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The Effects of Aging on the Rectal Mucosal CD4+ and CD8+ T cell Compartments and the Implications for HIV Transmission

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B.S., Shorter College, 2008
M.D., Medical College of Georgia, 2012

Advisor: Colleen F. Kelley, M.D., MPH

An abstract of
A thesis submitted to the Faculty of the
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Abstract

The Effects of Aging on the Rectal Mucosal CD4+ and CD8+ T cell Compartments and the Implications for HIV Transmission
By Cassie Grimsley Ackerley, M.D.

Men who have sex with men (MSM) remain disproportionately affected by the HIV epidemic in the US, and adolescent and young adult MSM represent a particularly high-risk group. As the vast majority of transmission in MSM occurs through receptive anal intercourse (RAI) (1), one potential reason for discrepant rates of HIV infection among adolescent compared to older MSM is a rectal mucosal immune environment that allows for more efficient viral transmission. In this study comparing immunologic characteristics of the rectal mucosal CD4+ and CD8+ T cell compartments between young MSM, adult MSM, and control males who had never engaged in receptive anal intercourse, we found that young MSM had overall higher levels of viral replication using an *ex vivo* rectal mucosal explant HIV-1 challenge model compared to adult MSM. Evaluation of CD4+ and CD8+ T cell subsets among the study cohorts revealed greater levels of memory CD4+ T cell proliferation and lower frequencies of IFN-γ -and TNF-α-producing CD4+ T cells in both the blood and rectal compartments of young MSM compared to older males, which were findings associated with higher HIV-1 viral replication. These findings suggest that young MSM have a distinct rectal mucosal immune environment that may facilitate HIV transmission.

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INTRODUCTION

Rates of HIV transmission for young men who have sex with men (YMSM), those ages 13 to 24 years, have remained relatively stable in recent years, yet they remain disproportionately high as 25% of new HIV cases among all men who have sex with men (MSM) in 2018 occurred among gay and bisexual males within this age group (2). HIV incidence and prevalence are disturbingly high for YMSM in certain areas (3, 4) despite similar reported rates of sexual activity compared to older MSM (5). Nonetheless, mucosal HIV transmission research has largely focused on vaginal transmission in women and non-human primates resulting in limited data regarding the mechanisms of rectal transmission, and no studies to date investigate the immunologic effects of aging on HIV transmission in the rectal mucosa (6).

The majority of HIV transmission events affecting MSM occur through receptive anal intercourse (RAI) (1); therefore, one potential contributor of disparate rates of HIV infection among adolescent compared to older MSM may be a rectal mucosal immune environment that facilitates HIV infection and viral replication. The adaptive immune system, comprised of CD4+ and CD8+ T cells, is of particular interest as both subsets are critical players involved in early HIV transmission events. CD4+ T cells are the main target cells for HIV infection and are rapidly depleted from the gut during early stages of viral spread (7). The phenotypic attributes of CD4+ T cells including activation/proliferation status, as well as HIV co-receptor and susceptibility marker expression have all been examined as potential determinants of HIV transmission and pathogenesis (8). Likewise, a high prevalence of highly susceptible T helper cell subtypes in the mucosa may also contribute to the risk of HIV acquisition. Alternatively, CD8+ lymphocytes play a critical role in combating intracellular infections through the utilization of both cytolytic and non-cytolytic effector mechanisms, and specifically function in early HIV infection to control HIV replication (9, 10). Therefore, potential age-related alterations in the frequencies of mucosal adaptive immune cell

subsets, in their activation status or functionality, or in the expression of HIV susceptibility markers, could have significant implications in terms of rectal HIV susceptibility.

Overall, the aim of this study was to analyze differences in the composition and phenotypic characteristics of the rectal CD4+ and CD8+ T cell compartments between HIV-negative YMSM, adult MSM, and control males who had never engaged in anal intercourse, and to determine whether age-related changes to the rectal mucosal immune environment were associated with HIV-1 viral replication utilizing an *ex vivo* rectal explant HIV challenge model.

BACKGROUND

Young men who have sex with men in the US are at high risk of acquiring HIV. Some individual risk behaviors of YMSM, including earlier sexual debut, partner concurrency, and selection of older partners, have been associated with increased HIV transmission risk in multiple studies (3, 11, 12), yet there is insufficient evidence to determine if there are also underlying biological determinants of HIV transmission affecting this population (13). An analysis of per contact risk (PCR) of HIV seroconversion, the probability of HIV acquisition per sexual act among high risk MSM with HIV seropositive partners, demonstrated a significantly higher per contact rate for YMSM (< 25 years) engaging in unprotected receptive anal intercourse (RAI) compared to older MSM (> 30 years), despite a lower mean number of reported sexual contacts: 7.1 vs 10.3 (13). These findings suggest there may be host mucosal factors that enhance the risk of HIV acquisition among YMSM.

The structure and cellular composition of the rectal mucosa likely influence HIV transmission. To date, the majority of mucosal HIV transmission research has focused on vaginal transmission in women and non-human primates, and the data have been extrapolated to characterize rectal transmission (14). Yet, there are significant differences in the microenvironments of the rectal mucosa and the female genital tract that likely influence HIV transmission at these sites (6, 14, 15). The rectal mucosa is lined by a single layer of columnar epithelial cells in contrast to the stratified squamous epithelium of the cervix and vagina, theoretically increasing the susceptibility to traumatic abrasions during intercourse that may facilitate viral transmission (6, 16). The cellular composition of the rectal mucosa is also distinct, containing the largest lymphoid compartment of the immune system accounting for more than 60% of all T cells in the body, many of which are primary HIV target cells (i.e. CD4+CCR5+ T cells) (17, 18).

As there is significant heterogeneity in the relative risk for HIV transmission for a given exposure event, efficient viral transmission may rely on the presence of a certain threshold of highly susceptible target cells within the mucosa to overcome host defense mechanisms (19). Productive viral infection in the rectal mucosa of rhesus macaques predominantly occurs in activated and proliferating CD4+ T cells, demonstrating their importance in the propagation of infection following mucosal HIV transmission (7, 20, 21). Not merely the predominance of these cells, but also their phenotypic features appear to be important, as certain characteristics can increase the relative susceptibility of a potential HIV target cell (7). Examination of the rectal mucosa in humans has demonstrated abundant expression of the HIV co-receptor, CCR5, on activated mucosal memory CD4+ T cells (18, 22). Likewise, α4β7, a homing integrin that facilitates migration of lymphocytes to the gut-associated lymphoid tissues, is expressed primarily by mucosal CD4+ T cells and, some argue, promotes attachment of the virus during HIV transmission (23, 24). Higher levels of expression of both CCR5 and α4β7 on the surface of CD4+ T cells is associated with increased HIV susceptibility in human and non-human primate HIV/SIV transmission models (18, 22-25). In addition, the predominance of certain highly susceptible mucosal T cell subsets may impact HIV viral propagation in the tissues. For example, IL-17-producing Th17 cells predominate at mucosal sites and play an important role in inducing mucosal inflammation for host protection against pathogens. Additionally, these cells appear to be highly susceptible to HIV as they are preferentially infected and subsequently depleted in the gut following HIV transmission (26). Collectively, these studies suggest that the activation/proliferation status, the predominance of certain subsets in the tissues, and the expression of susceptibility markers by mucosal memory CD4+ T cells may impact HIV acquisition. These immunologic factors likely contribute to the approximate 18-fold increase in transmission probability per exposure event for RAI compared to penile-vaginal intercourse (14, 16).

Engagement in RAI has been shown to influence the composition of rectal mucosal adaptive immune subsets which may influence HIV transmission risk. Prior work from our group demonstrated a higher proportion of mucosal Th17 cells, greater levels of CD8+ T-cell proliferation and increased pro-inflammatory cytokine production by CD8+ T cells in MSM engaging in condomless RAI compared to controls who had never engaged in anal intercourse (27). Likewise, the microbiota was also noted to be distinct between these groups with enrichment for *Prevotellaceae* among MSM compared to controls, who were enriched with *Bacteroidaceae*. Alterations in the microbiota were thought to likely represent a shift to organisms better able to metabolize products of mucosal injury incurred with repeated mechanical microtrauma from RAI (27, 28). These observations laid the groundwork for considering other demographic or sexual behavior characteristics that may also incite perturbation of the rectal mucosal immune environment, potentially leading to downstream effects that influence the immune response to rectal HIV transmission.

Observed differences in the per contact risk of HIV transmission between younger and older males engaging in RAI suggest that age may also be an important, yet unexplored, factor that impacts rectal HIV transmission (13). The majority of studies evaluating effects of aging on the immune system are primarily focused on immune senescence that occurs in the elderly; however, there may be altered immune responses earlier in life, as individuals move from childhood into early adulthood and beyond, that have the potential to influence host susceptibility to certain infections, including HIV. To date, despite a paucity of human studies investigating age-related changes to gut mucosal immunological processes, there is some evidence to suggest that aging is associated with decreases in systemic and mucosal naïve T cells resulting in a more restricted, terminally differentiated T cell repertoire (29-31), as well as a reduction in T cell cytokine and chemokine production and B cell activity (32). As the significance of these changes in terms of responsiveness

to HIV exposure remain unclear, further investigation is warranted to better understand whether age-related differences in the rectal mucosal T cell composition and the expression of certain HIV susceptibility markers influence HIV infectivity as these findings would have important implications for the advancement of biomedical HIV prevention modalities for YMSM.

METHODS

General Hypothesis. YMSM have a distinct rectal mucosal immune environment that contributes to increased risk of HIV transmission.

The clinical cohort. In this cross-sectional study, healthy HIV-negative MSM and control males were recruited from Atlanta, Georgia. Eligible participants included young MSM (18-21 years, n=32) following receptive anal sexual debut, adult MSM (≥35 years; n=33) who had engaged regularly in RAI, and control males (≥35 years; n=12) who had never engaged in anal intercourse. The decision to establish frequency of RAI as one lifetime partner for adolescents (<19 years) aligns with data suggesting that anal sexual debut most often occurs during late adolescence, typically between 16 and 18 years of age (33, 34). Frequent RAI was defined as at least 5 years with ≥ 12 episodes of RAI per year to ensure a regular pattern of sexual behavior in adult MSM that would support the study of a distinct rectal mucosal immune environment compared to young MSM with more recent sexual debut. The exclusion criteria included men currently on HIV pre-exposure prophylaxis (PrEP), a history of inflammatory bowel disease or other condition of the gastrointestinal tract, current diagnosis of a rectal STI, history of a bleeding disorder, recent major surgeries, and use of immunosuppressive agents. Men who were determined to be high risk for the rectal biopsy procedure based on medical comorbidities were also excluded. Informed consent was obtained for each subject.

All study participants presented for a screening study visit that included a brief medical history, physical examination, rapid HIV testing, and rectal STI testing for gonorrhea and chlamydia (GC/CT). A brief sexual history questionnaire was completed by all participants engaging in RAI and included timing of receptive anal sexual debut, frequency of sexual acts, and use of rectal products (e.g. enemas and lubricants). A screening CBC and coagulation tests were collected to ensure safety of rectal biopsy procedures.

Following this initial evaluation, participants were then scheduled for a subsequent study visit involving rectal biopsy sampling. A trained clinician performed rectal biopsies with all disposable materials via rigid sigmoidoscopy without sedation at the Hope Clinic of the Emory Vaccine Center. At this time, repeat rectal STI testing (GC/CT) with a swab was performed. Up to 10 pinch biopsies were collected using 3.7 mm forceps between 6 to 12 cm from the anal verge. As damage to the rectal mucosa could enhance risk of HIV acquisition, the men were counseled to abstain from anal intercourse for a least 7 days following the biopsy procedure to allow adequate time for healing of the mucosa.

