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Anthony Bennici Date

The Expression and Distribution of IL-17 and FOXP3 in the Rectal Mucosa of HIV-Negative MSM Engaging in Condomless Receptive Anal Intercourse

By

Anthony K. Bennici
Degree to be awarded: Master of Public Health

Epidemiology

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Dr. Colleen F. Kelley
Committee Chair

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By

Anthony K. Bennici

B.S.
College of William and Mary
2015

Thesis Committee Chair: Colleen F. Kelley, MD, MPH

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Abstract

The Expression and Distribution of IL-17 and FOXP3 in the Rectal Mucosa of HIV-Negative MSM Engaging in Condomless Receptive Anal Intercourse

By Anthony K. Bennici

In 2017, approximately 66% of new HIV infections in United States occurred in men who have sex with men (MSM). The majority of infections among MSM occur through exposure to the rectal mucosa, and few studies have investigated the effect of condomless receptive anal intercourse (CRAI) on the rectal mucosal immune environment. We assessed the expression and distribution of biomarkers associated with HIV target cell populations and inflammation based on CRAI. A cohort of 41 HIV-negative MSM engaging in CRAI and 21 HIV-negative men who never engaged in anal intercourse were recruited from the Atlanta community and enrolled in the study. We characterized the expression, measured in optical density, of IL-17, a pro-inflammatory cytokine produced by T helper cells, and FOXP3, a transcription factor of T regulatory cells, in the rectal mucosa using standardized automated immunohistochemistry and quantitative image analysis. The distributions of IL-17 and FOXP3 both showed trends of increased expression towards the colon lumen. The geometric mean of the difference in IL-17 expression between the upper 40% and middle 40% of the lamina propria was 57.8 (95% CI: 40.4, 82.7, p <0.001) and the geometric mean of the difference in FOXP3 expression between the upper 40% and the middle 40% of the lamina propria was 26.9 (95% CI: 22.6, 32.0; p <0.001). The upper 40% of the lamina propria accounted for a larger proportion of expression compared to the middle 40% for both biomarkers. Modeling results did not determine any statistically significant difference in overall IL-17 or FOXP3 expression between MSM engaging in CRAI and controls, though we detected an increase in IL-17 expression in MSM after engaging in CRAI. Our findings suggest that IL-17 and FOXP3 expression in the lamina propria of the rectal mucosa is concentrated towards the colon lumen regardless of engagement in CRAI. The localization of these regulators of inflammation towards the colon lumen and the increase in IL-17 expression in MSM after engaging in CRAI may influence the design of future preventative interventions against HIV.

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Background

Although new human immunodeficiency virus (HIV) infections and diagnoses have remained stable in the United States in recent years, HIV is still a major cause of morbidity and mortality. Approximately 1.1 million people were living with HIV in 2015 and over 38,500 new infections occurred in 2017 (1). Approximately 66% of these new infections occurred among men who have sex with men (MSM), making them the largest affected subpopulation in the U.S. The majority of HIV transmissions among MSM occur through exposure to the rectal mucosa and unprotected receptive anal intercourse carries the highest per exposure probability of infection among all mucosal transmission sites (2, 3).

The gastrointestinal tract functions as a barrier against the invasion of pathogenic microorganisms and prevents dissemination of intestinal microbiota. The rectal mucosa consists of a single layer columnar epithelium, lamina propria, and muscularis mucosa (Fig. 1). The epithelium forms crypts that are tightly packed parallel to each other, opening into the colon lumen and resting on a thin basement membrane which extends to the muscularis mucosa. The muscularis mucosa is an incomplete layer of smooth muscle cells that defines the lower boundary of the mucosa and submucosa. The lamina propria is a thin layer of loose connective tissue that lies between the epithelium and the muscularis mucosa.

Lymphocytes are a major component of the gut mucosa and are located between epithelial cells and in the lamina propria (4). Several subsets of T cells, including T helper (Th) cells, γδT cells, and natural killer (NK) T cells, reside in the mucosa and together contribute to the mucosal response to pathogens. T lymphocytes in the lamina propria consist of primarily CD4+ Th cells and CD8αβ+ T cells (5). CD4+ Th cells play

an important role in adaptive immunity by releasing cytokines that regulate immune responses. CD4 is also one of the co-receptors for HIV, and thus many of these lymphocytes in the mucosa are primary target cells for HIV infection. Untreated HIV infection is characterized by the depletion of CD4+ Th cells and progressive immune system dysregulation (6). The distal section of the rectum, which is exposed during receptive anal intercourse, contains an abundance of CCR5-expressing macrophages that are particularly vulnerable to HIV infection (7).

One of the more recently discovered subsets of CD4+ cells are Th17 cells, which are characterized by the production of pro-inflammatory cytokines, including interleukin (IL) -17, IL-22, and IL-26 (4). Th17 cells are also involved in epithelial regeneration and mucosal integrity. Th17 differentiation is controlled by transcription factor RORγt, which is specific for the Th17 lineage and induces transcription of the genes encoding IL-17 (8). Although IL-17 production has been most closely associated with Th17 cells, it is also produced by γδT and NK cells. As these cells are part of the innate immune response, their production of IL-17 may actually precede and in some cases may be more dominant than Th17 CD+ T cells (9). Deficient production of IL-17 may lead to increased susceptibility to bacterial and fungal infections (6). However, excessive production of IL-17 may result in autoimmunity and pathogenic inflammatory conditions. IL-17 also promotes neutrophil accumulation and protects mucosal barrier functions due to stimulation of tight junction formation and mucin secretion (10). A previous study of the rectal mucosa of MSM engaging in condomless receptive anal intercourse showed higher levels of Th17 cells, greater CD8+ T cell proliferation, and production of pro-inflammatory cytokines, compared to men who never engaged in receptive anal

intercourse, indicating IL-17 could be a useful biomarker to further the understanding of the immune environment in the rectal mucosa (11).

