SUPPLEMENTAL DIGITAL CONTENT

Comprehensive assessment of HIV target cells in the distal human gut suggests increasing HIV susceptibility toward the anus

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Supplemental Materials and Methods

Study Subjects and Clinical Specimens

The 29 study subjects were a subset of individuals recruited into the HVTN 905 protocol, an open specimen collection protocol for targeted immunologic studies in past vaccine trial participants. Specifically, all subjects in our immunohistological study were from HVTN 905 Project 01, a study examining long-term cellular immune responses at mucosal sites following immunization with HIV antigens previously delivered using an adenovirus serotype 5 vector, MRK HIV-1 gag/pol/nef (Step Study).[1, 2] All aspects of the study were approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center (protocol #1492) and the Regulatory Affairs Branch of the Division of AIDS at the National Institute for Allergy and Infectious Diseases (June 26th, 2008). No investigational agents were administered in HVTN 905 Project 01. All subjects provided informed consent to take part in the study and have intestinal biopsies taken. The median time period between administration of the last investigational vaccine dose during the Step Study and the intestinal biopsies taken from the 29 subjects in this study was 866 days (mean 918, σ 168). The 29 study subjects were healthy US men who have sex with men aged 25 to 53 years (median and mean 38). At the time of entry into the original Step Study the subjects were at high risk of HIV-1 acquisition on the basis of reported unprotected anal intercourse with a male partner or anal intercourse with two or more male partners in the 6 months before enrollment.[1] Risk was not an eligibility criteria for enrollment into the HVTN 905 protocol and thus was not assessed again before 905 study entry. Subjects who tested positive for HIV infection, rectal gonorrhea or chlamydia infection;

who had clinical suspicion of inflammatory bowel disease; who were diagnosed with a bleeding or other hematological disorder at the screening visit; who had inserted a foreign object into the anus within three days prior to the scheduled procedure; or who were not willing to abstain from receptive anal intercourse from 3 days prior to 7 days after the biopsy were excluded from the study.

Colon biopsies were obtained by flexible sigmoidoscopy approximately ~30 cm proximal to the pectinate line in the anal canal. Biopsies from the rectum were obtained from approximately 4 cm proximal to the pectinate line by either flexible sigmoidoscopy (in individuals who also had a colon biopsy) or by anoscopy (in individuals who only had rectum biopsies). Biopsies were taken with a 4 mm × 2 mm biopsy punch (Wallach Surgical, Trumbull, CT). All procedures were performed by trained physicians. Any incidental abnormal findings were reported to the study participants with the recommendation to seek care through their primary physicians.

Biopsy Processing and Immunofluorescence Staining

Biopsies were fixed for 4-7 days at room temperature in 10% neutral buffered formalin (VWR, Radnor, PA) and embedded in paraffin following standard histology procedures. Four-micron sections were cut on a Leica RM2255 Automated Rotary Microtome (Leica, Buffalo Grove, IL), mounted on positively charged EP-3000 slides (Creative Waste Solutions, Tualatin, OR), dried for 1 hr at 60°C, and stored until staining at 4°C. Slides were deparaffinized in xylene and rehydrated in graded dilutions of ethanol in water. Antigen retrieval was performed by heating the slides in Trilogy™ Pretreatment Solution (Cell Marque, Rocklin, CA) for 20 minutes at boiling temperature in a conventional steamer. Slides were then cooled for 20 minutes, soaked in Tris-buffered 0.15 M/L NaCl solution containing 0.05% Tween 20 (Wash Buffer; Dako North America, Carpinteria, CA) for at least 5 minutes before staining in an automated slide-processing system (Dako Autostainer Plus).

During automated slide processing, slides were blocked for 10 min in avidin solution and 10 min in biotin solution (Avidin-Biotin Kit, Biocare, Concord, LA), followed by 10 min in Serum-Free Protein Block (Dako), with Wash Buffer rinses between each step. The slides were then stained for one hour with the primary antibodies. Because of the high autofluorescence of intestinal mucosa in the green and red fluorescence spectra, we placed detection of the primary antibody combinations into the violet (350 nm) and far red (647 nm) spectra, and the nuclear counter-stain into the red spectrum (550 nm). Staining was performed with the following primary antibodies: anti-CD3 (RM9107S, rabbit clone SP7; Lab Vision Corporation, Fremont, CA), anti-CD68 (M0876, mouse clone Pg-M1; Dako), anti-CD209 (DCSIGN) (MAB161, mouse clone 120507; R&D Systems, Minneapolis, MN), and anti-CCR5 (mouse clone MC5[3-6]). Negative control primary immunoglobulins were either whole rabbit IgG (011-000-003; Jackson ImmunoResearch Laboratories, West Grove, PA) or mouse IgG (I-2000; Vector Laboratories, Burlingame, CA).

For the CD3/CCR5 staining combination, 15% goat and 5% human serum were added during blocking with Serum-Free Protein Block. Anti-CD3 was applied for 60 min at a dilution of 1:2000, endogenous peroxidase activity was blocked for 8 min in 3% H_2O_2, and biotinylated polyclonal goat-anti-rabbit (E0432; Dako) was added for 15 min at a 1:1500 dilution in Dako Antibody Diluent with Background Reducing Components (S3022; Dako). Readout signal was then amplified using enzymatic methods based on peroxidase catalyzed deposition of a biotinylated tyramide compound (Catalyzed Signal Amplification System kit, K1500; Dako), according to the manufacturer's instructions.[7-9] This was followed by incubation with streptavidin AlexaFluor350 (Invitrogen, Grand Island, NY) for 30 min at a dilution of 1:100 and then slides were blocked again with Serum-Free Protein Block containing 5% human serum. CCR5 mouse hybridoma supernatant was added at a dilution of 1:50 for 60 min, followed by

goat-anti-mouse AlexaFluor647 (A21236; Invitrogen) at a dilution of 1:200, then mouse anti-AlexaFluor647 (C1117; Sigma-Aldrich) at 1:500 for 30 min. Staining was completed by incubation with goat-anti-mouse AlexaFluor647 at a dilution of 1:200 for 30 min.