Blood and rectal mucosal mononuclear cell phenotyping. Blood samples collected in EDTA tubes were processed by Ficoll density gradient to separate peripheral blood mononuclear cells, and rectal mucosal pinch biopsies were processed by collagenase digestion to separate mucosal mononuclear cells as has been previously described (35). Isolated cells were then stained with LIVE/DEAD marker and antibodies to CD45, CD4, CD8, CD45RA, CCR7, CCR5, α4β7, Ki67, FOXP3, and CD25. Mononuclear cells were first identified with expression of CD45 and then subdivided into CD4+ and CD8+ subsets. Naïve and memory subsets of CD4+ cells were identified based on expression of CCR7 and CD45RO. CD69 and CD103 markers were used to define tissue resident and non-tissue resident CD4+ and CD8+ T cell subsets. Subsequently, these cell subsets were examined for phenotypic markers for proliferation (Ki67+), HIV co-receptor expression (CCR5+), and HIV susceptibility marker expression (α4β7+). Cell populations were acquired by flow cytometry on the LSR-Fortessa platform and analyzed with Flowjo software (Treestar Inc., CA). Appropriate positive and negative controls were conducted with all assays to ensure reliable results. If there were fewer than 800 memory CD4+ or CD8+events acquired, the subsequent subpopulation data from that specimen was excluded from all further analyses.

Intracellular cytokine staining. A minimum of 1 million peripheral blood and 500,000 rectal mucosal mononuclear cells were stimulated with 25 ng/ml of PMA and 500 ng/mL of Ionomycin in the presence of Brefeldin A (5 μg/ml; Sigma-Aldrich; St. Louis,MO) and Golgi stop (0.5 μl/ml; BD Pharmingen; San Jose, CA) and incubated for 4 h at 37° C in the presence of 5% CO_2. At the end of stimulation, cells were stained with LIVE/DEAD marker and surface marker antibodies CD3, CD4, and CD8. Following fixation and permeabilization, cells were stained for intracellular cytokines with IL-17A, IFN-γ, and TNF-α antibodies. Cytokine secretion from unstimulated and stimulated cells was assessed using flow cytometry. If fewer than 500 CD4+ or CD8+ events were acquired from the stimulated specimens, the specimen was classified as a non-response to stimulation and excluded from all further analyses.

Explant challenge experiments. Three rectal pinch biopsies from each participant were weighed and then placed in complete medium with antibiotics at 37°C in a cell culture incubator. Within 24 hours, each biopsy was inoculated with 250 μL of HIV-1 BaL virus ($10^{2.8}$ $TCID_{50}$) for 2 hours (37° C, 5% CO_2). Following viral exposure, each biopsy was serially washed in sterile phosphate-buffered saline and placed on a pre-wet gelfoam raft in 1 ml complete media (RPMI 1640 with 10% FBS, Gentamicin Sulfate, and Zosyn). Culture supernatant was collected from each well at days 3, 7, 10, 14, and 18 for p24 concentration analysis, and the well was replenished with 700 μl of fresh complete medium. The p24 concentrations were quantified using an HIV p24 Antigen Capture Assay ELISA kit according to the manufacturer's instructions, and the values were normalized to biopsy weight.

Statistical Analyses. Demographic and sexual behavior characteristics were compared between study groups using Mann Whitney U tests for continuous variables and chi square or Fisher's exact test for proportions. To analyze differential distributions of CD4+ and CD8+ T cell subsets of interest between groups, differences in the subset frequencies and percentages of cellular marker expression were compared using a two-sample t-test for normally distributed data and the Mann-Whitney U test for nonparametric data. Age-adjusted associations between the subsets of interest and HIV viral replication were analyzed by calculating the residual values for both the immune subset percentage and the normalized median log of the area under the longitudinal p24 curves (logAUC) and using these values in Spearman's rank-order correlation analyses. To be able to include all participants in the analyses and to compare differences in the immune phenotypes from participants with low or undetectable p24 concentrations versus those with high p24 concentrations, an imputed value for median logAUC was derived for six participants with undetectable p24 concentrations over the 18-day explant challenge time course. For these individuals, imputed minimal log p24 values were derived by calculating the AUC with p24 set at the limit of the detection (50 pg/ml) and then normalized by the biopsy weight. Data analyses were performed using SAS 9.4 (SAS Institute Inc, NC) and GraphPad PRISM 8 (GraphPad Software, CA).

Approval and Funding: The Institutional Review Board at Emory University reviewed the protocol and approved this study. The project described was primarily supported through a grant from the National Institute of Allergy and Infectious Diseases (R01 AI128799-01, C.F.K.). Cassie Grimsley Ackerley received support to complete this work from Award Number T32DK108735 from the National Institute of Diabetes and Digestive and Kidney Diseases and also from the National Institutes of Health (UL1 TR002378).

RESULTS

The Clinical Cohort. Thirty-two HIV-negative YMSM and thirty-three HIV-negative adult MSM engaging in RAI, along with 12 HIV-negative control males who had never engaged in anal intercourse were enrolled and underwent peripheral blood and rectal biopsy sampling. Demographic and clinical characteristics are presented in **Table 1**. The median age of subjects from the YMSM group was 20 years of age (interquartile range (IQR): 19-21 years), in contrast to the median age of 49 years (IQR: 39-50 years) in the adult MSM group and 48 years (IQR: 40-52 years) in the control male group. Notably, there were higher proportions of Black/African American subjects in the young MSM and control cohorts (88% and 83%, respectively) compared to the adult MSM cohort (52%; $p<0.0001$). As the sexual questionnaires were completed exclusively by participants engaging in RAI, the sexual behavior characteristics were only compared between the young and adult MSM groups. The age at time of RAI sexual debut was earlier for young (18 years; IQR: 16.5-19) compared to adult MSM (23 years; IQR 18-28), respectively ($p<0.0001$). The YMSM group did report a lower number of lifetime sexual partners ($p<0.0001$) and a reduced number of sexual encounters involving RAI during the 12 months and 30 days prior to the screening visit compared to adult MSM ($p<0.008$ for both comparisons), which suggests a pattern of less frequent RAI encounters among the younger males in this study. There were no significant differences in reported use of lubricants or in douching before or after RAI between young and adult MSM.

Blood and rectal mucosal CD4+ memory T cell subsets from YMSM demonstrate greater levels of proliferation compared to the older male cohorts. To understand age-related differences in the frequency of primary HIV target cells from the blood and rectal mucosal compartments, we measured the expression of CCR5 (HIV co-receptor), Ki67 (indicator of proliferation status) and $\alpha 4\beta 7$ (gut homing marker associated with HIV susceptibility) by blood and

mucosal CD4+ memory T cell subsets. Representative gating strategies for blood and gut are presented in **Supplementary Figure 1** and **Figure 1**, respectively. We found there were lower frequencies of total memory CD4+ T cells among YMSM compared to the older male cohorts in the blood (YMSM 59.5% vs AMSM 71.6%, p=0.008; YMSM vs controls 77.4%, p=0.07; **Supplementary Figure 2a** and **Supplementary Table 1**) and rectal mucosal compartments (YMSM 96.7% vs AMSM 98.4%, p=0.001; YMSM vs Controls 98.8%, p=0.02; **Figure 2a** and **Table 2**). Despite no significant difference in the frequencies of rectal mucosal CD4+ tissue resident (Trm) or non-tissue resident subsets (non-Trm) between groups, there was heterogeneity in the expression of CCR5 and Ki67 by the CD4+ T cell subsets of younger and older males. YMSM showed lower percentages of blood and rectal CCR5-expressing CD4+ memory T cells compared to adult MSM (Blood: 11.6% vs 15.0%, p=0.005; rectal mucosal (RM): 31.7% vs 45.5%, p=0.006), and this CCR5 expression trend was also observed among mucosal CD4+ memory Trm and non-Trm subsets. Contrastingly, the YMSM demonstrated greater expression of Ki67 by blood and rectal mucosal CD4+ memory T cell subsets compared to adult MSM and controls (Blood: YMSM 2.5% vs AMSM 2.2%, p=0.03; YMSM 2.5% vs Controls 1.4%, p=0.0002; RM: YMSM 2.2% vs AMSM 1.3%, p=0.005; YMSM 2.2% vs Controls 0.8%, p<0.0001). Rectal mucosal memory CD4+ T cells with co-expression of CCR5 and Ki67 were higher among YMSM compared to adult MSM (0.82% vs 0.71%, p=0.06) and both MSM cohorts had greater frequencies of this subset compared to control males (YMSM 0.82% vs Controls 0.35%, p<0.0001; AMSM 0.71% vs Controls 0.35%, p=0.008; **Table 2**). Likewise, there were higher frequencies of rectal T regulatory (Treg; CD25+FoxP3+CD4+) cells among the groups of men engaging in RAI compared to control males (YMSM 2.8% vs Controls 1.2%, p=0.008; AMSM 2.6% vs Controls 1.2%, p =0.02). Overall, these findings suggest that blood and rectal mucosal memory CD4+ T cells from YMSM have higher levels of cellular activation/proliferation but lower CCR5 expression, compared to the older male

cohorts. Although there was a trend toward greater expression of α4β7 by CD4+ memory T cell subsets of YMSM, the findings were not statistically significant between study groups in either the blood or rectal mucosal compartments.

In the rectal CD8+ T cell compartment, YMSM were observed to have a lower frequency of mucosal CD8+CD69+CD103+ tissue resident T cells compared to older males (YMSM 35.8% vs AMSM 43.4%, p=0.04; YMSM 35.8% vs 57.2%, p=0.047; **Figure 2b** and **Table 2**) despite similar frequencies of rectal CD8+ memory T cell populations among the study groups. Ki67 expression by CD8+ memory T cell subsets did not differ significantly between groups (data not shown).

Blood and rectal mucosal CD4+ and CD8+ T cells from YMSM show lower levels of pro-inflammatory cytokine expression compared to adult MSM. Next, we investigated the function of CD4+ and CD8+ T cells from the blood and rectal mucosal compartments (representative gating presented in **Supplementary Figure 3** and **Figure 3**, respectively), specifically evaluating the production of cytokines IL-17A, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) following stimulation with phorbol myristate acetate (PMA) and Ionomycin. Blood CD4+ T cells from YMSM demonstrated reduced TNF-α (YMSM 1.8% vs AMSM 3.3%, p=0.0002; YMSM 1.8% vs Controls 4.2%, p=0.02) and IFN-γ production (YMSM 17.7% vs AMSM 26.1%, p=0.004; YMSM 17.7% vs Controls 28.4%, p=0.02; **Supplementary Figure 4a** and **Supplementary Table 1**) compared to the older male cohorts, and there was a similar trend observed among the rectal CD4+ TNF-α- (YMSM 5.5% vs AMSM 7.7%, p=0.08; YMSM 5.5% vs Controls 7.0%, p=0.09) and IFN-γ-producing subsets (YMSM 29.3% vs AMSM 36.6%, p=0.008; YMSM 29.3% vs Controls 33.3%, p=0.15; **Figure 4a** and **Table 2**). Similarly, blood and rectal mucosal CD8+T cells from YMSM also showed a trend toward reduced secretion of IFN-γ and TNF-α cytokines following

stimulation compared to adult MSM (**Figure 4b** and **Supplementary Figure 4b**, respectively). No significant differences were observed between the study groups in the production of IL-17A by blood or rectal CD4+ T cells between the groups.