Another subset of CD4+ T cells include regulatory T (Treg) cells, whose development and function are controlled by transcription factor forkhead box protein P3 (FOXP3). Treg cells are important in suppressing autoreactive cells as well as limiting immune activation during infection (12). CD4+CD25+ Treg cells express both CXCR4 and CCR5 coreceptors and are also susceptible to HIV infection (13). FOXP3 is able to override other developmental T-cell pathways, including Th17, and is a determinant of functional differentiation and maintenance of Treg cells (14). FOXP3+ Treg cells secrete anti-inflammatory cytokines, express co-inhibitory molecules, and can modulate the activity of antigen-presenting cells (15). The mutation of the FOXP3 gene that renders it non-functional has been shown in both mice and humans to be associated with immune dysregulation and the inability to maintain suppressive functions over pro-inflammatory cytokines (15).

The lamina propria contains both Th17 and Treg cells, and each play important roles in maintaining gut inflammation. Under normal conditions, the lamina propria is enriched for both FOXP3+ Treg and Th17 cells, and the balance between pro- and anti-inflammatory responses is largely regulated by transforming growth factor (TGF) –β (16). TGF-β induces Th17 differentiation in the presence of the pro-inflammatory cytokine IL-6, whereas retinonic acid secreted by mucosal dendritic cells will enhance Treg induction and inhibits Th17 development (10, 17, 18, 19). The accumulation of Th17 and Treg cells in the lamina propria may also be partly regulated by commensal bacteria. The presence of commensal bacteria can induce Th17 and the production of IL-17 while limiting Treg conversion (20, 21).

It is necessary to better characterize the immune environment of the rectal mucosa to understand the mechanisms by which HIV can cause infection through rectal exposure and the risks for men engaging in condomless receptive anal intercourse (CRAI). While the 2017 Kelley et. al. study described higher levels of Th17 cells and the production of pro-inflammatory cytokines, the techniques were unable to provide an indication of where the cells were distributed throughout the rectal mucosa. We utilized quantitative immunohistochemistry techniques to measure the expression of IL-17 and FOXP3 in rectal mucosa biopsies to better understand the location and distribution of susceptible cells as well as the concentrations of inducers and suppressors of inflammation. Together, we aim to provide a more comprehensive assessment of the immune environment of the rectal mucosa in MSM who engage in CRAI to describe factors that may contribute to the increased risk of transmission.

Methods

The clinical cohort.

HIV-negative MSM aged 18-45 years in good health who were engaging in condomless receptive anal intercourse with an HIV-negative partner were recruited from the Atlanta community. HIV-negative men aged 18-45 years in good health who had never engaged in anal intercourse, regardless of sexual orientation, were recruited as controls. Inclusion criteria for MSM engaging in CRAI included HIV-negative status, being in a monogamous relationship for at least 45 days with an HIV-negative man, and reporting a minimum of four episodes of CRAI in the last month. MSM engaging in CRAI were also asked to keep an electronic or paper sex diary for the duration of the study that recorded each episode of receptive anal intercourse, whether a condom was used, whether ejaculation occurred during penetrative intercourse, type of lubricant used (if any), and type of enema used (if any). Men in both study groups who were determined by the principal investigator to be high risk for rectal biopsy procedures due to medical comorbidities or who intended to take pre-exposure prophylaxis during the study were not enrolled.

The study consisted of three visits. During the screening study visit, eligible participants provided informed consent, underwent a brief sexual and medical history and physical examination, rapid HIV testing, and blood collection. The next study visit (visit 1) occurred 1–6 weeks later (median 2.6 weeks) and participants underwent peripheral blood and rectal biopsy sampling via rigid sigmoidoscopy with no prior bowel preparation. All biopsies were collected approximately 3–10 cm from the anal verge. MSM who were engaging in CRAI were asked to abstain from CRAI for at least 72 hours before study visit 1. The final study visit (visit 2) occurred 8–16 weeks after study visit 1

(median 9 weeks) and participants again underwent peripheral blood and rectal biopsy sampling. The intestinal epithelium is a rapidly self-renewing tissue and when injury occurs, it is quickly repaired (22). We hypothesized that some effects of CRAI may be transient; therefore, MSM engaging in CRAI were asked to engage in CRAI within 24 hours of study visit 2. During each study visit, participants were also tested for rectal STIs, including chlamydia, gonorrhea, and shedding of herpes simplex virus 2. The Institutional Review Board (IRB) at Emory University approved this study.

Rectal biopsy collection and biomarker quantification.

Biopsies were collected from normal-appearing rectal mucosa at baseline and the final study visit without any prior bowel preparation. Details of the rectal biopsy procedure and immunohistochemistry protocol were previously published (23). Briefly, three approximately 1 mm thick biopsy specimens were taken from the rectal mucosa 3-10 cm from the anal verge via rigid sigmoidoscope. Biopsies were placed onto a strip of bibulous paper and immediately placed in PBS, oriented, transferred to 10% normal-buffered formalin for 24 hours, and then transferred to 70% ethanol. Within a week, biopsies were processed and embedded in paraffin blocks with three biopsies per block. For each biomarker, five slides with three levels of 3 µm-thick biopsy sections taken 40 µm apart were prepared, yielding a total of 15 levels per participant per visit. Antigen retrieval was performed in a Lab Vision™ PT Module (Lab Vision Corp., Fremont, CA) in a 1X citrate buffer solution (Thermo Scientific, Cat. #TA-250-PM1X, Waltham, MA). Slides were immunohistochemically processed using a Dako Autostainer Plus Staining System (Agilent, Santa Clara, CA) by applying anti-IL-17 antibody, polyclonal goat antibody (R&D System, Cat. # AF-317-NA, dilution 1:100, Minneapolis, MN) and anti-FOXP3 antibody, mouse monoclonal antibody (Abcam, Cat. #ab20034, dilution 1:300,

Cambridge, UK). For IL-17 processing, donkey anti-goat IgG HRP (Abcam, Cat. # ab6885, dilution 1:250, Cambridge, UK) was used as the secondary antibody. Pre-made solution UltraVision Large Volume Detection System (Thermo Scientific, Cat. #TP-125-HL, Waltham, MA) was used for the detection of FOXP3. Baseline and follow-up biopsy slides for individual study participants were included in the same immunohistochemistry batch, and each batch included a balance of participants from each study group.

