.4 (19.5–42.1), 0.02	26.2 (16.3–38.0), 0.05
CD28 <sup>-</sup>	60.5 (46.5–72.9)	52.1 (45.5–62.0), 0.06	48.7 (36.1–61.1), 0.24
CD27 <sup>+</sup> CD28 <sup>+</sup> CD45RA <sup>+</sup>	11.3 (6.8–14.8)	23.9 (14.1–31.9), 0.05	19.1 (10.7–34.5), 0.02
CD27 <sup>-</sup> CD28 <sup>-</sup> CD45RA <sup>-</sup>	35.0 (19.9–37.8)	17.2 (8.9–27.5), 0.18	20.0 (12.2–31.1), 0.07
CCR5 <sup>+</sup>	43.1 (36.6–60.2)	29.0 (23.3–36.2), <0.001	27.5 (23.4–42.2), 0.003
Percentage of CD4 <sup>+</sup> T cells			
CD57 <sup>+</sup>	7.4 (3.9–17.3)	3.7 (1.2–14.4), 0.15	5.0 (1.7–10.4), 0.32
CD28 <sup>-</sup>	9.1 (5.0–20.9)	4.8 (1.5–17.0), 0.09	5.3 (1.8–12.1), 0.20
CD27 <sup>+</sup> CD28 <sup>+</sup> CD45RA <sup>+</sup>	23 (14.3–34.0)	38.8 (20.8–56.2), 0.33	28.0 (17.7–42.0), 0.05
CD27 <sup>-</sup> CD28 <sup>-</sup> CD45RA <sup>-</sup>	6.7 (3.9–20.4)	2.9 (0.5–8.7), 0.08	4.0 (1.3–9.8), 0.09
CCR5 <sup>+</sup>	16.3 (9.4–28.9)	8.2 (5.5–15.3), 0.19	12.2 (8.0–17.7), 0.1

when looking at measures of activation, proliferation, telomere length, and telomerase activity.

## Discussion

The incidence and prevalence of systemic, life-threatening Kaposi's sarcoma has declined dramatically since the advent of highly effective ART. Despite this unquestioned success, some apparently well treated individuals still develop Kaposi's sarcoma, although the clinical presentation is much less aggressive than that which was observed among untreated individuals with advanced disease. Indeed, as we have previously described, the Kaposi's sarcoma that occurs during effective therapy has many characteristics of that seen in elderly HIV-uninfected men (i.e. classical Kaposi's sarcoma). Given that HIV affects the immune system in a manner comparable to that observed in aging, we hypothesized that certain markers of immunologic aging (immunosenescence) may be associated with Kaposi's sarcoma in the context of effective therapy. Here, we present data that are largely consistent with this hypothesis.

Specifically, we observed increased frequencies of T cells with an immunosenescent phenotype (CD28<sup>-</sup> and CD57<sup>+</sup>) in those with Kaposi's sarcoma vs. those without Kaposi's sarcoma. We also found evidence of lower frequencies of naive T cells and higher frequencies of effector T cells in those with Kaposi's sarcoma than those without Kaposi's sarcoma. These associations were preserved even when controlling for effects of age (age was controlled via both regression and quartile analysis with preservation of results). These associations remained significant regardless of HHV8 status. This suggests that HHV8 infection itself does not explain the difference in immunosenescence patterns observed in this comparison of cases with clinical Kaposi's sarcoma vs. Kaposi's

sarcoma-negative controls. These findings support the hypothesis that immune aging may play a role in increasing vulnerability to and/or as a consequence of Kaposi's sarcoma.

While this study begins to look at the relationship between immune aging and Kaposi's sarcoma, further investigation is needed to see how duration and severity of HIV infection may be influencing immune aging in these cohorts. At this time, very few cohorts can provide precise information regarding the duration of infection – mainly those cohorts enrolling individuals with primary infection. These cohorts examining primary infection, at this time, are too small to address the pathogenesis of relatively more unusual events such as development of clinical Kaposi's sarcoma. CD4 nadir was thought of as a possible surrogate for duration or severity of HIV infection but a significant proportion of our Kaposi's sarcoma-positive cases were HIV-infected prior to widespread clinical availability of CD4 cell count assay, and were, thus, already immune reconstituted prior to our being able to accurately assess their CD4 cell count.

Another way to isolate the effect of HIV from the acquisition of clinical Kaposi's sarcoma would be to include non-HIV-infected patients with Kaposi's sarcoma in these analyses. Comparisons involving this HIV-negative, 'classical Kaposi's sarcoma' group would certainly be of great interest, but this is a rare group of patients in the United States. It is hoped that this work may provide the impetus to explore issues of immunosenescence in other Kaposi's sarcoma cohorts.