Greater levels of cellular proliferation and lower frequencies of pro-inflammatory memory CD4+ T cells are associated with increased HIV replication in the rectal mucosal explant challenge model. The *ex vivo* rectal explant challenge model provides a unique opportunity to better understand the adaptive immune response to early HIV transmission events. Rectal mucosal biopsies designated for the *ex vivo* explant challenge model were exposed to HIV-1 BaL virus and p24 concentrations were longitudinally assessed over an 18-day period. From the challenge experiments, p24 concentrations were used to derive the median logAUC, which was utilized in our analyses as a surrogate marker for viral replication, allowing for the evaluation of associations between rectal mucosal immune cell subset frequencies and HIV-1 viral replication. Comparing the normalized median logAUC values for p24 concentration between study groups, we found higher levels of viral replication among YMSM compared to adult MSM (**Figure 5**; p=0.03). There was no significant difference in viral replication between the control males and the MSM cohorts.
In analyzing the age-adjusted associations between p24 production and frequencies of rectal CD4+ and CD8+ T cell subsets of interest, higher levels of Ki67 expression by memory CD4+ T cells ($r = 0.21$, $p = 0.07$) and by CD4+CD69+ tissue resident cells ($r = 0.23$, $p = 0.046$) were positively correlated with viral replication in our model. Notably, following age adjustment, neither expression of CCR5 nor $\alpha4\beta7$ by CD4+ memory T cells was associated with HIV infectivity. Increased frequencies of rectal CD4+ T cells with the potential to produce pro-inflammatory cytokines, IFN-γ ($r = -0.32$, $p = 0.005$) and TNF-α ($r = -0.22$, $p = 0.06$), were negatively correlated with viral replication (**Figure 6**). There were no significant associations between viral replication and the

frequencies of CD8+ T cell subsets, including CD8+ pro-inflammatory cytokine-producers (data not shown).

DISCUSSION

In this study comparing the rectal mucosal T cell compartments between young and adult MSM engaging in RAI, and control males who had never engaged in anal intercourse, we found that YMSM have a distinct rectal mucosal adaptive immune environment characterized by higher levels of CD4+ T cell proliferation, lower expression of CCR5 by memory CD4+ T cells, fewer mucosal CD8+ Trm cells, and decreased frequencies of pro-inflammatory CD4+ and CD8+ T cell populations compared to older males. Notably, the associations between greater CD4+ T cell proliferation and lower frequencies of mucosal pro-inflammatory CD4+ T cells with higher HIV-1 viral replication in our model provide evidence that the rectal mucosal immunological milieu of YMSM may facilitate HIV transmission.

Our finding that YMSM have a lower proportion of memory CD4+ T cells, including Th1 cytokine-producers, as well as CD8+ Trm cells compared to the older cohorts likely is related to the younger age of these individuals. In healthy persons, memory CD4+ and CD8+ T cells are predominant at mucosal sites throughout all stages of life, but there is a trend toward lower frequencies of memory CD4+ and CD8+ T cells in the colonic mucosa of younger individuals (<20 years), compared to older individuals (>30 years), and the same pattern is observed in the peripheral blood and lymphoid tissues (30). Naïve T cells, those with the capacity to respond to new antigens, become activated and differentiate into effector cells that then migrate to various sites in order to promote antigenic control (36). A small proportion of these effector cells survive as memory T cells responsible for re-activation upon recurrent antigenic exposure. Thus, younger individuals with lower cumulative antigenic exposures compared to older individuals have lower frequencies of memory T cells systemically and throughout many lymphoid and mucosal tissue compartments. Notably, naïve T cells are less susceptible to HIV infection compared to memory CD4+ T cells (37); however, the difference in the proportion of rectal memory CD4+ T cells between the younger and

older cohorts in this study is minimal and presumably would not significantly impact the necessary threshold of HIV target cells within the rectal mucosal tissues to propagate infection following HIV exposure.

One potential reason for increased HIV-1 viral replication among YMSM compared to adult MSM in this study is the discordant proportion of proliferating CD4+ T cells in the rectal tissues of these young men. HIV preferentially infects activated, proliferating CD4+ T cells, as these cells can accommodate higher levels of viral replication and more efficiently amplify viral spread (7, 38). Our findings, therefore, underscore the importance of CD4+ T cell activation and proliferation in promoting mucosal HIV transmission. As there are no known prior studies characterizing the typical rectal mucosal immune environment of young, HIV-negative men, it is unclear whether a greater proportion of proliferating CD4+ T cells would be seen among adolescents generally or whether this finding may be related to RAI. As there were notable differences in the reported sexual behaviors between YMSM and adult MSM cohorts in this study, whereby the older MSM reported a more regular pattern of RAI, it is possible that a more frequent pattern of RAI encounters may induce some immune tolerance over time due to repeated, frequent mucosal microtrauma. This recurrent mucosal injury may result in lower levels of inflammation and immune activation at this site. Moreover, sporadic RAI, as reported by the majority of YMSM in our cohort, may promote robust inflammatory responses to infrequent traumatic abrasions during sexual intercourse (39). The immune response to RAI has yet to be fully examined and may be essential to better understanding the observed differences in the rectal mucosal immune cell composition and levels of viral replication between the younger and older MSM cohorts.

Prior studies have demonstrated the significance of CCR5 expression for R5 HIV-1 entry into CD4+ T cells, allowing for rapid viral replication and dissemination (18, 22). In our explant challenge model, CCR5 expression by CD4+ memory T cell subsets was not correlated with HIV-1

viral replication following adjustment for age, an unexpected yet potentially significant observation. T cells are essential in providing immunologic defenses in response to pathogenic antigen exposure and subsequently develop into memory subsets that confer long-term protective immunity in the case of recurrent infection. The memory CD4+ T cell population is diverse in its comprisal of distinct subsets, including tissue resident memory, central memory (Tcm), effector memory (Tem), terminally differentiated T cells that re-express CD45RA (Temra), and regulatory T cells (Treg), which are distinguished by specialized functions within lymphoid and mucosal tissues (40). Therefore, the CCR5+CD4+ population found within a mucosal tissue site is likely to constitute a heterogenous composition of memory subsets that are differentially susceptible to HIV despite their shared immune phenotype (41). Oswald-Richter *et al* (37) demonstrated differential CCR5 expression among naïve and memory CD4+ subsets from HIV-uninfected individuals, with Tem and Temra expressing the highest levels of CCR5 (46.7% and 78.1%, respectively) compared to significantly lower expression by Tcm (15.4%) and naïve (0.3%) T cells. They further showed that the levels of CCR5 expression did not necessarily correlate with HIV infectivity, as the Tem and Tcm populations from HIV-negative subjects were more susceptible to HIV infection compared to naïve or Temra cells. Likewise, despite their expression of CCR5, Treg cells are not preferentially targeted for infection by HIV, unlike many of the other memory CD4+ T cell subtypes (42), possibly in part due to FoxP3-mediated inhibition of HIV-1 transcription (43, 44). Therefore, we hypothesize that CCR5 expression, while important for R5 HIV-1 infection of CD4+ T cells, must be considered in conjunction with other factors, including the memory cellular subtype and the activation status of a cell, which may further impact the cell's overall susceptibility to HIV. Further investigation into the composition of the CD4+ memory T cell compartment of the rectal mucosa is warranted to better characterize the complex, immune phenotypes of highly HIV-susceptible CD4+ target cells at this site.

The majority of work addressing HIV risk and tissue inflammation derive from non-human primate and female genital tract studies, whereby elevated concentrations of pro-inflammatory cytokines are thought to enhance the risk of HIV acquisition. It has been hypothesized that secretion of pro-inflammatory cytokines within genital tissues induces the recruitment of innate and adaptive immune cell subsets to the site, including CD4+ HIV target cells, promotes immune cell activation and differentiation, as well as disrupts tight junctions between epithelial cells leading to reduced barrier integrity (45). In this study, however, higher frequencies of CD4+ T cells with the potential to produce the pro-inflammatory cytokines, IFN-γ and TNF-α, was associated with reduced HIV-1 viral replication in our model. We hypothesize that these CD4+ T cells with a Th1-like phenotype may also be secreting other cytokines or chemokines with the ability to inhibit HIV viral replication. Three chemokines that have previously been described as having suppressor activity against macrophage tropic HIV-1 isolates are RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β. These are β chemokines that bind to CCR5 and competitively block viral interaction with the HIV co-receptor and may also promote the down-regulation of CCR5 from the host-cell surface (46). Evidence from prior studies demonstrates that multiple cytokines/chemokines are upregulated in response to HIV infection (47), and it is likely that these factors have some capacity to limit HIV viral propagation at mucosal transmission sites. In a study evaluating cytokine and chemokine responses from CD4+ T cells during acute simian immunodeficiency virus infection of non-human primates, both TNF-α and RANTES were upregulated by peripheral blood T cells following infection, and CD4+ T cells from the bone marrow demonstrated increased secretion of MIP-1α, MIP-1β, and RANTES (48). Similarly, in the female genital tract, greater expression of RANTES by CD8+ T cells was identified in HIV-negative commercial sex workers compared to lower levels in HIV-negative women with low-risk of HIV acquisition (49). Taken together, the impact of cytokine/ chemokine secretion at mucosal sites during HIV-1 exposure may have both

enhancing and inhibitory consequences depending on the type and concentration of the signaling molecules being produced and also possibly depending on the particular site of transmission. Currently, there remains a paucity of information about early HIV-1 transmission events in the rectum, and ongoing evaluation into the specific secretory functions of adaptive immune subsets in the rectal mucosa may lead to the identification of HIV suppressive factors that could become critical targets in the development of future HIV prevention interventions.

An important limitation in this study is the absence of a young male control group not engaging in anal intercourse. Without this group, we are unable to more definitively confirm that the rectal mucosal immune cell subset characteristics that differentiate YMSM from the older male cohorts in this study are secondary to age rather than to an alternative etiology, such as a less frequent, more sporadic pattern of RAI. Due to sample size limitations, we did not control for race in our analyses, and the unequal racial/ethnic distribution between the cohorts could explain some of the mucosal immunological differences that were observed. Other potential viral suppressive factors that may be secreted by CD4+ and CD8+ T cells were not evaluated here yet will be important in further analyses in order to better understand the full arsenal of rectal mucosal defenses against HIV. Additionally, survival bias is certainly a consideration in this study as it is possible that the adult MSM cohort who have remained HIV-negative for an extended period of time compared to the younger males, may benefit from inherent host protective factors against HIV infection.

In summary, we have identified a distinct rectal mucosal immune environment among a young MSM cohort characterized by high levels of CD4+ T cell proliferation and lower frequencies of pro-inflammatory cytokine-producing CD4+ T cells, and these findings were associated with greater levels of viral replication in our HIV explant challenge model. These results suggest that the mucosal immunologic milieu of YMSM may play a role in facilitating rectal HIV transmission. It is unclear whether age or possibly less frequent, sporadic RAI encounters may be contributing to the

immunological differences observed among the study cohorts, and further investigation will be necessary to better characterize how certain demographic and sexual behavior characteristics impact rectal HIV transmission. Furthermore, it will also be critical to further describe the immune phenotype of HIV-susceptible target cells within the rectum and to explore the role of secreted HIV suppressive cytokines/chemokines that may also influence HIV transmission at this site. Nevertheless, to our knowledge, this is the first study of its kind to comprehensively evaluate the rectal mucosal immune environment of HIV-negative at-risk YMSM and to compare those results directly with older male cohorts. These findings are valuable as they have future implications for mucosal vaccine responsiveness and could inform the development and optimization of biomedical HIV prevention interventions that provide a direct, protective benefit for YMSM.