A quantitative image analysis method ("scoring") was used to evaluate the biomarker expression in the rectal biopsies. Digital images of baseline and follow-up slides for the 62 participants were reviewed using CellularEyes software (DivEyes LLC, Atlanta, GA). Technicians blinded to study group identified only "scorable" hemicrypts, which were defined as one side of a crypt bisected from base to colon lumen surface, intact, and extending from the muscularis mucosa to the colon lumen. However, the biomarkers of interest, IL-17 and FOXP3, are expressed in lymphocytes that exist in the lamina propria, not the colon crypts (24). Thus, scoring for these biomarkers occurred in the lamina propria adjacent to scorable hemicrypts. If the width of the lamina propria between a scorable hemicrypt and other crypts was less than the width of the cells in the crypt, the region was not scored. The width of the area in the lamina propria was approximated to equal the width of half of the scorable crypt. Once a scorable crypt and appropriate lamina propria region was identified, the technician traced the borders of the lamina propria using a digital drawing board. Starting at the middle of the base of the crypt while avoiding any epithelial cells, the technician traced along the curve of the hemicrypt up to the epithelium and colon lumen. Keeping the width of the selected region relatively consistent, the technician would then trace through the lamina propria

back towards the base of the hemicrypt and the muscularis mucosa. The program then divided the outlined area into 50 equally-spaced segments ("bins") and measured the background-corrected optical density (OD) of the biomarker labeling across the full length of the hemicrypt, as well as within each bin. For each biomarker, the technician identified and outlined scorable lamina propria regions until at least 8 and at most 40 regions were scored per participant per visit.

Statistical analysis.

Demographic and clinical characteristics were compared between study groups (MSM engaging in CRAI and controls) with Wilcoxon rank-sum test for continuous variables. Observations that contained fewer than three scorable hemicrypts per participant per visit and observations in which the participant mean biomarker density sum (primary measure of biomarker expression) was at least three standard deviations away from the visit mean biomarker density sum were excluded from analysis. The distribution of mean biomarker density sums were right-skewed by study group and visit number and were log-transformed for modeling analysis to produce geometric means. To compare the ratio of IL-17 to FOXP3 expression, the cleaned IL-17 and FOXP3 datasets were merged by participant identification number (ID). Merged observations that did not contain mean biomarker density sums for both IL-17 and FOXP3 by participant ID and visit number were also excluded.

We plotted the distribution of expression of each biomarker through the length of the lamina propria based on median biomarker density sum in each of the 50 equally spaced bins created by the scoring software. These sections began with Bin 1 in the area adjacent to the base of a crypt and the muscularis mucosa and ended with Bin 50 adjacent to the lumen epithelium. The average of the medians of each bin across the lamina

propria was predicted by the LOESS procedure and plotted. Geometric means of the bins could not be calculated due to the presence of zeros for some observations, and the medians provided the most similar estimates. The area under the curve (AUC) for these distributions was calculated to provide a cumulative comparison of the difference between the upper and the lower regions of the lamina propria. The first 10 scored bins were dropped for these calculations, to create an equal number of bins when comparing the upper and lower regions. The new region created by dropping the lowest 10 bins from the lower 60% (bins 1-30) will be referred to as the middle 40% (bins 11-30) of the lamina propria. Biomarker distributions and areas under the curves were evaluated with RStudio (Version 3.5.2, including packages "rio", "ggplot2", "MESS", and "cowplot").

In conjunction with biomarker expression distribution plots, we compared the difference in the geometric means of the biomarker density sums in the upper 40% (bins 31-50) of the lamina propria compared to the middle 40% using a paired sample t-test on the log-transformed values. Additionally, we calculated the percent of total expression, excluding the lowest 10 bins, that was contributed by the upper 40% of the scored regions of the lamina propria [(sum of upper bins 31-50) / (sum of middle and upper bins 11-50)].

To evaluate the longitudinal relative effects between MSM engaging in CRAI and controls, repeated-measures analyses for differences in IL-17 and FOXP3 expression were performed with a means model via the SAS MIXED Procedure (version 9.4), providing separate estimates of the geometric means by time on study (baseline and follow-up) and study group (MSM engaging in CRAI or controls). The model included six predictors: study group, time on study, the statistical interaction between study group and time on study, age, race, and the batch in which the assay was performed. We evaluated changes in expression of IL-17, FOXP3, and the ratio of IL-17/FOXP3 in the

full length of the lamina propria as well as the lower 60% (adjacent to the bottom of scorable crypts and the muscularis mucosa) and the upper 40% (adjacent to the epithelium and colon lumen). We decided *a priori* to use these within-mucosa distributions of the biomarkers (upper 40%, differentiation zone; lower 60%, proliferation zone) because they represent the ratios of well-recognized functional or exposure zones in colon crypts (25). Relative and absolute study group effects were calculated: relative = [(CRAI follow-up geometric mean) / (CRAI baseline geometric mean)] / [(control follow-up geometric mean) / (control baseline geometric mean)], absolute = [(CRAI follow-up geometric mean) – (CRAI baseline geometric mean)] – [(control follow-up geometric mean) – (control baseline geometric mean)]. A relative effect of 1.25 would indicate a 25% increase in biomarker expression in the CRAI group relative to the control group from baseline to follow-up.

A priori-selected biologically plausible confounders were assessed to determine if there were any imbalances in their distribution across treatment groups. White blood cell count, hemoglobin concentration, and the number of days between biopsies were not found to differ significantly between treatment groups and inclusion in the models did not materially affect the estimated treatment effects. Other potential variables of interest, including sex frequency per week, lube use, and enema use were only collected for MSM engaging in CRAI and were not included in the models. The use of lubricants could not be examined given that nearly all MSM engaging in CRAI reported lubricant use. A two-sided p-value ≤ 0.05 was considered statistically significant.