Although many of our measurements of immunologic aging were higher in our cases than controls, there were exceptions. Specifically, telomere length and telomerase activity did not differ between the groups. Our analysis was limited, however, by the use of total PBMCs for these analyses. It is possible that telomeric effects may be

predominant in certain T cell or other cellular subpopulations not evaluated. Although not directly related to our study question, we found that having a higher frequency of more differentiated CD28<sup>-</sup> CD8<sup>+</sup> T cells was associated with shorter telomere length. This observation is consistent with the known biology of CD8<sup>+</sup> T cells, and is consistent with findings in uninfected individuals [22]. This study is one of the first to demonstrate this association in persons with HIV disease.

The elevated CCR5 expression in the Kaposi's sarcoma cohort carries potential implication for Kaposi's sarcoma disease pathogenesis. CCR5, a chemokine receptor, has been shown to interact with viral macrophage inflammatory protein (vMIP), one of the proteins encoded by HHV8, the virus necessary for Kaposi's sarcoma development [23–25]. The interaction of CCR5 with vMIP has been shown in monocytes to effect calcium influx [26], which may indicate there are potential downstream effects to the inflammatory process involved with Kaposi's sarcoma lesion formation and persistence.

This study does not address how the relationship between ART (or lack thereof), duration and severity of HIV infection, duration and severity of HHV8 infection, potential interaction between HIV and HHV8, and the nonlinear inflammation associated with Kaposi's sarcoma oncogenesis, might affect these immunosenescence and activation markers – arenas that should be addressed and controlled for in future studies. Although we did subgroup analysis involving HHV8 status, it is important to note that this study was not originally powered for these tertiary outcome analyses and, thus, conclusions with regard to lack of differences found between these groups must be carefully considered. As well, other future studies would include investigation of other HIV malignant disease states with immunosenescence markers, as well as prospective tracking of these immunosenescence markers during changes in disease course. Finally, exploration of CCR5's potential role in Kaposi's sarcoma pathogenesis and in other modes of Kaposi's sarcoma oncogenesis (e.g. classical Kaposi's sarcoma, low CD4 cell count Kaposi's sarcoma, and transplant Kaposi's sarcoma) may yet shed more light on the mechanisms behind Kaposi's sarcoma oncogenesis.

This is one of the first studies documenting an association between immunosenescence and Kaposi's sarcoma even when controlling for confounders including age, CD4 cell count, gender, and HHV8 status. Our findings add to the increasing evidence supporting the notion that HIV disease may constitute a model of accelerated aging [27]. Our data also suggest that immunosenescence may be causally associated with development of Kaposi's sarcoma in uninfected elderly adults. Future studies in that population are needed.

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## Conflicts of interest

The authors have no conflicts of interest.

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# **Chapter 5**

## **Conclusions**



## CONCLUSIONS

Immunosenescence is the global term used to describe the observed age-associated decline in immune competence characterized by functional and phenotypic alterations to the immune system as a whole [1]. The motivation for the research described herein is based on findings, many made by this laboratory, that a major feature of immunosenescence is the accumulation of late-differentiated memory CD8 T cells with features of replicative senescence. These include inability to proliferate, absence of CD28 expression, shortened telomeres, loss of telomerase activity, enhanced activation, and increased secretion of inflammatory cytokines [2, 3]; and the association of these cells with age- and HIV-mediated morbidity [4-6]. A caveat to our understanding of CD8 T cell replicative senescence is that most studies have been done on peripheral blood, which contains only 2% of total body lymphocytes, whereas little is known concerning the gut-associated lymphoid tissue (GALT), which contains 40-65% of lymphocytes and is an area of high antigenic exposure [7, 8].

In Chapter 2, we describe our initial efforts to fill this knowledge gap, by comparing CD8 T cells from peripheral blood and GALT (taken from rectosigmoid colon biopsies), focusing on age-related phenotypic and functional alterations that had been previously linked to senescence in peripheral blood. Because the gut is a primary line of defense that routinely faces antigenic challenges, as opposed to the blood, which is primarily a conduit for trafficking lymphocytes, we hypothesized that gut CD8 T cells would have undergone a greater degree of antigenic driven proliferation and differentiation, and be more senescent than their blood counterparts. This hypothesis is consistent with early work looking at intestinal lamina propria T cells [9], previous