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TABLES/FIGURES

Table 1: Demographic and clinical characteristics of young MSM, adult MSM, and men who never engaged in anal intercourse (controls) included in this study.

Characteristic	YMSM (n = 32)	Adult MSM (n = 33)	Control Males (n = 12)	*P* value
Median age in years (25th, 75th).	20 (19, 21)	46 (39, 50)	48 (40, 52)	
Race *n* (%)				
White	4 (12)	15 (44)	2 (17)	**<0.0001**
Black	28 (88)	17 (50)	10 (83)	
Other	0 (0)	2 (6)	0 (0)	
Sexual characteristics:				
Median age at time of sexual debut (25th, 75th)	18 (16.5, 19)	23 (18, 28)	--	**<0.0001**
Total number of sexual partners with whom the participant engaged in RAI during their lifetime, *n* (%)				**<0.0001**
0 to 1	4 (12.5)	0 (0)	--	
2 to 5	17 (53.1)	4 (12.5)	--	
6 to 20	7 (21.9)	12 (37.5)	--	
21 to 50	3 (9.4)	11 (34.4)	--	
51+	1 (3.1)	5 (15.6)	--	
Total number of sexual partners with whom the participant engaged in RAI during the previous 12 months, *n* (%)				**0.005**
0 to 1	10 (31.3)	10 (30.3)	--	
2 to 5	17 (53.1)	12 (36.4)	--	
6 to 20	5 (15.6)	9 (27.3)	--	
21 to 50	0 (0)	0 (0)	--	

51+	0 (0)	2 (6.1)	--	
Total number of RAI sexual encounters during the previous 12 months, *n* (%)				**<0.0001**
0 to 1	10 (31.3)	2 (6.1)	--	
2 to 5	14 (43.8)	6 (18.2)	--	
6 to 20	6 (18.8)	16 (48.5)	--	
21 to 50	0 (0)	6 (18.2)	--	
51+	2 (6.3)	3 (9.1)	--	
Total RAI episodes in previous 30 days, *n* (%)				**0.007**
0	15 (48.4)	10 (30.3)	--	
1 to 5	14 (45.2)	17 (51.5)	--	
6 to 20	2 (6.5)	4 (12.1)	--	
21 to 50	0 (0)	2 (6.1)	--	
51+	0 (0)	0 (0)	--	
Lubricant use, *n* (%)				0.51
Yes	31 (96.9)	32 (97.0)	--	
No	1 (3.1)	1 (3.0)	--	
Douching before or after RAI, *n* (%)				0.38
Yes	19 (59.4)	23 (69.7)	--	
No	13 (40.6)	10 (30.3)	--	

Abbreviations: YMSM, young men who have sex with men, MSM, men who have sex with men; RAI, receptive anal intercourse. Significant p-value < 0.05 for chi square or Fisher's exact test for proportions.

Table 2: The distribution of rectal mucosal CD4+ and CD8+ memory T cell subsets among YMSM, AMSM and control males.

Immunological index	*n*	Median (25^{th}, 75^{th})	*P-value (vs YMSM)*
Rectal memory CD4 + cells			
% $CD4^+$ memory			
YMSM	31	96.7 (90.9, 98.0)	--
AMSM	30	98.4 (96.7, 99.0)	**0.001**
Control males	10	98.8 (93.9, 99.0)	**0.02**
% $CD69^+$			
YMSM	30	53.7 (43.1, 68.3)	--
AMSM	30	56.3 (45.1, 66.0)	0.83
Control males	10	75.4 (38.3, 81.9)	0.20
% $CD69^-$ $CD103^-$			
YMSM	30	38.9 (26.4, 50.4)	--
AMSM	30	36.7 (26.8, 53.5)	0.82
Control males	10	19.8 (14.1, 62.3)	0.33
% $CD25^+$ $FoxP3^+$			
YMSM	31	2.8 (1.9, 4.0)	--
AMSM	30	2.6 (1.5, 4.4)	0.91
Control males	10	1.2 (0.5, 2.3)	**0.008**
% $\alpha4\beta7^+$			
YMSM	30	5.6 (1.0, 10.3)	--
AMSM	30	3.5 (1.4, 6.4)	0.25
Control males	10	3.1 (2.2, 7.5)	0.28
% $CCR5^+$			
YMSM	30	31.7 (27.1, 44.0)	--
AMSM	30	45.5 (33.2, 52.6)	**0.006**
Control males	10	33.7 (27.9, 48.0)	0.75
% $Ki67^+$			
YMSM	30	2.2 (1.4, 4.2)	--
AMSM	30	1.3 (0.9, 2.4)	**0.005**
Control males	10	0.8 (0.6, 1.0)	**<0.0001**
% $CCR5^+$ $Ki67^+$			
YMSM	30	0.82 (0.6, 1.8)	--
AMSM	30	0.7 (0.4, 1.1)	0.06
Control males	10	0.3 (0.2, 0.5)	**<0.0001**
% $\alpha4\beta7^+$ $CD69^+$			
YMSM	30	2.6 (0.2, 5.5)	--
AMSM	30	1.7 (0.6, 2.9)	0.30
Control males	10	1.1 (0.7, 5.7)	0.59
% $CCR5^+$ $CD69^+$			
YMSM	30	23.1 (18.4, 31.9)	--
AMSM	30	30.8 (21.9, 42.0)	**0.043**
Control males	10	29.4 (12.6, 51.1)	0.61
% $Ki67^+$ $CD69^+$			
YMSM	30	1.1 (0.7, 1.6)	--
AMSM	30	0.7 (0.4, 1.3)	0.09
Control males	10	0.5 (0.1, 0.9)	**0.01**

% $\alpha4\beta7^+$ $CD69^-$ 103^-			
YMSM	30	3.0 (0.6, 5.0)	--
AMSM	30	1.8 (0.7, 2.8)	0.24
Control males	10	1.7 (1.1, 2.5)	0.18
% $CCR5^+$ $CD69^-$ 103^-			
YMSM	30	9.2 (5.1, 12.3)	--
AMSM	30	10.3 (7.7, 15.5)	**0.048**
Control males	10	4.6 (2.6, 14.5)	0.20
% $Ki67^+$ $CD69^-$ 103^-			
YMSM	30	1.1 (0.7, 1.9)	--
AMSM	30	0.7 (0.5, 1.2)	**0.01**
Control males	10	0.5 (0.3, 0.6)	**<0.0001**
Rectal memory $CD8^+$ cells			
% $CD8^+$ memory			
YMSM	31	99.1 (97.4, 99.6)	--
AMSM	30	99.2 (98.8, 99.6)	0.14
Control males	10	99.2 (98.8, 99.7)	0.22
% $CD69^+103^+$			
YMSM	29	35.8 (26.9, 41.1)	--
AMSM	28	43.4 (33.0, 53.4)	**0.040**
Control males	10	57.2 (29.5, 70.8)	**0.047**
% $CD69^-CD103^-$			
YMSM	29	31.1 (21.6, 38.1)	--
AMSM	28	21.4 (17.0, 36.0)	0.13
Control males	10	16.0 (8.3, 36.6)	**0.049**
Stimulated rectal $CD4^+$ cells			
% IL-$17A^+$			
YMSM	30	8.2 (6.6, 10.7)	--
AMSM	26	7.6 (5.5, 10.3)	0.62
Control males	10	7.0 (4.8, 7.5)	0.12
% $IFN\gamma^+$			
YMSM	30	28.5 (22.5, 33.6)	--
AMSM	26	38.4 (27.8, 48.1)	**0.01**
Control males	10	34.7 (26.6, 43.9)	0.08
% $TNF\alpha^+$			
YMSM	30	5.4 (3.5, 8.2)	--
AMSM	26	7.7 (4.5, 9.8)	0.07
Control males	10	7.7 (4.5, 8.6)	0.12
Stimulated rectal $CD8^+$ cells			
% $IFN\gamma^+$			
YMSM	22	49.6 (37.6, 55.6)	--
AMSM	24	58.2 (46.6, 67.5)	0.05
Control males	7	60.8 (30.8, 71.9)	0.25
% $TNF\alpha^+$			
YMSM	22	3.8 (2.7, 6.3)	--
AMSM	24	7.0 (4.1, 10.7)	**0.01**
Control males	7	6.1 (4.6, 8.3)	0.08

Abbreviations: YMSM, young men who have sex with men, AMSM, adult men who have sex with men. Bolded items are significant *P*-values (<0.05) for Mann Whitney U test for differences in median cell subset frequencies between study groups.

Figure 1: Representative gating strategy for rectal mucosal mononuclear cells. Lymphocytes were identified by forward and side scatter and CD45+ cells were isolated. This subset was divided into CD4+ and CD8+ subsets. Memory CD4 cells were identified by excluding CD45RA+ cells. CD69 marker was then used to divide CD4+ cells into tissue resident and non-tissue resident populations. Memory CD8 + cells were designated as being $CCR7^{-}$ and $CD45RA^{+/-}$. Among memory CD8+ T cells, both CD69 and CD103 markers were used to designate these populations. Memory CD4+ and CD8+ populations, including tissue resident and non-tissue resident subsets were then assessed for expression of CCR5 (HIV co-receptor), α4β7 (gut homing and HIV co-receptor) and Ki67 (proliferation).