Results

The clinical cohort baseline characteristics.

Selected baseline characteristics of the biomarker study participants are presented in Table 1. Forty-one HIV-negative MSM engaging in CRAI with an HIV-negative partner and 21 HIV-negative men who never engaged in receptive anal intercourse (controls) were enrolled into the study, with biopsy sampling timed with acts of CRAI for MSM engaging in CRAI. For baseline biopsy visit 1, MSM engaging in CRAI were asked to abstain from CRAI for at least 72 hours before the visit. For the follow-up biopsy visit 2, MSM engaging in CRAI were asked to engage in CRAI within the 24 hours preceding the visit. The median age of MSM engaging in CRAI was slightly higher than controls (28 and 24 years, respectively). The majority of participants in both groups were white (80.5% MSM engaging in CRAI, 66.7% controls). During study visit 1, one MSM engaging in CRAI was diagnosed with rectal chlamydia and one was positive for herpes simplex virus type 2 shedding. During study visit 2, three MSM engaging in CRAI were diagnosed with rectal chlamydia, one with rectal gonorrhea, and one was positive for herpes simplex virus type 2 shedding.

The size of the analyzed dataset varied by biomarker, and the number of participants for each study group and study visit are shown in Table 2. No scorable hemicrypts were available from either visit for five participants for both IL-17 and FOXP3. Participant observations were excluded from analysis if there were no available biopsies, if fewer than three scorable hemicrypts per participant per visit were observed, or if the participant's mean biomarker density sum was greater than three standard deviations from the visit mean biomarker density sum.

Distribution of biomarkers through the lamina propria.

The distributions of IL-17 and FOXP3 staining densities ("expression") through the lamina propria for men remaining after exclusion procedures are shown in Figures 2-4. Both biomarkers show trends of increased expression towards the colon lumen (Fig. 2). Among MSM, IL-17 expression throughout the lamina propria appears to increase after engaging in CRAI (AUC Follow-up – AUC Baseline = 16.32) (Fig.3). There did not appear to be any appreciable difference in FOXP3 expression from baseline to follow-up among MSM (AUC Follow-up – AUC Baseline = 5.18) (Fig.4). FOXP3 expression appears to increase from baseline to follow-up among controls, though there is considerable variation due to limited sample size.

The geometric mean of the difference in IL-17 expression between the upper 40% and middle 40% of the lamina propria was 57.8 (95% CI: 40.4, 82.7; p <0.001) (Fig.2). The geometric mean of the difference in FOXP3 expression between the upper 40% and middle 40% of the lamina propria was 26.9 (95% CI: 22.6, 32.0; p <0.001) (Fig.2). Additionally, the upper 40% accounted for approximately 59.3% (95% CI: 57.6, 61.0) of the IL-17 expression and 70.3% (95% CI: 67.8, 72.8) of the FOXP3 expression, excluding the first 10 bins (Fig.2).

Follow-up results.

Using regression models controlling for study group, time on study, the interaction between study group and time on study, age, race, and the batch in which the assay was performed, we produced estimated relative and absolute effects on IL-17 and FOXP3 expression comparing MSM engaging in CRAI and controls between baseline and follow-up. In the CRAI group relative to the control group, IL-17 expression was an estimated 14% higher (p = 0.42; Table 2) in the full length of the crypts. Expression of

IL-17 was 25% higher ($p = 0.23$, Table 2) in the lower 60% of the lamina propria, but there was a minimal, not-statistically significant increase in IL-17 expression in the upper 40% of the lamina propria in the CRAI group relative to the control group. Although the overall geometric mean of IL-17 expression increased from visit 1 to visit 2 among MSM engaging in CRAI, this change was not significant ($p = 0.32$).

There was minimal, statistically not-significant change in FOXP3 expression in the CRAI group relative to the control group in the lower 60%, upper 40%, and throughout the full length of the lamina propria from baseline to follow-up visits. The overall geometric mean FOXP3 expression also increased from visit 1 to visit 2 among MSM engaging in CRAI, but this change was not significant ($p = 0.23$).

The ratio of IL-17/FOXP3 expression suggested that OD measurements for IL-17 were approximately 4 times higher than the OD measurements for FOXP3. There was minimal, statistically not-significant change in the ratio of IL-17/FOXP3 expression in the CRAI group relative to the control group in the lower 60%, upper 40%, and throughout the full length of the lamina propria from baseline to follow-up.

Discussion

The immune environment of the rectal mucosa serves as a barrier against foreign pathogens and prevents excessive immune responses against existing microbiota. A variety of lymphocytes occupy the mucosal tissue to maintain this balance. Th cell subsets, including Th17 cells, produce pro-inflammatory responses, while Treg cells maintain homeostasis and control over excessive inflammation (5). IL-17, a cytokine produced by Th17 cells, γδT cells, and NK cells, contributes to the pro-inflammatory response against pathogens, while transcription factor FOXP3 controls the development of Treg cells and consequently, the secretion of anti-inflammatory cytokines (15, 30). Our preliminary findings suggest that the expression and distribution of IL-17 and FOXP3 in the rectal mucosa do not vary statistically significantly based on engagement in or abstention from CRAI. For both MSM and controls, we observed greater expression of both IL-17 and FOXP3 in the upper 40% of the lamina propria closest to the colon lumen epithelium.

In a previous study involving the same cohort of MSM and controls, CRAI was characterized by the expression of genes associated with mucosal injury and repair (11). Neutrophil and Th17 cell activity were most pronounced in MSM after engaging in CRAI in the 24 hours preceding the study visit. The study also found higher levels of mucosal CD8+ T cell proliferation and pro-inflammatory cytokine production in the rectal mucosa and a distinct microbiota enriched for *Prevotellaceae*, which did not differ based on the timing of CRAI, suggesting that CRAI may be associated with chronic changes in the rectal mucosal immune environment. Our findings did not show any statistically significant difference in IL-17 levels based on engagement in CRAI. Although Th17 cells are known as the major source of IL-17, γδT cells and NK cells also express IL-17

to mediate pro-inflammatory immune responses (30). The IL-17 that we observed through these immunohistochemistry studies may not necessarily be associated with the Th17 cells previously observed.