unpublished observations from our laboratory, and mouse studies looking at age-associated alterations in the mouse GALT [10-16]. Overall, our results support the hypothesis that gut CD8 T cells have profiles suggestive of greater “aging” than those in peripheral blood. Indeed, compared to blood from the same individuals, there is a significant increase within the GALT in RA-CD8<sup>+</sup> (memory), CD28-CD8<sup>+</sup> (differentiated), RA-CD28-CD8<sup>+</sup> (differentiated memory), DR+CD38+CD8<sup>+</sup> (activated), CD8  $\alpha/\alpha$ , and RO+PD-1+CD8<sup>+</sup> CD3 T cells. There is also a (non-significant) decrease in baseline telomerase activity. However, based on other criteria, CD8 gut cells may not necessarily be more senescent than blood. Specifically, gut CD8 T cells have lower levels of CD57 surface expression, an increase in KI-67<sup>+</sup> (proliferation) expression, and demonstrate proliferative potential that is similar to those in the blood. In addition, the inverted CD4:8 ratio observed in older persons with high proportions of senescent CD8 T cells is not observed in the gut, at least in the relatively young population used in this study.

In Chapter 3 we describe the development and optimization of a novel, reproducible experimental protocol to test our hypothesis regarding compartment-specific differences of *in vitro* proliferative dynamics of CD8 T cells. The final protocol involves a 5 day culture of mononuclear leukocyte populations, from blood and gut respectively, labeled with CFSE and BrdU and stimulated with anti-CD2/3/28-linked microbeads. Variables that were tested and optimized include: mode of T cell stimulation, CFSE concentration, inclusion of second proliferation marker BrdU, culture duration, initial culture concentration and inclusion of autologous irradiated feeder cells. Perhaps the biggest challenge in developing this protocol, which originally included only a CFSE label, was that mucosal T cells yielded wide CFSE fluorescence distributions, presumably due to variable CFSE uptake, that made definitive demarcation between proliferating and non-proliferating cells problematic. Our solution to this

problem was to incorporate a 2<sup>nd</sup> marker, BrdU, allowing us to separate proliferating (CFSE<sup>lo</sup>BrdU<sup>+</sup>) and non-proliferating (CFSE<sup>hi</sup>BrdU<sup>-</sup>) populations using 2 dimensional analysis. Ultimately, we were able to successfully address this, among other, challenges in facilitating mucosal T cell stimulation and short term culture, and develop the first detailed protocol for evaluating proliferative dynamics of blood and rectal mucosal T cells *in vitro*.

Chapter 4 outlines a separate project examining a possible link between peripheral blood T cell senescence and a mild presentation of Kaposi's sarcoma [termed classical Kaposi's sarcoma (KS) and generally associated with advanced age] in treated HIV-infected subjects who had long-term suppression of HIV replication. Despite undetectable plasma HIV RNA levels and relatively high CD4+ counts in this cohort, evidence supporting the concept of HIV "accelerated" immune senescence (even in subjects that have not progressed to AIDS) [17, 18], suggests that the presentation of Kaposi's sarcoma may be an indication of immune senescence in this cohort. Our data indicate these individuals exhibit some features of immunosenescence relative to treated controls without sarcoma. These include increased frequency of CD57+ and CD28- CD4 and CD8 T cells, decreased proportion of naïve CD27+CD28+CD45RA+ CD4 and CD8 cells, increased expression of CCR5 in CD4 and CD8 T cells, indicating immunologic perturbations associated with immune senescence may play a causal role in this form of KS..

Our work, therefore, provides significant advances in our understanding of CD8 T cell replicative senescence, in the GALT (rectosigmoid colon) of young, healthy donors, and in the peripheral blood of HIV subjects exhibiting classical presentation of Kaposi's sarcoma. Ongoing research is addressing the effects of aging and HIV infection on the gut senescence profile. Interestingly, initial results suggest that, compared to the blood, the gut acquires a "more

senescent” phenotype (i.e., higher expression of CD45RA+, HLA-DR+38+) in young age, possibly as part of normal immune homeostasis, and that advanced age and HIV infection have a less dramatic effect on many of these phenotypic markers (unpublished observations). Additional work on GALT senescence, currently being initiated by our collaborators, is examining other regions of the GALT and cell types other than cytotoxic T lymphocytes. Indeed, pilot studies by our group indicate the small intestine has a more advanced senescence profile than the colon (i.e., lower CD4:CD8 ratio and upregulation of HLA-DR+38+ T cells) and CD4 T lymphocytes also exhibit advanced feature of replicative senescence in the gut. The long term goal of these studies is to provide a more comprehensive understanding of the process of CD8 T cell replicative senescence and immunosenescence throughout the entire immune system, which may ultimately lead to more effective therapeutic interventions.

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