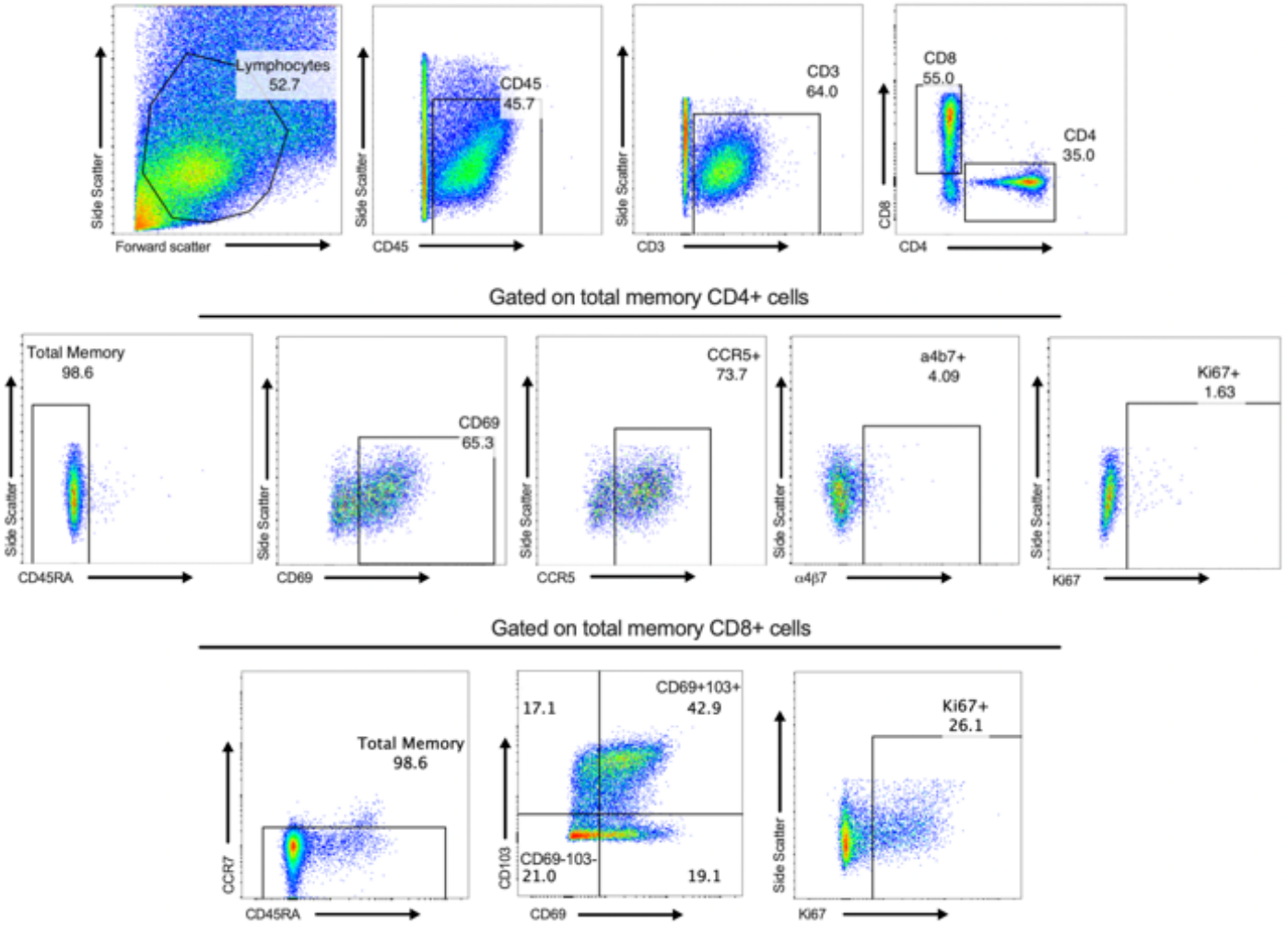
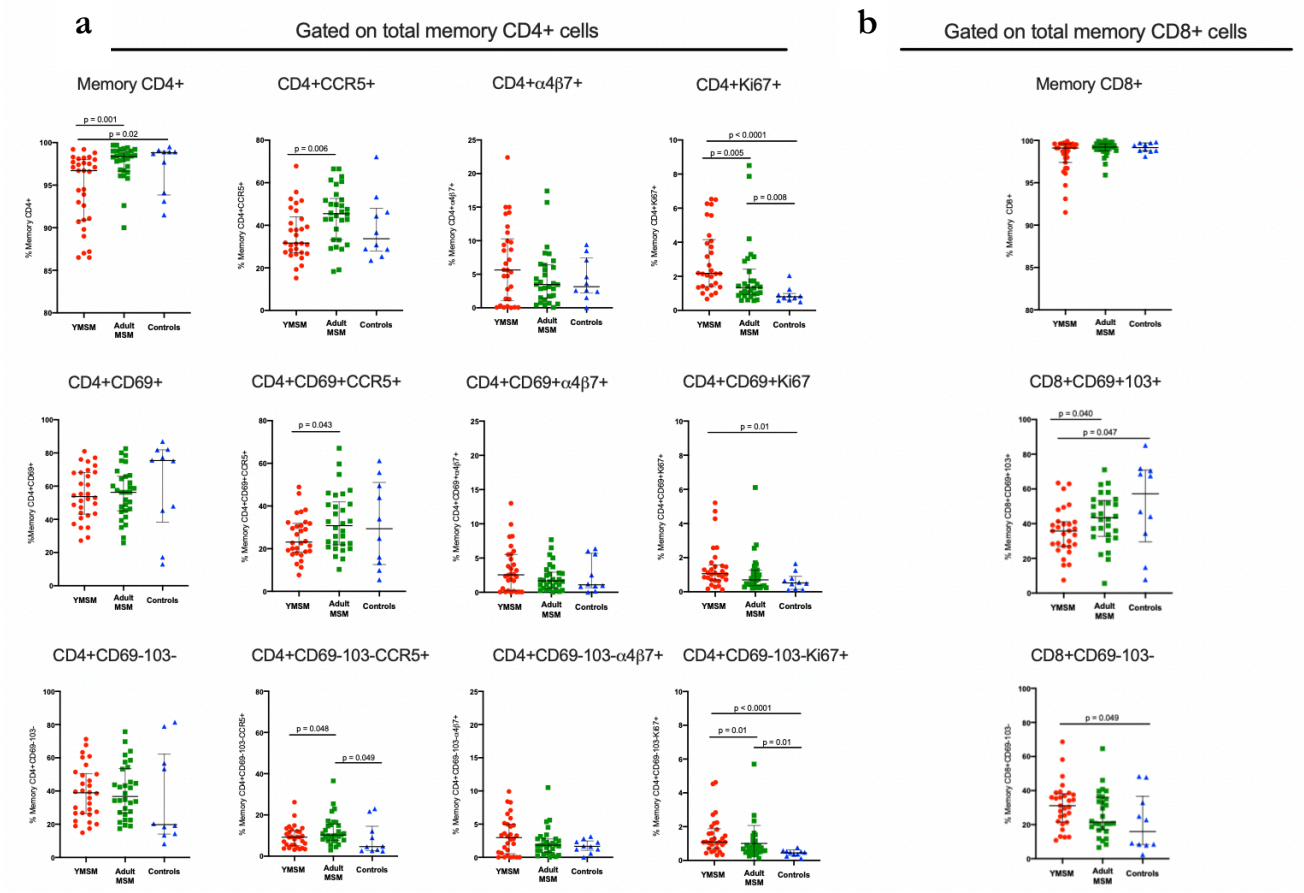
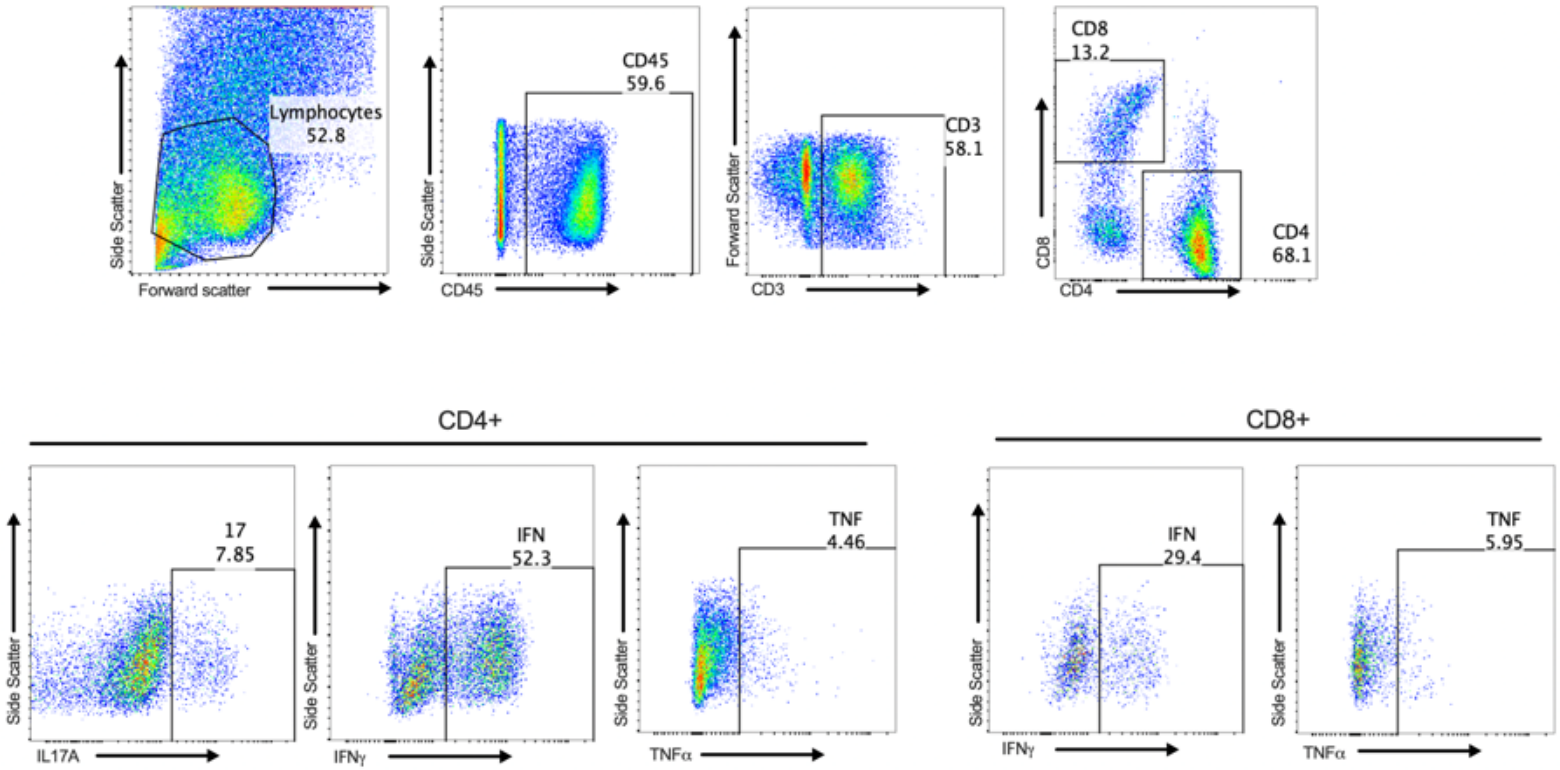


Figure 2: Phenotype of rectal MMCs demonstrates elevated levels of Ki67 expression but decreased CCR5 expression by CD4+ memory subsets among YMSM compared to AMSM. (**a**) Results of rectal mucosal CD4+ cell phenotyping for YMSM, AMSM, and Controls. (**b**) Results of rectal mucosal CD8+ cell phenotyping among YMSM, AMSM and controls. Black horizontal lines and vertical ranges represent the median and interquartile range as reported in **Table 2**.