We also investigated the distribution of IL-17 and FOXP3 within the lamina propria, from the border with the muscularis mucosa to the colon lumen. Our findings suggest that in both MSM engaging in CRAI and men that do not engage in CRAI, IL-17 and FOXP3 are enriched towards the luminal surface. This observation is consistent with our hypothesis that these immune regulators would have greater expression by the colon lumen, as the epithelial layer is subject to potential mechanical trauma and is the first line of defense against infection. Additionally, overall IL-17 levels were shown to increase among MSM following CRAI. CRAI could potentially influence inflammation in the rectal mucosa in response to the mechanical trauma, the presence of semen, or changes in the rectal microbiota. Although the geometric means of baseline and follow-up were not statistically significantly different, larger sample sizes may increase the statistical power to determine the true effect of CRAI on IL-17 expression.

This study had several strengths and limitations. The strengths include: novel application of image analysis software to quantify biomarker distributions in the rectal mucosa, follow-up on prior study to characterize the rectal immune environment, and the ability to visualize the distribution of HIV target cell populations with rectal mucosa. This was the first study to use immunohistochemistry to investigate the rectal immune environment in HIV-negative MSM and men who have never engaged in AI. The study was constrained by relatively small sample size, which limited the statistical power to detect small differences in biomarker expression and to conduct subgroup analyses. The majority of study participants were white, reducing our ability to detect any biomarker

differences that may occur by race. IL-17 and FOXP3 can also be produced by more than one type of cell, so this analysis does not reveal what specific types of cells were stained and scored. Additionally, 20% of the data from the area closest to the muscularis mucosa was dropped from the distribution plots to allow equivalent comparisons of the upper and lower regions of the lamina propria. Thus, the expression of IL-17 and FOXP3 in the lowest region of the lamina propria is still undetermined.

In summary, we have identified a trend in both MSM engaging in CRAI and men who have never engaged in AI, characterized by increased expression of IL-17 and FOXP3 in the upper region of the lamina propria closest to the colon lumen. This gradient could influence susceptibility to infection or mucosal immune response to vaccines. Further immunohistochemistry studies are ongoing to investigate the distribution of other biomarkers, including Ki67, MPO, and e-cadherin.

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Tables

Table 1. Demographic and Clinical Characteristics of 41 HIV-Negative MSM Engaging in CRAI and 21 Men Who Never Engaged in AI (controls) Included in the Study, Atlanta, GA, 2013-2015.

Characteristic	MSM engaging in CRAI (n = 41)	Men never engaged in AI (n = 21)	*P* value
Median age in years (25th, 75th)	28 (25.5, 33.9)	24 (23.5, 30.0)	0.02
Race, n (%)			
White	33 (80.5)	14 (66.7)	
Black	6 (14.6)	2 (9.5)	
Other	2 (4.9)	5 (23.8)	
Lubricant use, n (%)	39 (95.1)	NA	
Enema use, n (%)	18 (43.9)	NA	
Median CRAI episodes in previous month (25th, 75th)	5 (5, 8)	NA	

Abbreviations: AI, anal intercourse; CRAI, condomless receptive anal intercourse; MSM, men who have sex with men.

Table 2. Modeling results for comparison of IL-17 and FOXP3 expression in the lamina propria of the rectal mucosa between MSM engaging in CRAI and men who have never engaged in AI[a].

Immunologic index	Visit 1			Visit 2			Adjusted model-based Mean[b] (95% CI)		Relative Effect[c]		Absolute Effect[d]
	n	Geometric Mean[b] (95% CI)	*P*	n	Geometric Mean[b] (95% CI)	*P*		*P*	Mean (95% CI)	*P*	
IL-17											
Overall											
CRAI	32	404.2 (309.2, 528.2)		33	443.1 (340.8, 576.3)		423.2 (330.1, 542.5)				
Control	18	461.9 (325.9, 654.5)	0.50	14	443.6 (307.1, 640.8)	1.00	452.7 (324.9, 630.7)	0.71	1.14 (0.82, 1.58)	0.42	57.23
Lower 60%											
CRAI	32	181.7 (135.7, 243.4)		33	207.2 (155.7, 275.9)		194.1 (148.3, 254)				
Control	18	250.3 (171.3, 365.7)	0.14	14	227.7 (152.4, 340.2)	0.67	238.7 (166.7, 341.8)	0.30	1.25 (0.86, 1.82)	0.23	48.07
Upper 40%											
CRAI	32	190.2 (143.7, 251.7)		33	204.6 (155.5, 269.3)		197.3 (152.4, 255.3)				
Control	18	183.6 (127.6, 264.2)	0.86	14	190.2 (129.3, 279.6)	0.73	186.9 (132.5, 263.6)	0.77	1.04 (0.72, 1.49)	0.83	7.90
FOXP3											
Overall											
CRAI	31	80.0 (60.8, 105.3)		33	91.1 (69.9, 118.6)		85.4 (66.7, 109.3)				
Control	17	61.7 (42.7, 89.1)	0.19	13	67.9 (46.1, 100.1)	0.14	64.7 (46.1, 90.9)	0.11	1.03 (0.70, 1.53)	0.87	4.79
Lower 60%											
CRAI	31	28.3 (19.8, 40.3)		31	37.1 (26.5, 52.1)		32.4 (23.8, 44.1)				
Control	17	23.2 (14.5, 37.3)	0.44	13	28.4 (17.1, 46.9)	0.30	25.7 (16.8, 39.2)	0.27	1.07 (0.60, 1.91)	0.80	3.71
Upper 40%											
CRAI	31	39.9 (29.5, 53.9)		31	40.6 (30.4, 54.2)		40.3 (30.7, 52.8)				
Control	17	30.6 (20.5, 45.9)	0.23	13	32.0 (20.9, 48.9)	0.27	31.3 (21.6, 45.4)	0.19	0.97 (0.63, 1.50)	0.91	-0.64