For the CD68/CCR5 and the CD209/CCR5 staining combinations, slides were stained with anti-CCR5 (1:2000), followed by 3% H_2O_2, Catalyzed Signal Amplification, and streptavidin AlexaFluor350. After Serum-Free Protein Block with 5% human serum, slides were incubated with the second primary antibody, either anti-CD68 (1:50) or anti-CD209 (1:500), followed by goat-anti-mouse AlexaFluor647. For CD209 visualization, the secondary signal was amplified with mouse anti-AlexaFluor647, followed by a second incubation with goat-anti-mouse AlexaFluor647 as described above. Controls for all staining combinations were run with identical procedures but replacing the primary antibodies with either rabbit IgG or mouse IgG, as appropriate, at calculated or estimated matching concentrations. All sections were counter-stained for 20 min with Sytox Orange (S11368; Invitrogen) at a dilution of 1:20,000. Slides were coverslipped in ProLong Gold antifade reagent (P36930; Invitrogen).

Acquisition and Analysis of Stained Tissue Sections

A sequence of slightly overlapping 20x images covering each stained tissue section in its entirety was acquired on a Zeiss Imager Z2 microscope using TissueFAXS Image Acquisition and Management Software (TissueGnostics, Vienna, Austria). Each stained marker was acquired separately as a monochrome image. Overlapping images were stitched together using the TissueStitching function of TissueFAXS (Figure 1A). All acquired images were exported to the TissueQuest Cell Analysis Software (TissueGnostics). Monochrome images were superimposed and rendered in pseudo-colors as depicted in Figure 1B. Individual cells were identified based on the nuclear counter-stain by the TissueQuest software, with comparable accuracy of nuclei counts by the software and a human operator (Figure 1C and D). Cell

outlines were superimposed on the acquired images of the violet and far red stains and assessed for expression of the phenotypic markers (CD3, CD68 or CD209) and CCR5. Measurements of individual cell fluorescence were displayed in two-dimensional dot plots, with each cell defined by a combination of its specific mean (x axis) and maximum (y axis) fluorescence intensity (Figure 2B). Mean fluorescence intensity for each cell is calculated as the average of all measured pixels of that cell. Maximum fluorescence intensity for each cell corresponds to the pixel with the highest fluorescence intensity measured in that cell. Positive-negative cut-offs for phenotypic marker or CCR5 expression were based on isotype control stains. For any given marker, only cells above the positive-negative cut-off for both mean and maximum fluorescence intensity were considered positive, ensuring a conservative assessment of marker positivity. Cells positive for either CD3, CD68 or CD209 were gated and displayed on a downstream scatterplot depicting their mean and maximum fluorescence intensity in the CCR5 staining channel (Figures 3A and 4A). Cell counting was performed automatically on the entire slide for each tissue section, permitting a more robust and objective analysis than traditional manual screening and cell counting methods that rely on the analysis of a limited number of user-selected fields of view. On average, the total area of mucosal epithelium analyzed per slide was 3.1 mm^2 (median 3.0 mm^2; range 0.8 - 7.2 mm^2), which corresponded to an average of 16,494 analyzed cells per slide (median 15,770, range 5117 – 52,997). Of note, we analyzed the epithelium and lamina propria of each biopsy but excluded deeper regions as well as follicle-like lymphoid aggregates.

Isotype control slides were used to determine the overall background in our data set. Double isotype controls were negative (Figure 2A), and single isotype controls for the phenotypic markers also showed only negligible background (not shown). Single isotype controls for CCR5, a representative set of which is shown in Figure 2A, displayed the following background levels across all control slides tested: (1) CCR5 isotype-positive cells among $CD3^+$ T cells (n=11

slides): median 0.155% (mean 0.146, σ 0.134) in colon, and median 0.103% (mean 0.171, σ 0.16) in rectum. (2) CCR5 isotype-positive cells among $CD68^+$ macrophages (n=30 slides): median 0% (mean 0.117, σ 0.343) in colon, and median 0% (mean 0.534, σ 1.075) in rectum. (3) CCR5 isotype-positive cells among $CD209^+$ DCs (n=24 slides): median 0.176% (mean 0.403, σ 0.688) in colon, and median 0% (mean 0.642, σ 1.095) in rectum.

CCR5 Genotyping

We genotyped all participants for hetero- or homozygosity of the CCR5Δ32 mutation by PCR and DNA restriction fragment length analysis.[10] Among the 29 subjects, we identified one CCR5Δ32 homozygote and one CCR5Δ32 heterozygote. The CCR5Δ32 homozygote individual tested completely negative for CCR5 expression in his colon and rectum biopsies, which provided an additional specificity control for the anti-CCR5 antibody. This individual was excluded from statistical analysis. The CCR5Δ32 heterozygote individual did not exhibit unusually low CCR5 expression levels and was not excluded from analysis.

Data Analysis

The numbers of total nucleated, single marker positive and double marker positive cells, as well as the acquired area in mm^2, were exported to the Statistical Center for HIV/AIDS Research and Prevention (SCHARP), which independently from the laboratory operator calculated cell percentages and densities/mm^2 and conducted the statistical analysis. Data sets were statistically compared between rectum and colon by Mann-Whitney U test when using all data in each comparison group, and by Wilcoxon signed-rank test when using only paired data where each data pair was derived from a colon and a rectum biopsy obtained from the same individual at the same time point.

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