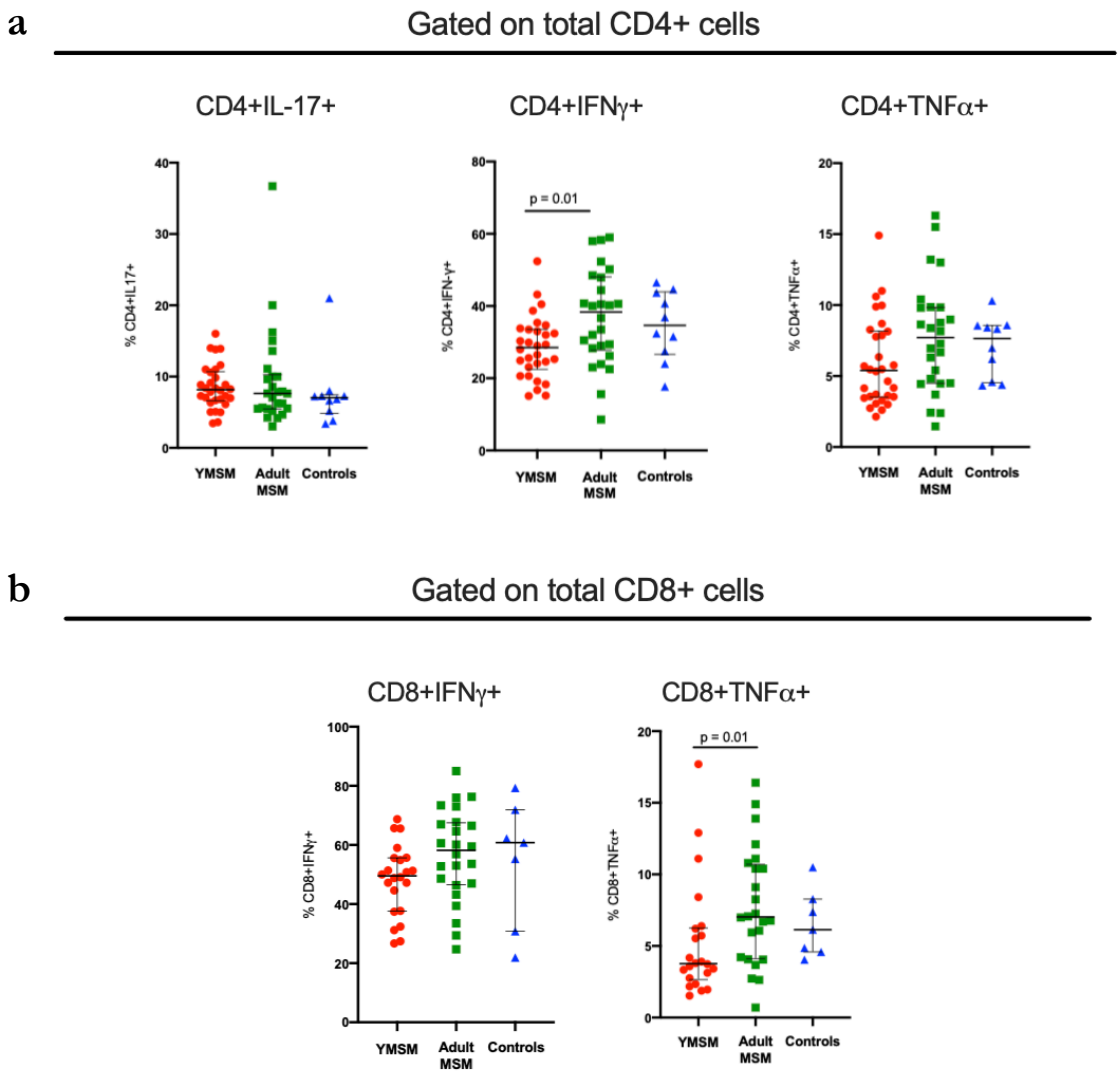
Abbreviations: MMC, mucosal mononuclear cells, YMSM, adolescent men who have sex with men, AMSM, adult men who have sex with men, Controls, control males not engaging in anal intercourse. P-values shown are significant at <0.05 for Mann Whitney U test for differences in median expression between groups.

Figure 3: Representative gating strategy to detect cytokine-positive rectal mucosal CD4+ and CD8+ T cells. Rectal CD4+ and CD8+ MMCs were stimulated for 4 hours with PMA/Ionomycin and stained for indicated cytokines. Live cells were identified by live/dead staining (not shown) and lymphocytes were identified by forward and side scatter. CD45+ cells were then isolated, followed by CD3+ cells which were separated into CD4+ and CD8+ subsets. Stimulated CD4+ T cells were assessed for IL-17A, IFN-γ and TNF-α cytokine production and stimulated CD8+ T cells for IFN-γ and TNF-α.



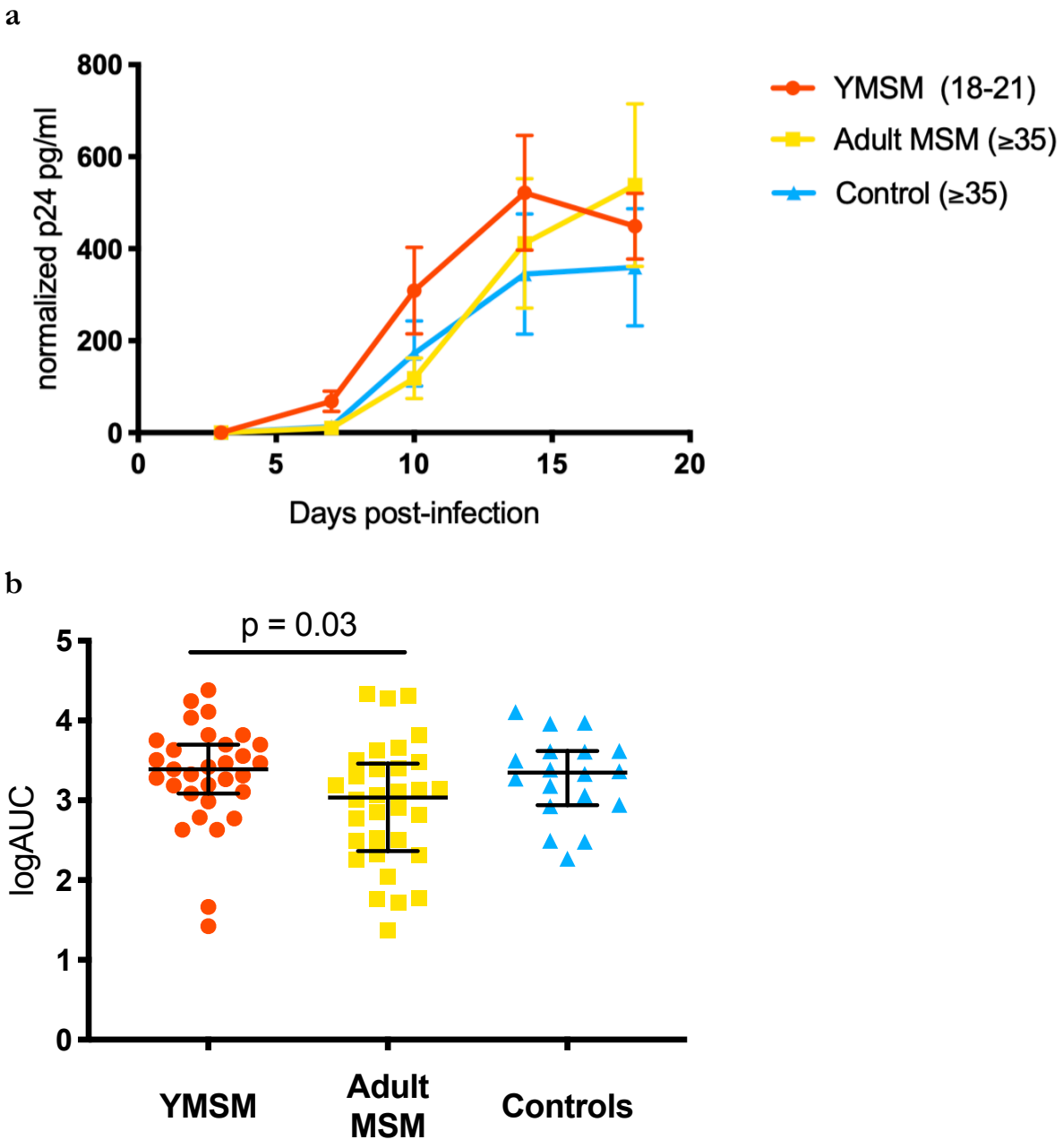
Abbreviations: MMC, mucosal mononuclear cells, PMA, phorbol myristate acetate, IFN-γ, interferon gamma, TNF, tumor necrosis factor.

Figure 4: Cytokine production on mitogen-stimulated rectal CD4+ and CD8+ mucosal mononuclear cells demonstrates a trend toward lower levels of pro-inflammatory cytokine production in YMSM compared to older males. (**a**) Results of cytokine-positive CD4+ T cells for YMSM, AMSM, and Controls. (**b**) Results of cytokine-positive CD8+ T cells for YMSM, AMSM, and Controls. Black horizontal lines and vertical ranges represent the median and interquartile range as reported in **Table 2**.



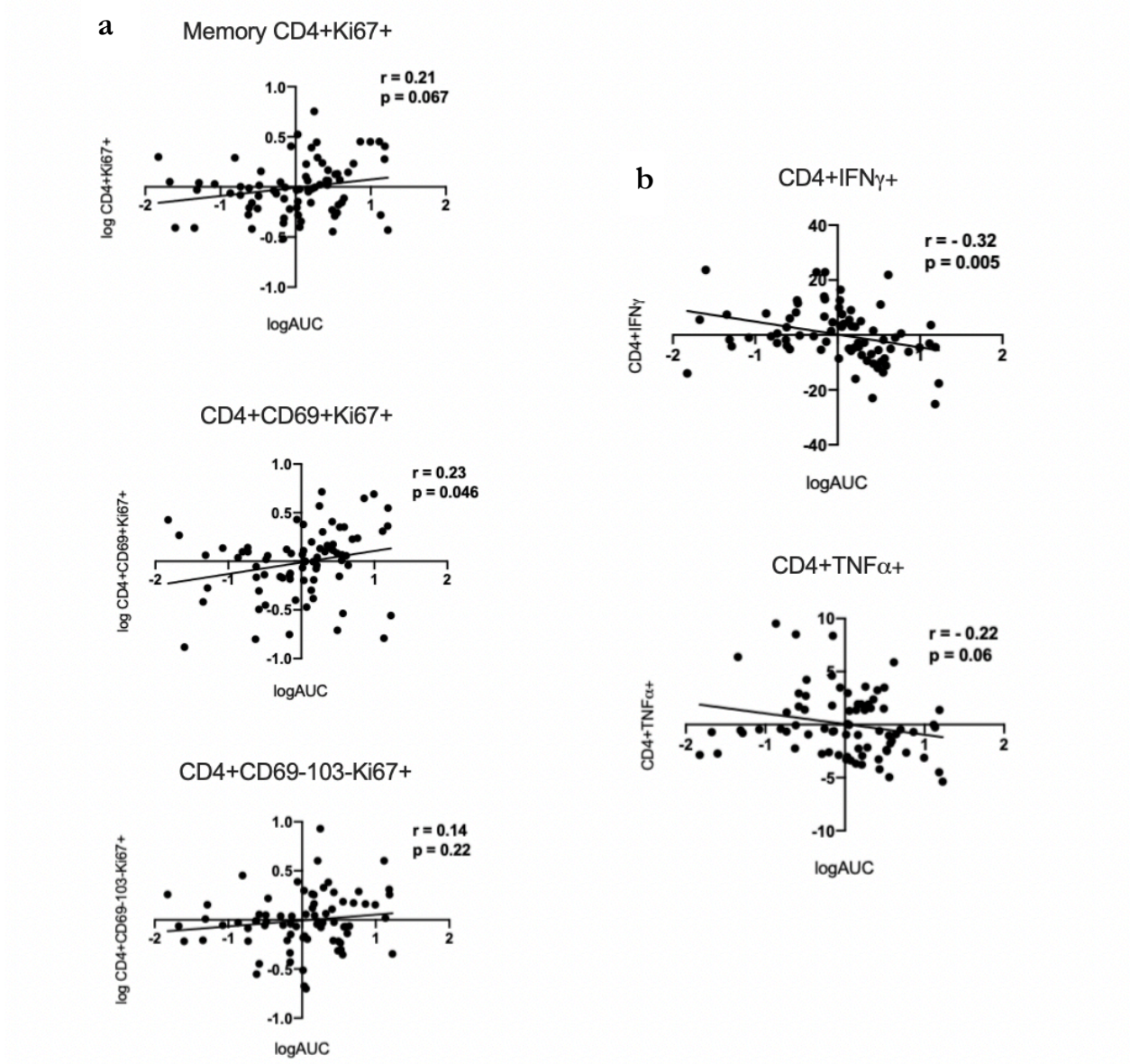
Abbreviations: YMSM, adolescent men who have sex with men, AMSM, adult men who have sex with men, Controls, control males not engaging in anal intercourse. P-values shown are significant at <0.05 for Mann Whitney U test for differences in median expression between groups.