IL-17/FOXP3[e]											
Overall											
CRAI	28	3.9 (3.4, 4.5)		29	4.0 (3.5, 4.5)		3.9 (3.5, 4.4)				
Control	17	4.5 (3.8, 5.4)	0.13	11	4.5 (3.8, 5.4)	0.16	4.5 (3.8, 5.3)	0.09	1.02 (0.84, 1.23)	0.86	0.06
Lower 60%											
CRAI	28	4.8 (3.7, 6.2)		29	4.7 (3.6, 6)		4.7 (3.8, 5.9)				
Control	17	5.9 (4.1, 8.5)	0.26	11	5.8 (4, 8.4)	0.25	5.9 (4.3, 8.1)	0.16	1.00 (0.64, 1.55)	1.00	0.02
Upper 40%											
CRAI	28	4.2 (3.5, 4.9)		29	4.5 (3.9, 5.3)		4.4 (3.7, 5.1)				
Control	17	4.9 (3.9, 6.2)	0.18	11	5.0 (4.0, 6.4)	0.37	5.0 (4.0, 6.2)	0.21	1.06 (0.85, 1.33)	0.60	0.24

Abbreviations: CI, confidence interval; CRAI, condomless receptive anal intercourse.

Results of repeated-measures modeling analysis for rectal mucosa immunohistochemistry staining for MSM engaging in condomless receptive anal intercourse (CRAI) and men who never engaged in anal intercourse (controls). MSM abstained from CRAI for > 72 hours prior to visit 1 and engaged in CRAI < 24 hours prior to visit 2. Mixed-effects models controlled for study group, time on study, time by group interactions, age, race, and the batch in which the assay was processed.

[a]The effect of study group on biomarker level was modeled using mixed linear models, implemented using PROC MIXED in SAS 9.4 (Cary, NC).

[b]Data were log-transformed; reported values are optical density geometric means.

[c]Relative effect = [(CRAI group follow-up) / (CRAI group baseline)] / [(control group follow-up) / (control group baseline)].

[d]Absolute effect = [(CRAI group follow-up) - (CRAI group baseline)] - [(control group follow-up) - (control group baseline)].

[e]Observations that did not include biomarker estimates for both IL-17 and FOXP3 for a participant by study visit were excluded.

Figures

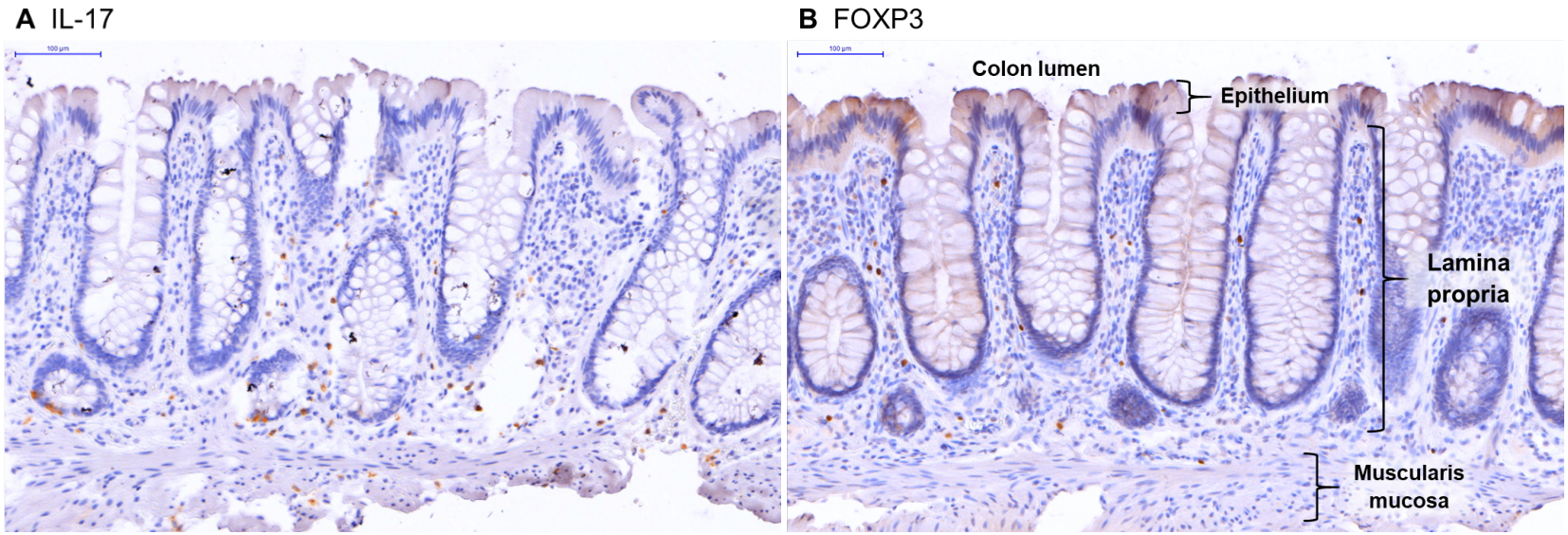

Figure 1. Representative image of rectal mucosa immunohistochemically processed for (**A**) IL-17 and (**B**) FOXP3. Scale bars indicates 100 µm. Cells stained brown are considered positive for the respective biomarker. Layer composition of mucosa labeled in (**B**).