Figure 5: A comparison of HIV viral replication from the HIV-1 explant challenge experiments between YMSM, AMSM and Control males. (a) Longitudinal normalized median p24 curves demonstrate that YMSM have higher median p24 concentrations on days 3 through 14 post-infection compared to older males. (b) A comparison of median logAUC values between groups shows higher viral replication among YMSM compared to adult MSM.



Abbreviations: YMSM, young men who have sex with men, AMSM, adult men who have sex with men, Controls, control males not engaging in anal intercourse, AUC, area under the curve. P-values shown are significant at <0.05 for Mann Whitney U test for differences in median logAUC values between groups.

Figure 6: Ki67 expression by memory CD4+ T cell subsets and pro-inflammatory cytokine-producing CD4+ T cells are associated with viral replication in the explant challenge model. (**a**) Ki67-expressing CD4+ T cell subsets demonstrated a trend toward a positive correlation with viral replication (median logAUC). (**b**) Higher frequencies of pro-inflammatory cytokine-producing CD4+ T cells was negatively associated with viral replication (median logAUC) from the HIV-1 explant challenge model.



Abbreviations: r, correlation coefficient, AUC, area under the curve. P-values significant at < 0.05 for Spearman rank-order correlation between frequency of CD4+ T cell subset and median logAUC.

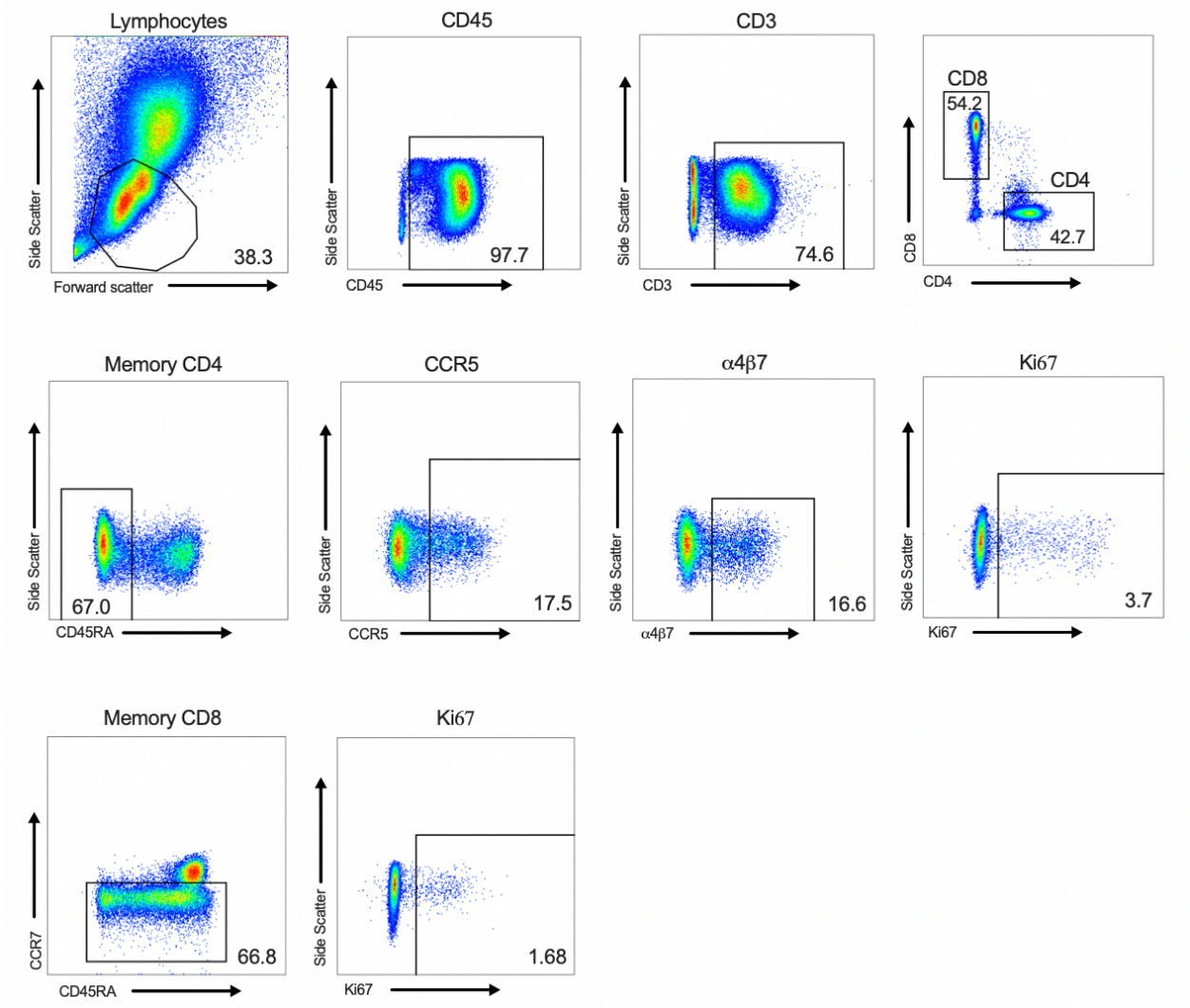
Supplementary Table 1: The distribution of peripheral blood CD4+ and CD8+ memory T cell subsets among YMSM, AMSM and control males.

Immunological index	*n*	Median (25^{th}, 75^{th})	*P-value (vs YMSM)*
Blood memory CD4 + cells			
% $CD4^+$ memory			
YMSM	32	59.5 (52.3, 68.0)	--
AMSM	33	71.6 (61.7, 81.1)	**0.008**
Control males	11	77.4 (55.3, 83.8)	0.07
% $CD25^+$ $FoxP3^+$			
YMSM	32	3.1 (2.4, 3.7)	--
AMSM	33	3.4 (2.5, 4.2)	0.54
Control males	11	2.6 (2.4, 2.9)	0.06
% $\alpha4\beta7^+$			
YMSM	32	8.7 (6.1, 12.6)	--
AMSM	33	7.4 (4.6, 8.8)	0.07
Control males	11	8.3 (4.3, 9.4)	0.42
% $CCR5^+$			
YMSM	32	11.6 (9.3, 14.5)	--
AMSM	33	15.0 (13.2, 19.5)	**0.005**
Control males	11	10.4 (7.7, 20.7)	0.98
% $Ki67^+$			
YMSM	32	2.5 (1.9, 3.4)	--
AMSM	33	2.2 (1.5, 2.6)	**0.03**
Control males	11	1.4 (1.3, 1.7)	**0.0002**
% $CCR5^+$ $Ki67^+$			
YMSM	32	1.0 (0.6, 1.5)	--
AMSM	33	0.9 (0.6, 1.1)	0.30
Control males	11	0.5 (0.4, 0.9)	**0.03**
Blood memory $CD8^+$ cells			
% $CD8^+$ memory			
YMSM	32	77.2 (64.6, 89.6)	--
AMSM	33	80.2 (76.0, 90.2)	0.14
Control males	12	79.7 (59.0, 86.1)	0.87
% Ki67+			
YMSM	32	1.6 (0.9, 2.4)	--
AMSM	33	1.3 (1.0, 2.2)	0.71
Control males	12	1.1 (0.9, 2.0)	0.34
Stimulated blood $CD4^+$ cells			
% $IL\text{-}17A^+$			
YMSM	32	1.6 (1.0, 2.6)	--
AMSM	33	1.2 (0.9, 2.9)	0.57
Control males	11	1.6 (0.9, 2.6)	0.65
% $IFN\gamma^+$			
YMSM	32	17.7(10.0, 23.0)	--
AMSM	33	26.1 (14.9, 41.9)	**0.004**
Control males	11	28.4 (18.0, 40.7)	**0.02**
% $TNF\alpha^+$			
YMSM	32	1.8 (1.2, 3.3)	--

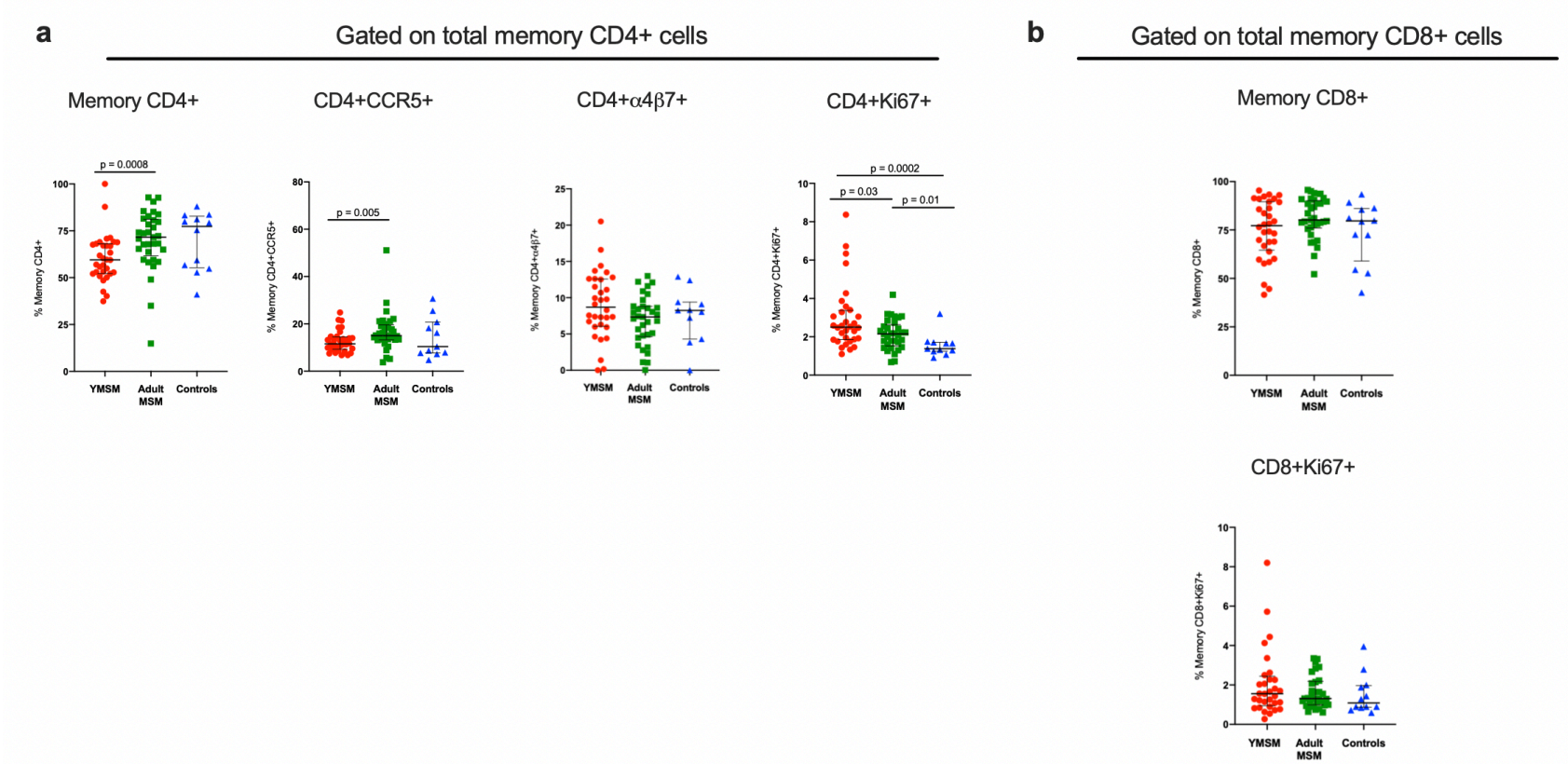
AMSM	33	3.3 (2.6, 5.6)	**0.0002**
Control males	11	4.2 (1.8, 6.3)	**0.02**
Stimulated blood CD8⁺ cells			
% IFNγ⁺			
YMSM	32	51.5 (35.2, 62.5)	--
AMSM	33	60.1 (54.2, 77.1)	**0.009**
Control males	12	66.8 (33.5, 77.9)	0.11
% TNFα⁺			
YMSM	32	5.2 (2.3, 10.5)	--
AMSM	33	8.1 (4.5, 12.4)	0.10
Control males	12	7.5 (4.3, 13.2)	0.21

Abbreviations: YMSM, young men who have sex with men, AMSM, adult men who have sex with men. Bolded items are significant *P*-values (<0.05) for Mann Whitney U test for differences in median cell subset frequencies between study groups.