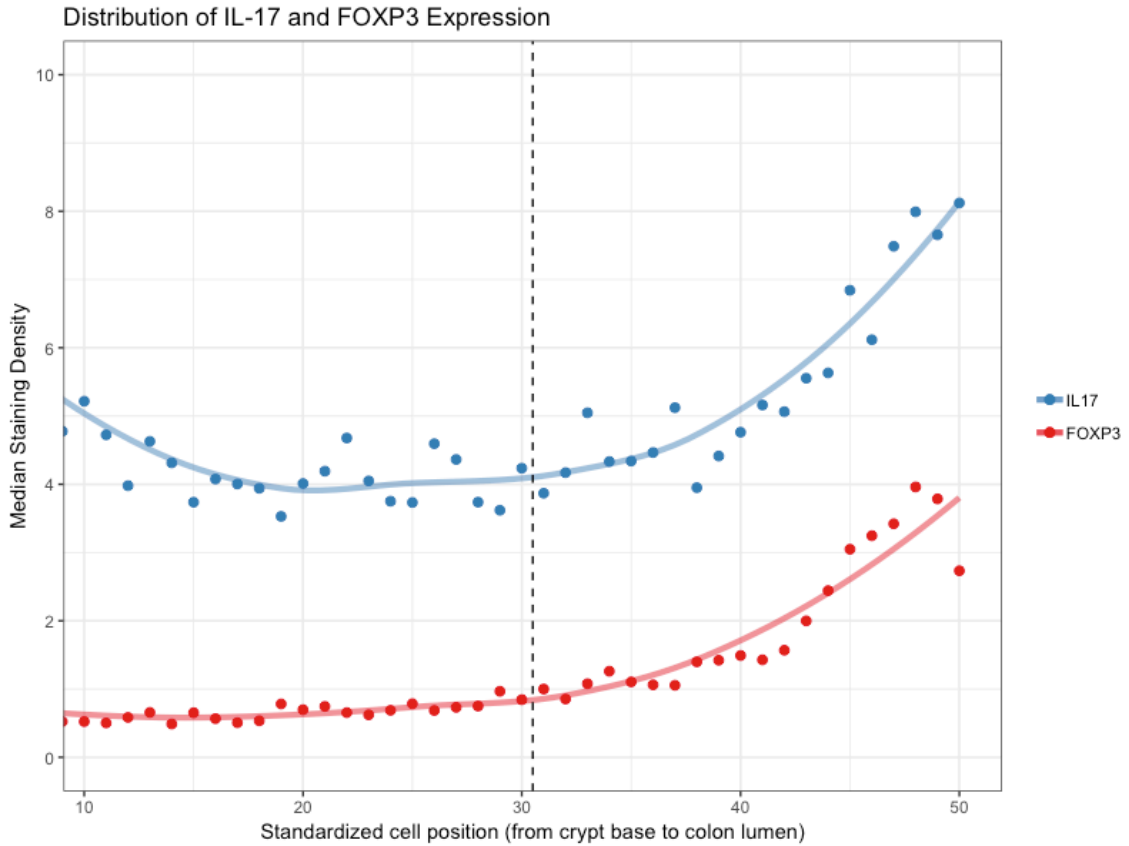

Figure 2. Distribution of median IL-17 (n = 97) and FOXP3 (n = 94) biomarker staining densities, including both visits and both study groups together for each biomarker. The scoring program divided the scorable area into 50 equally-spaced segments ("bins"). The first 10 bins were excluded from the plot to allow equivalent comparisons of the upper (bins 31-50) and middle (bins 11-30) regions of the lamina propria. The average of the medians of each bin was predicted by the LOESS procedure. The difference of the geometric means of IL-17 expression between the upper and middle regions of the lamina propria was 57.8 (95% CI: 40.4, 82.7; p <0.001). The difference of the geometric means of FOXP3 expression between the upper and middle regions of the lamina propria was 26.9 (95% CI: 22.6, 32.0; p <0.001). The upper region accounted for approximately 59.3% (95% CI: 57.6, 61.0) of IL-17 expression and 70.3% (95% CI: 67.8, 72.8) of FOXP3 expression, excluding the first 10 bins.