Supplementary Figure 1: Representative gating strategy for blood CD4+ and CD8+ mononuclear cells. Representative gating strategy for blood mononuclear cells. Lymphocytes were first identified by forward and side scatter. Next, CD45+ cells were isolated followed by the CD3+ subset. This subset was then divided into CD4+ and CD8+ subsets. Memory CD4 cells were identified by excluding CD45RA+ cells. For CD8 + cells, the memory CD8+ population was designated as $CCR7^{-}$ and $CD45RA^{+/-}$. Memory CD4+ T cells were then assessed for expression of CCR5, α4β7, and Ki67. Memory CD8+ T cells were evaluated for expression of Ki67.

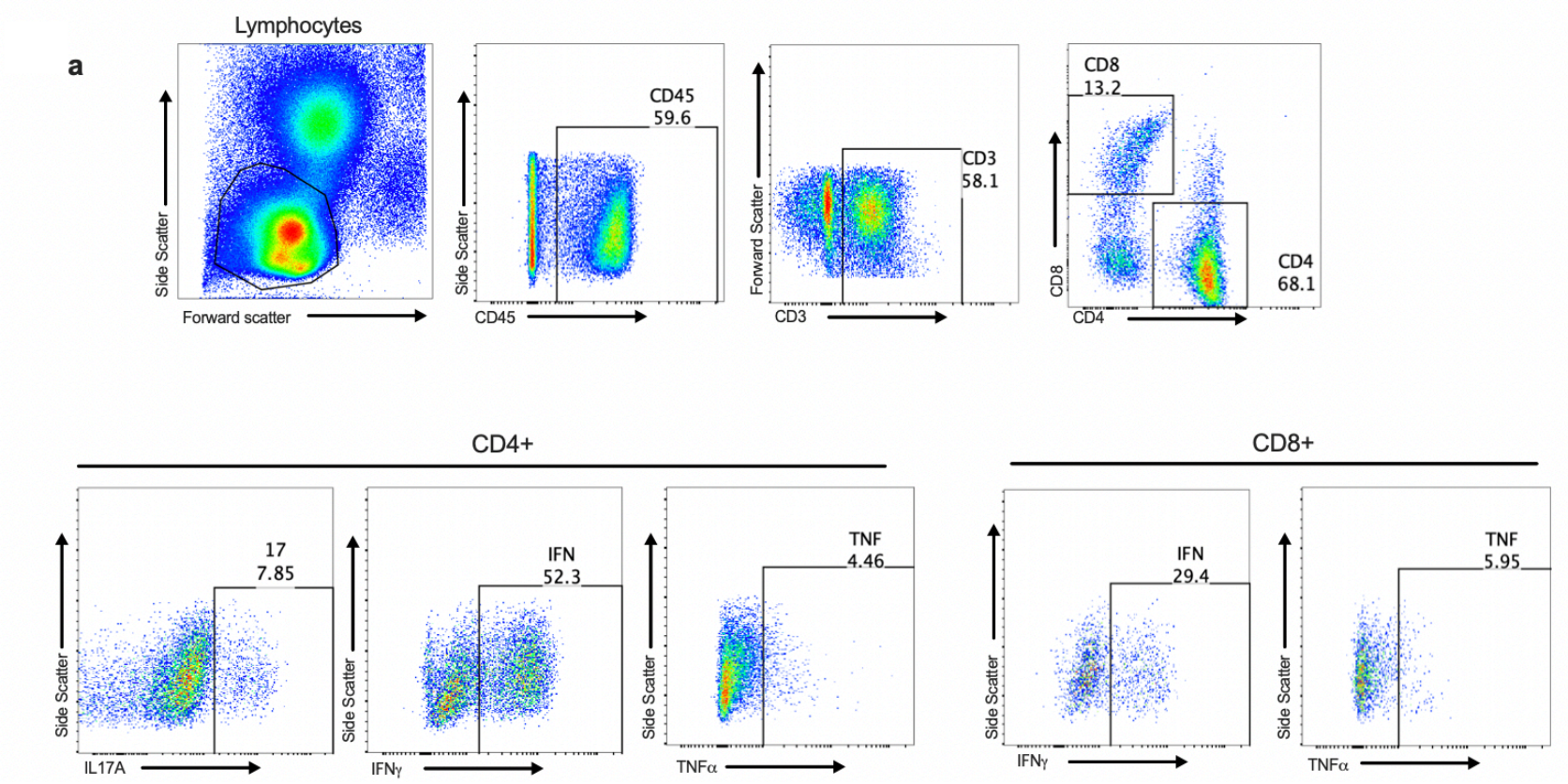


Supplementary Figure 2: Distribution of peripheral blood mononuclear CD4+ and CD8+ T cells among YMSM, AMSM, and control males. (**a**) Results of PBMC CD4+ cell phenotyping for YMSM, AMSM, and Controls. (**b**) Results of CD8+ cell phenotyping among YMSM, AMSM and controls. Black horizontal lines and vertical ranges represent the median and interquartile range as reported in **Supplementary Table 1**.



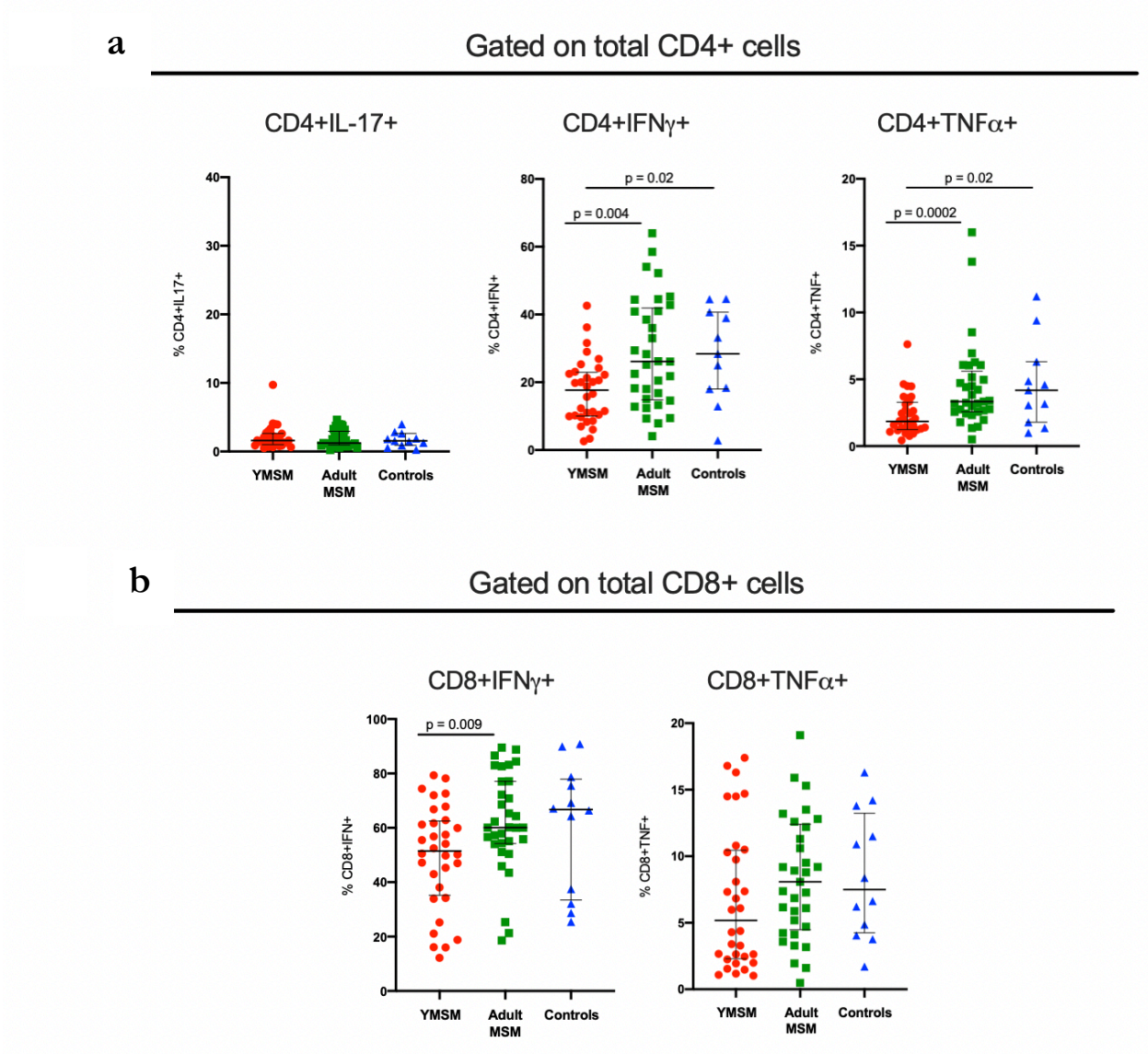
Abbreviations: PBMC, peripheral blood mononuclear cells, YMSM, adolescent men who have sex with men, AMSM, adult men who have sex with men, Controls, control males not engaging in anal intercourse. P-values shown are significant at <0.05 for Mann Whitney U test for differences in median expression between groups.

Supplementary Figure 3: Representative gating strategy to detect cytokine-positive peripheral blood mononuclear CD4+ and CD8+ T cells. Peripheral blood mononuclear cells were stimulated for 4 hours with PMA/Ionomycin and stained for indicated cytokines. Live cells were identified by live/dead staining (not shown) and lymphocytes were identified by forward and side scatter. CD45+ cells were identified, followed by CD3+ cells which were separated into CD4+ and CD8+ subsets. Stimulated CD4+ and CD8+ cells were assessed for indicated cytokine production.



Abbreviations: PMA, phorbol myristate acetate.

Supplemental Figure 4: Cytokine production on mitogen-stimulated peripheral blood CD4+ and CD8+ mononuclear cells demonstrates a trend toward lower levels of pro-inflammatory cytokine production in YMSM compared to older males. (**a**) Results of peripheral blood cytokine-positive CD4+ T cells for YMSM, AMSM, and Controls. (**b**) Results of peripheral blood cytokine-positive CD8+ T cells for YMSM, AMSM, and Controls. Black horizontal lines and vertical ranges represent the median and interquartile range as reported in **Supplemental Table 1**.



Abbreviations: YMSM, adolescent men who have sex with men, AMSM, adult men who have sex with men, controls, control males not engaging in anal intercourse. P-values shown are significant at <0.05 for Mann Whitney U test for differences in median expression between groups.