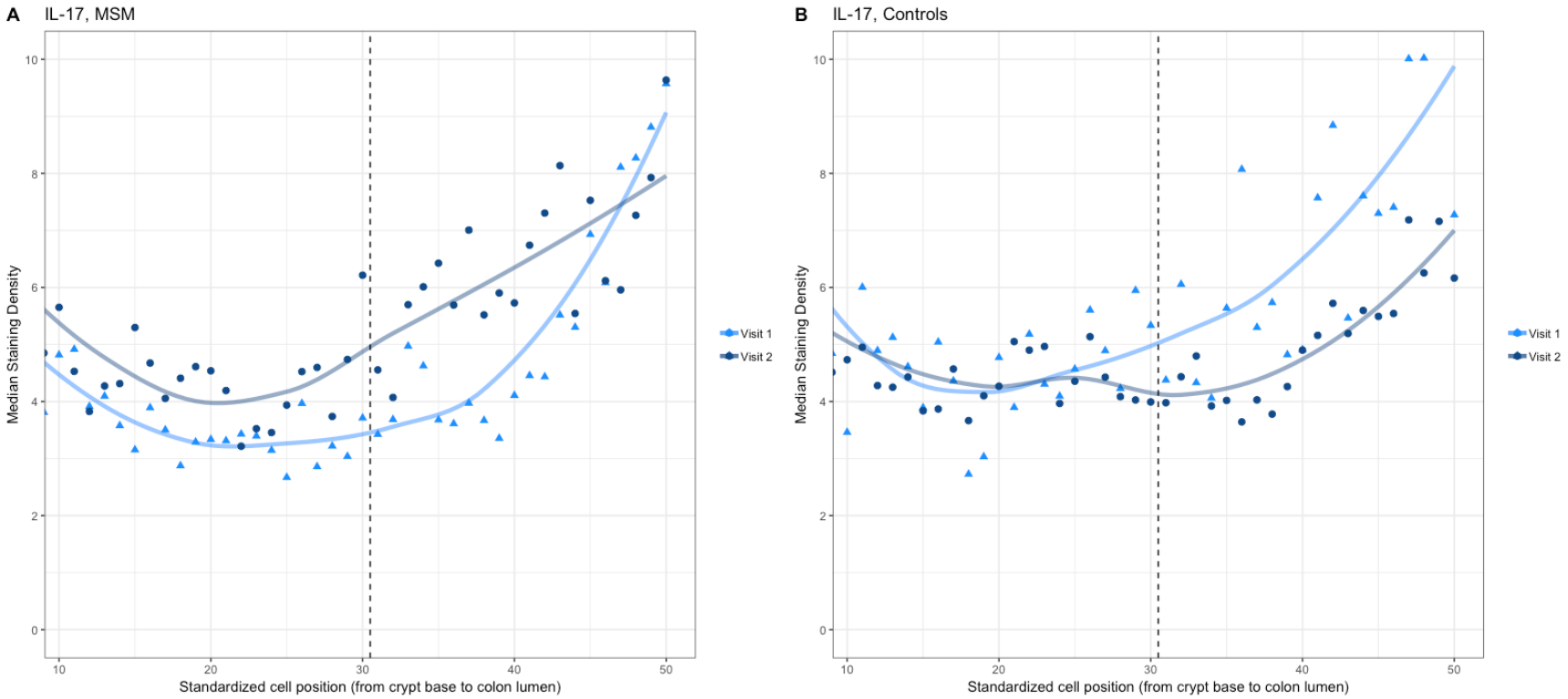

Figure 3. Distribution of median IL-17 biomarker staining densities stratified by study group and visit. The average of the medians of each bin was predicted by the LOESS procedure. The area under the curve (AUC) was used to compare expression by visit. (**A**) MSM group. Participants were asked to abstain from CRAI $\geq$ 72 hours preceding visit 1 (n = 32) and to engage in CRAI $\leq$ 24 hours preceding visit 2 (*n* = 33). IL-17 expression appears to increase after engaging in CRAI (AUC Visit 2 – AUC Visit1 = 16.32). (**B**) Control group. Visit 1 *n* = 18, Visit 2 *n* = 14.

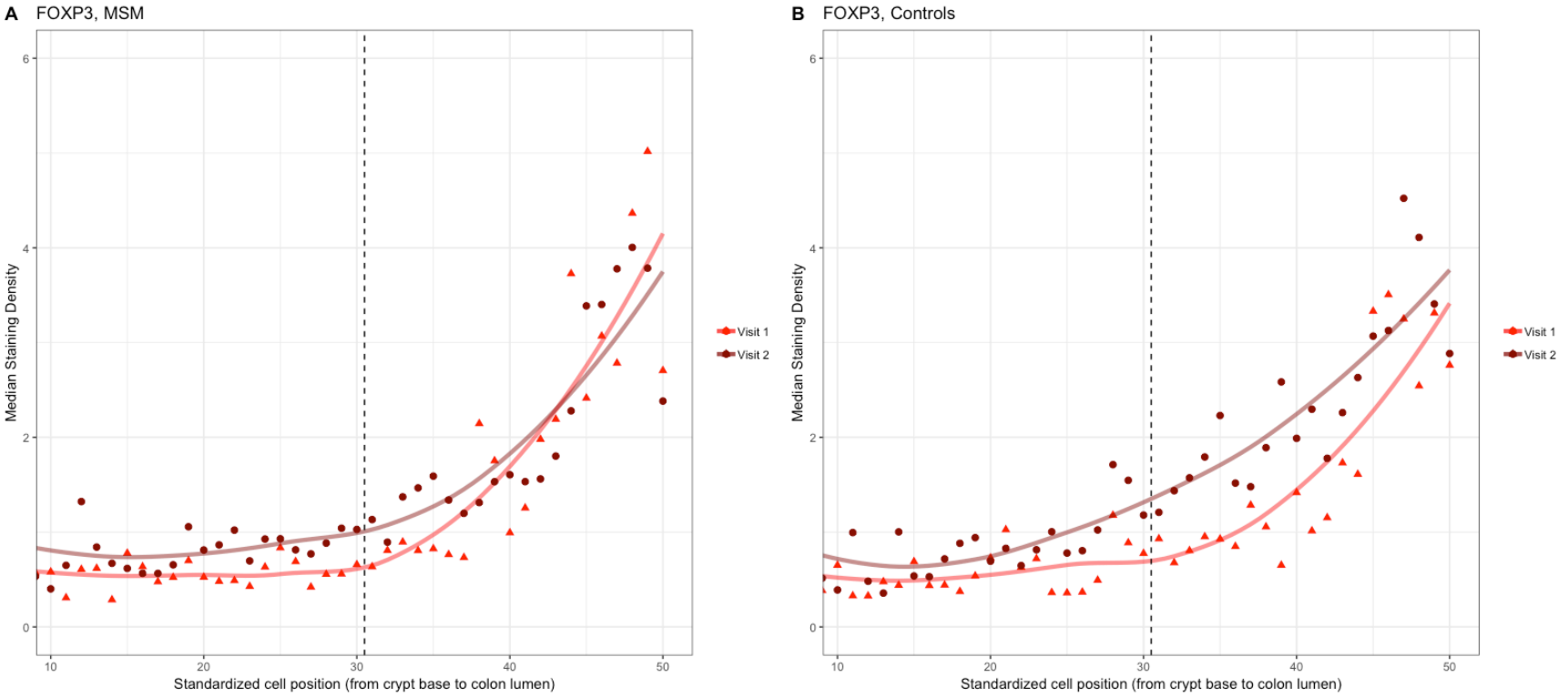

Figure 4. Distribution of median FOXP3 biomarker staining densities stratified by study group and visit. The average of the medians of each bin was predicted by the LOESS procedure. The area under the curve (AUC) was used to compare expression by visit. (**A)** MSM group. Participants were asked to abstain from CRAI $\geq$ 72 hours preceding visit 1 (n = 31) and to engage in CRAI $\leq$ 24 hours preceding visit 2 (*n* = 33). FOXP3 expression did not appear to change after engaging in CRAI (AUC Visit 2 – AUC Visit1 = 5.18). (**B**) Control group. Visit 1 *n* = 17, Visit 2 *n* = 13.